# Some Secondary Metabolites in Leaves of *Echinodorus grandiflorus* (Cham. & Schldl.) Micheli in Brazil

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**Abstract:** The paper aimed to study the influence of naphthalene acetic acid (NAA) and ethephon on total phenols, flavonoids and tannins as a function of seasonality in *Echinodorus grandiflorus* plants. The experiment used adventitious plantlets from the flower stem with 4 to 5 leaves, planted in 10-liter pots. The leaf spray treatments applied were: control, NAA 100 mg $L^{-1}$, and ethephon 100 mg $L^{-1}$. Treatments were applied fifteen days after collecting the leaf blades, once at each season of the year. The results showed that plant growth regulators did not influence *E. grandiflorus*' phenology and morphology. The ethephon did not affect synthesis of total phenols and flavonoids. The NAA caused a decrease in the synthesis of total phenols and flavonoids in some periods. Tannin synthesis is not expressive in *E. grandiflorus*. The study presented information about ecological aspects and chemical and mechanical defenses of *Echinodorus grandiflorus*.

**Keywords:** Ethephon, flavonoids, NAA, phenols, tannins, secondary metabolites, plant growth regulators.

## INTRODUCTION

*Echinodorus* is a genus that belongs to family Alismataceae, which is found exclusively in the Americas, with a center of dispersion in South America, occurring in several regions of Brazil. Besides *Echinodorus*, the family is also represented among us by the genus *Sagittaria* [1]. Popularly known as "chapéu-de-couro", it is frequently used by the population as diuretic, antirheumatic, antiinflammatory, and as an agent to fight uric acid and skin problems [2]. For this reason, it has been the target of extractivism.

With regard to ecological aspects, *Echinodorus grandiflorus* (Cham. & Schldl.) 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 [1].

Because synthetic medicines cost so much the population has resumed the use of medicinal plants due to their low cost and easy access, and also because this practice is a tradition, passed from generation to generation. This fact has called the attention of researchers seeking to obtain information of a scientific nature [3], both for a knowledge of their therapeutic action and their implications, and for the establishment of cropping conditions, which would make plants accessible to populations in urban centers that generally have no contact with natural vegetation. In addition, the large-scale growing of medicinal plants is one of the basic steps, highly important for the quality of the herbal medicine, since it is so hard to find satisfactory and homogeneous quantities of these plants under natural conditions. However, literature regarding agronomic aspects is scarce for most plants, or merely only a few aspects about cultivation are mentioned, as is the case with *Echinodorus grandiflorus* (Cham. & Schldl.) Micheli ("chapéu-de-couro", Amazon swordplant, burhead).

The action of plant growth regulators on adventitious plantlets originated on the flower stem of the plant, a reproductive strategy for the perpetuation of the species, would be an interesting approach, since there are no papers in the literature relating the action of plant growth regulators on contents of total phenols, flavonoids, and tannins in leaves of *E. grandiflorus* plants.

Both total phenols and flavonoids and tannins are secondary metabolites with great potential against herbivory, also acting as fungicides and having a number of therapeutic uses.

In view of the lack of information concerning the action of plant growth regulators on adventitious plantlets of *E. grandiflorus*, we gave priority in this work to an approach about the influence of the application of naphthalene acetic acid (NAA) and ethephon (ET) over the contents of total phenols, flavonoids, and tannins as a function of seasonality, in the plant's leaf blades. We also aimed to evaluate the influence of plant growth regulators on aspects of the plant's phenology. The importance of these studies in *Echinodorus grandiflorus* plants can be justified by a lack of studies with the objectives here proposed and because the secondary metabolites studied play a role against herbivory.

## MATERIALS AND METHODS

The experiment was conducted at the Medicinal Plants Garden, at Universidade do Vale do Paraíba - UNIVAP

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Campus, São José dos Campos (SP, Brazil), in the period from March 2002 to February 2003 (23°14' S, 45°51' W).

The *Echinodorus grandiflorus* experiment was installed in a nursery 20 meters long by 4 meters wide, at a 2-meter height, totaling an area of 100 $m^2$, completely covered with a black polypropylene shading (50% shade cloth), up to a height of 30 cm above the ground [4], thus providing the most suitable light intensity for the development of *E. grandiflorus* adventitious plantlets. Three benches 4 meters long by 1.30 meter wide were constructed inside the nursery, spaced at 3 meters from one another.

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 mother 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 - UNESP, Brazil.

*E. 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 [4].

Treatments consisted of: control; NAA (naphthalene acetic acid) at 100 mg $L^{-1}$; and ethephon (2- chloroethylphosphonic acid) at 100 mg $L^{-1}$. The treatment solutions were added of 0.5 mL nonionic surfactant adjuvant (Extravon®), and the leaf-spray treatments were applied with a hand sprayer 40 days after the plantlets were planted in the pots. The other plant growth regulator applications were performed at every 15 days from the leaf blade collections, which were made at the 4 seasons of the year.

The first collection of leaf blades from *E. grandiflorus* plants was made 15 days after the treatments, for chemical analysis of total phenols, flavonoids, and tannins. All leaf blades collected for the phenols and flavonoids analyses 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 leaf blades used in the tannin studies were packaged in aluminum foil and stored in a freezer at the Chemistry Laboratory.

The dry and frozen material was taken to the Department of Botany's Phytochemistry Laboratory, of Instituto de Biociências at Universidade de São Paulo - USP, São Paulo, Brazil, where phytochemical analyses of the leaf blades of *E. grandiflorus* were performed.

In the total phenols and flavonoid studies, the dehydrated material of each treatment was ground in a household blender, packaged in paper bags, and stored in the refrigerator.

The extraction of phenolic substances was performed according to a methodology modified from Phillips and Henshaw [5], and the extracts were submitted to the total phenols quantification method of Folin-Ciocalteu [6]. Absorbance readings were done in a spectrophotometer at 760 nm, according to a methodology proposed by Waterman and Mole [6]. A standard curve, prepared with gallic acid solutions at 50, 100, 150, 200, 250, 300, and 350 µg $mL^{-1}$ was plotted to determine the concentration of total phenols in the sample. This method is based upon the reducing effect of phenolic hydroxyls present in the extract.

The methodology proposed by Deutsches Arzneibuch (1978), cited by Motta [7] was used to dose flavonoids. A standard curve prepared with quercetin solutions at 20, 40, 60, 80, 100, 120, 140, and 160 µg $mL^{-1}$ was used to determine the concentration of flavonoids present in the samples.

Tannins were dosed according to the modified colorimetric method of Watermann and Mole [6], which is based on the precipitation of tannin with BSA (bovine serum albumin solution). A standard curve prepared with tannic acid solutions at 0.075, 0.150, 0.250, 0.300, 0.375, 0.450, and 0.60 $mL^{-1}$ was used to determine the concentration of tannins present in the samples.

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

A completely randomized experimental design was used, containing 3 treatments with 5 replicates of 5 pots each, totaling 75 pots. The results were subjected to analysis of variance and the means of the treatments were compared by Tukey's test at 0.05 significance level.

## RESULTS AND DISCUSSION

### Flowering and Emission of Adventitious Plantlets of *Echinodorus grandiflorus*

During the experiment, it was observed in all treatments that the *Echinodorus grandiflorus* plants began flowering in spring (end of October), ending practically in the beginning of March (summer), with a significantly higher intensity from November to January.

In Rio Grande do Sul (southern region of Brazil), Rego [1] reported that an interruption of *E. grandiflorus* flowering was observed in winter, followed by significant senescence of leaves; the same was verified by Vieira and Lima [8] in Viçosa (Minas Gerais State), in the southeastern region of Brazil. The latter authors reported that in Viçosa, *E. grandiflorus* flowered from October to January, during the rainy season, reaching a flowering peak in November.

Pimenta [9] 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.

Therefore, *E. grandiflorus* shows a flowering peak from November to January. It is suggested that translocation of metabolites occurs from the leaf 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 winter. With regard to phenological aspects, no influence of plant growth regulators on the plants seems to have occurred, since flowering occurred simultaneously in all treatments, and no morphological changes whatsoever were observed in leaves and inflorescences.

In recent studies, Pimenta [10] 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 *Echinodorus grandiflorus* in the present work, under the conditions to which plants were submitted, corroborate the data described above and were not influenced by the treatments.

The study showed that the plant growth regulators at the concentrations used in the experiment did not cause plant morphology changes; the simultaneous emission of adventitious plantlets on the flower stem was observed in all treatments, just like in control plants.

**Total Phenols and Flavonoids Contents**

In the fall the total phenol and flavonoids contents in the *E. grandiflorus* leaves was higer than in the other seasons. The treatment with ethephon and control plants showed the highest contents these secondary compounds (Table **1**).

By analyzing Table **1**, it can be noted that in all treatments there was a decrease of about 40% in total phenols contents in the winter, spring, and summer months, when compared with the fall, when contents in all treatments were significantly higher in relation to the others.

Pip [11] studied phenolic compounds in aquatic plants and filamentous algae and verified that the amount and types of these substances varied among the species studied depending on their habitats, plant organs, and seasons of the year; however, the contents of phenolic compounds did not vary with plant age. In addition, submerged plants showed reduced total phenols contents, while plants with emersed and floating leaves showed high quantities of total phenols.

In general, total phenols contents were smaller in NAA-treated plants, regardless of season of the year. According to Galston and Davies [12], the plant's response to exogenous or endogenous auxin depends both on its concentration and on the nature of the tissue. Hoque and Arima [13] observed that 2,4-D influenced the contents of phenolic substances in the aquatic plant *Trapa japonica* grown *in vitro*, reducing the accumulation of phenols and increasing the induction of callus in culture medium.

The small increase in total phenols content in plants submitted to the ethephon treatment could be due to the fact that ethylene, at the concentration used in this work (100 mg $L^{-1}$), activates synthesis of the PAL enzyme, which takes an active part in the biosynthesis of phenolic compounds. Ecker and Davis [14] and Ked Salyveit Jr. [15] also reported that the application of exogenous ethylene is quite effective, because it induces the synthesis of several enzymes; in studies with pea seedlings, they observed that ethylene induced the synthesis of PAL, a key enzyme for the biosynthesis of phenylpropanoids. Abeles *et al.* [16] showed that ethylene is biologically active at low concentrations (less than 1 mg $L^{-1}$). The authors also reported that papers have demonstrated the participation of ethylene as a second messenger in physiological processes.

According to Kanmegne and Omokolo [17], plants undergo changes in their enzyme expressions, regulated by different metabolic pathways such as the metabolism of phenols, and also by genetic expression. Phenolic compounds constitute a group of substances with a wide spectrum of physiological activities, and are the main substrates for peroxidase. They participate in processes of reduction and defense reactions in plants, and without a doubt interfere with the regulation of the development process, such as organogenesis, by means of their interaction with plant growth regulators.

From the flowering behaviour and emission of adventitious plantlets observed during the experiment, it can be suggested that the variation in total phenols contents in different seasons of the year could be related to the plant's phenology. The *E. grandiflorus* plants begin flowering during spring, finishing at the end of the summer; during this period, the plant may be mobilize photoassimilates for flower development, decreasing the amount of substrates available for the synthesis of secondary metabolites, in this case phenolic substances. In addition, in the same period the plants produce adventitious plantlets, another process that must consume a great deal of photoassimilates, reducing even more the availability of substrates for the synthesis of these metabolites. It can be seen that all processes were finished before the fall season. During this period, photoassimilate availability is higher and the synthesis of phenolic compounds reaches its maximum peak.

It can be seen that in the winter, spring, and summer the flavonoid contents found both in control plants and in those treateds remained at about one half of values found in the fall. In the fall the ethephon treatment did not influence flavonoid contents compared from the flavonoid contents of control plants.

In the fall season, we verified that flavonoid contents were higher in all treatments, when compared with other seasons of the year, differing from results obtained by Pimenta [10], where the highest contents were obtained during spring and in the full sun. We remind that the contents in the present study were obtained under 50% shading.

The level of flavonoids in the plant is a reflex of the influence in its biosynthesis [18]. Because flavonoids correspond to one of the great classes of phenolic substances, it is quite consistent that they have their maximum synthesis peak in the fall, when PAL, a key enzyme for the biosynthesis of phenylpropanoids [12, 19] possibly has its highest synthesis.

Mc Clure [18] commented that light intensifies the production of PAL in corn and bean epicotyls, producing a significant increase of flavonoids and, according to the author, this would explain why PAL could be a limiting factor for the synthesis of flavonoids in some plants. Dixon and Paiva [20] reported that anthocyanins and flavones increased in response to an increase in the incidence of light in the visible range, and the same occurred with some flavonoids in response to UV-B radiation. Under different climates and UV-B radiation, *Betula pendula* and *B. resinifera*

**Table 1. Mean Total Phenols, Flavonoid, and Tannin Contents (%) in Leaves of *Echinodorus grandiflorus* Plants Treated with Plant Growth Regulators in Different Seasons of the Year**

| Phenols Contents (%) | Treatment | | |
|---|---|---|---|
| **Season** | **NAA** | **Ethephon** | **Control** |
| Fall | 3.4 ± 0.23 Ab | 4.2 ± 0.12 Aa | 4.0 ± 0.08 Aa |
| Winter | 2.0 ± 0.07 Cb | 2.4 ± 0.05 Ca | 2.4 ± 0.23 Ba |
| Spring | 1.8 ± 0.09 Cb | 2.2 ± 0.03 Ca | 1.9 ± 0.10 Cab |
| Summer | 2.4 ± 0.08 Bb | 2.8 ± 0.21 Ba | 2.4 ± 0.11 Bb |
| **Flavonoid Contents (%)** | | | |
| Fall | 2.9 ± 0.12 Ab | 3.5 ± 0.16 Aa | 3.3 ± 0.35 Aa |
| Winter | 1.3 ± 0.02 Cb | 1.5 ± 0.08 Ca | 1.6 ± 0.06 Ba |
| Spring | 1.6 ± 0.03 Ba | 1.8 ± 0.05 Ba | 1.6 ± 0.02 Ba |
| Summer | 1.3 ± 0.02 Cb | 1.6 ± 0.09 Ba | 1.5 ± 0.07 Bab |
| **Tannin Contents (%)** | | | |
| Fall | $6.3x10^{-4} \pm 1.87x10^{-5}$ Ab | $7.2x10^{-4} \pm 2.55x10^{-5}$ Aa | $6.9x10^{-4} \pm 4.98x10^{-5}$ Aa |
| Winter | $4.7x10^{-4} \pm 1.22x10^{-5}$ Cb | $6.3x10^{-4} \pm 1.79x10^{-5}$ Ca | $6.2x10^{-4} \pm 3.94x10^{-5}$ Ba |
| Spring | $5.1x10^{-4} \pm 2.28x10^{-5}$ BCc | $7.5x10^{-4} \pm 1.79x10^{-5}$ Aa | $6.5x10^{-4} \pm 2.41x10^{-5}$ ABb |
| Summer | $5.4x10^{-4} \pm 2.61x10^{-5}$ Bc | $7.8x10^{-4} \pm 2.92x10^{-5}$ Aa | $6.8x10^{-4} \pm 2.77x10^{-5}$ ABb |

Means followed by the same upper case letter vertically, and lower case letter horizontally are not significantly different by Tukey test ($p < 0.05$).

showed high concentrations of several types of condensed flavonoids and tannins in the leaves, when compared with plants that did not receive UV-B radiation [21]. Ryan *et al*. [22] verified that flavonoid levels doubled when *Arabidopsis mutans* plants were treated with UV-B radiation, suggesting that flavonoids protect the plant against UV-B radiation. Samson *et al*. [23] observed that *Vigna unguiculata*, *Glycine max*, and *Phaseolus vulgaris* exposed to moderate and high UV-B radiation showed flavonoid and anthocyanin content increases in all plants.

Pimenta [10] observed greater production of flavonoids and arylpropanoids in *E. grandiflorus* in the sunny days and during spring, suggesting that both flavonoids and arylpropanoids may be involved in the defense against photooxidation. He also reported that in *E. grandiflorus*, flavonoids and arylpropanoids vary together, showing the same pattern of response to growing conditions (50% shading, full sun, moderate flooding levels, with or without suppression of the inflorescence).

The *E. grandiflorus* plants in this study were grown at 50% shading, suggesting that maybe on the full sun or under a shade cloth that would provide a lower shading rate the flavonoid contents could have been higher, because, as previously mentioned, photocontrol of the PAL enzyme does occur. The higher production of flavonoids in the spring, according to Pimenta [10], was not observed in this work. However, flavonoid contents decreased by one-half in the winter in relation to the fall, and remained at that level until the summer.

With respect to the action of plant growth regulators on flavonoid contents, Mc Clure [18] stated that it is quite variable. In *Echinodorus grandiflorus*, it was observed that during the fall, a season of the year when there was a pronounced increase in flavonoid contents, the NAA treatment caused a decrease of approximately 15% in that quantity, while the ethephon treatment did not affect the production of flavonoids (Table **1**). Possibly, ethephon (100 mg $L^{-1}$) sprayed on *E. grandiflorus* leaf blades was not sufficient to promote significant modifications in the flavonoid contents.

Flavonoids interfere with plant growth and development control mechanisms, for example, acting as regulators of enzymatic activity, IAA-oxidase, and also on the seed dormancy of several species [18, 24]. IAA-oxidase control by flavonoids does not reduce the possibility that the flavonoids may also interact with other responses, normally attributed to auxins. Effects associated with IAA were related by Stafford [25], who observed that B-ring monohydroxylated flavonoids were identified as peroxidase cofactors, activating the IAA-oxidase system that would destroy IAA, while B-ring dihydroxylated forms inhibited IAA's degrading activity.

### Tannin Contents

The effects of treatments on tannin contents was extremely low in *E. grandiflorus* leaves; therefore, the total phenols contents found in these plants are not represented by tannins (Table **1**). It can be seen that tannin contents practically are not influenced by seasons of the year; this is probably due to the fact that synthesis of these secondary metabolites in *E. grandiflorus* is not very expressive. However, variations can be observed in the quantities of tannins in the plants treateds in this work.

The NAA treatment provided a slight decrease in the production of tannins in all seasons of the year, when compared with the ethephon treatment (Table **1**). The ethephon treatment did not influence in the tannins synthesis in the fall

and winter (dry seasons), while there was a significant increase in the contents of these secondary compounds ($7.5.10^{-4}$% and $7.8.10^{-4}$%, respectively) in the rainy seasons (spring and summer), when compared with control plants.

When the quantities of tannins obtained by treating *E. grandiflorus* plants with NAA are compared, it can be noticed that the greater effect of this hormone occurs in winter, when tannin content is lowest ($4.7.10^{-4}$%); in the spring and summer there is also a decline in the synthesis of tannins ($5.1.10^{-4}$% and $5.4.10^{-4}$%, respectively); the smallest influence occurs in the fall ($6.3.10^{-4}$%).

The ethephon treatment promoted a slight increase in the tannin content, since the lowest values also occurred in plants harvested during the winter, with a tannin content of $6.3.10^{-4}$%, when compared with plants submitted to the same treatment in other seasons of the year; no statistically significant differences in tannin values were noticed in the fall, spring, and summer (Table **1**).

The results demonstrating low tannin contents in *E. grandiflorus* plants that it is supported by the results obtained in Pimenta's work [10], who reported that studies have demonstrated a low relative content of condensed tannins in this species. These plants produce latex, which consists, among other substances, of diterpenes, an effective class of metabolites against herbivory, just like tannins. Maybe suppression in the synthesis of these compounds could be explained by the plant's priority in the latex production.

The importance of verifying whether the treatments used in this work (ethephon and NAA) increased or not the contents of phenolic substances, flavonoids, and tannins, is also due to the fact that these substances present great therapeutic potential, because when this medicinal plant is controlled agronomically (by treating the medicinal plant as a crop), standardization of the drug will become possible.

## CONCLUSIONS

Under the study conditions and considering the proposed objectives, it can be concluded that the plant growth regulators (NAA and ethephon) did not promote alterations in the morphology and phenology of *Echinodorus grandiflorus* in the different seasons of the year; the ethephon treatment, in general, did not affect the synthesis of total phenols and flavonoids in different seasons; the NAA treatment caused a decrease in the synthesis of total phenols and flavonoids in some seasons of the year (spring and summer), and synthesis of tannins in *Echinodorus grandiflorus* is insignificant.

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