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Qualitative characterization of secondary metabolites of *Paspalum virgatum* weed under different water conditions

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Abstract

The presence of weeds amid pastures has caused significant damage to cattle farmers due to the difficulty in controlling these species. *Paspalum virgatum* stands out as an aggressive weed in Amazonian agroecosystems. Proper management of weed species is threatened by their aggressiveness, adaptation to low fertility soils and morphological, physiological and biochemical similarities with the grasses used as fodder. It is also possible that part of these characteristics of aggressiveness occur due to the ability of weeds to produce allelochemicals, resulting in damage to surrounding plants. The objective of this study was to qualitatively verify the presence of secondary metabolites in *P. virgatum* species under different water availability. The analyzed species were initially grown separately under three conditions of water availability: water deficit, field capacity and flooded environment. The water deficit treatment was conducted to verify weed ability to develop under conditions of extreme water shortage. This is a characteristic related to its rusticity, as found in grasses in the Amazon region. Excess water treatments were also conducted, since this is a common condition found in areas with partial flooding of a few months during the rainy season in the Amazon. After 120 days, weeds were collected and separated into roots, stems and leaves. The material was subsequently dried, ground and subjected to procedures to determine secondary metabolites. Phenolic compounds, flavonoids and alkaloids were identified in all conditions of plant development. The flavonoid detection test presented an intense yellow color. This is a strong indication of the presence of this class in stems and leaves of plants under flood and in leaves of plants under field capacity soil conditions. An intense presence of phenolic compounds was verified in stems and leaves, regardless of treatment. General alkaloids were found in great intensity in all samples. Saponins were found in leaves of *P. virgatum* under water stress (flooding and water deficit). All the metabolites were found in the plants, diverging between treatments and parts of the plant. It was concluded that the species presented higher steroid content in the roots when growing in places with excess of water. In conditions of full water supply, the presence of steroid was less intense in the roots, leaves and stems, result also found in roots and leaves of plants under water deficit. Alkaloids were less intensely present in the roots, regardless of the water treatment in which the plant was submitted. Results demonstrated that there are secondary metabolites responsible for some physiological ability of the weed to withstand excess or water deficit, indicating the need for further studies.

Keywords: Allelochemicals; Phytochemistry; Secondary Metabolites; Spontaneous Plant; Weed

Introduction

Weeds emerged from natural environmental disturbances and agriculture development. These conditions modified the habitat of native vegetation and provided conditions for the evolution of species more adapted to these environments. The spread of weeds occurs in several ways, including man's actions for the purpose of ornamentation, medicine, forage or unconsciously through machinery contaminated with seeds (Carvalho, 2013).
Cattle farmers in the northern region of Mato Grosso state, comprising the Southern Amazon area in Brazil, have had difficulty in renewing pastures. This is due to the predominant presence of weeds, among which it is identified *Paspalum virgatum*, popularly known as Naval Grass. The difficulty in managing this particular weed is based on its characteristics of aggressiveness, adaptation to soils with low permeability and morphological, physiological and biochemical similarity with grasses used as fodder. These characteristics hamper weed management by conventional methods (Sistachs and Leon, 1987).
Secondary metabolism has important ecological functions in plants. It protects the plants against herbivores, infection by pathogenic microorganisms, attracts pollinators and seed

dispersers and acts as agent in plant - plant competition and plant-microorganisms symbioses. The same compounds that increase the reproductive performance of plants may also become undesirable as food for humans, when acting in defense against fungi, bacteria and herbivores (Taiz and Zeiger, 2009). The determination of appropriate weed management methods is facilitated by knowledge of the types of secondary compounds in plants (Shah et al., 2016). The identification of the presence of allelochemicals in plants has become an option for a more rational management; use of agrochemicals may be reduced, presenting a more ecological alternative in weed control (Rigon et al., 2013). The identification and evaluation of chemical compounds in a certain plant species can be carried out through phytochemical prospecting. Preliminary phytochemical analysis can determine the existence of groups of relevant secondary metabolites when there are no chemical studies of the species (Falkenberg et al., 2010). The referred method consists in carrying out chemical reactions to obtain prior knowledge of the chemical behavior of plant extracts, being a valuable tool for such purpose (Matos, 1988).

Secondary metabolites are classified according to their structure and properties. These compounds are divided into the following classes: phenolic compounds, flavonoids, coumarins, terpenes, alkaloids, fatty acids and saponins. Within these classes, the most well known allelochemicals are straight-chain alcohol, water soluble organic acids, aliphatic ketones and aldehydes, single lactones, polyacetylenes and long-chain fatty acids, quinine (anthraquinones, enzoquinones, and quinine complexes), cinnamic acid and their derivatives, phenolics (caffeic acid, coumaric acid, vanillic acid, syringic acid, ferulic acid), coumarins, tannins, flavonoids, terpenoids and steroids (Shah et al., 2016).

When the plant is in a stressful condition, the concentration of secondary metabolites may vary, affecting the ability of the plant to stand out in relation to others. These conditions include lack or excess of water, attacked by microorganisms or insects, lacking essential nutrients, in shading and / or excessive luminosity (Souza-Filho and Alves, 2002).

The objective of this research was to verify the presence and quantify the intensity of the secondary metabolites in *P. virgatum* plants under different water availability conditions.

Results and discussion

Phytochemical prospecting

Several secondary metabolites were identified in the phytochemical prospecting of *P. virgatum*. Phenolic compounds, flavonoids and general alkaloids were the ones that stood out; they were present in all samples and with strong intensity in most of them. These metabolites were observed both under conditions of excess and lack of water.

Phenolic compounds and flavonoids

When using the 5% sodium hydroxide test, a strong intensity of flavonoids was found in the stems and leaves of plants under flooding conditions, and in leaves of plants under field capacity. Medium strength intensity was found in the stem of plants under field capacity, and in the stems and leaves of plants under water deficit. When using ferric chloride, strong intensity of flavonoids was found only in *P. virgatum* leaves that were submitted to field capacity. However, a medium strength intensity of the compound was found in the stems and leaves under all water conditions. For the other treatments, we found a weak intensity of the secondary compound (Table 1).

According to Taiz and Zeiger (2009), flavonoids perform different functions in plants, such as pigmentation and defense. These, together with phenolic compounds and tannins, are normally associated with responsibility for the potential allelopathic effect of plants. However, its toxicological effects vary according to its solubility in water. These chemical substances can inhibit the growth of seedlings of the same species (autotoxicity) or of other species (heterotoxicity). The presence of phenolic compounds and flavonoids in annoni grass (*Eragrostis plano*), considered an aggressive weed, was also verified (Fiorenza et al., 2016). Latter authors attributed the allelopathic effect to the presence of the referred metabolites. They reduced or inhibited the germination and development of seeds of bioindicator species. Oliveira et al. (2013) observed that leaves of *P. maximum* also presented phenolic compounds. However, the latter authors did not report the presence of tannins, saponins, flavonoids, steroids and free triterpenes.

When using the ferric chloride test, a strong intensity of phenolic compounds was found in the stems and leaves (intense yellow color); while a medium strength intensity was found in the roots, regardless of the water treatment. When using tests with 5% sodium hydroxide, differences were found only in plants under flooding: a strong intensity presence in the roots, medium strength intensity in the leaves, and weak intensity in the stems (Supplementary Figure 1). Studies point to phenolic compounds as being directly responsible for the allelopathic activity of the plant, inhibiting growth or germination of adjacent species (Fiorenza et al., 2016). Taiz and Zeiger (2009) confirm that phenolic compounds, such as those found in *P. virgatum* samples, exhibit a range of functions. They have a variety of chemical substances, and many of them act in favor of plant's defense mechanisms against herbivores and pathogens. Other substances can serve as attractive to pollinators or fruit dispersers, mechanical support or to reduce the growth of nearby competing plants.

Most compounds that are capable of inhibiting or stimulating the surrounding plants correspond to the phenolic compounds class (Rice, 1984). Its presence was verified in all plant parts of *P. virgatum* in strong intensity regardless of the water availability treatment, as pointed by the phytochemical analysis.When using ferric chloride, strong intensity of tannins was not found in tests performed to identify this metabolite (Supplementary Figure 2). When using the gelatin test, medium strength intensity of tannins was found in stems and leaves of plants only in treatments under field capacity (Supplementary Figure 3). By using copper acetate reagent, medium strength intensity was found in plants under water deficit and in stems under field capacity (Supplementary Figure 4).

Tannins, flavonones, steroids and saponins

Tannins inhibit seed germination and plant growth, standing out mainly concerning its phytopathogenic functions. These substances were identified in *P. virgatum*. According to Taiz and Zeiger (2009), the presence of these compounds can significantly reduce the growth and survival of herbivores that feed on such plants. They also act as a food repellent for animals like cattle, deer and monkeys. They avoid plants and its parts that have high levels of tannins (Camarão et al., 1990).

Pereira (2017), in a phytochemical study of *P. maritimum* grass (same family and genus as *P. virgatum*), identified the presence of tannins, flavonones, steroids and saponins when using ethanolic extracts from shoots and fractions with several solvents.

Saponins were evaluated using three different concentrations. As the concentration increased, so did the amount of foam. A strong intensity of saponins was identified in the highest concentration in the samples, of 0.1 gram. Leaves of *P. virgatum* showed a strong intensity of this metabolite in flooding and water deficit treatments. The stem and leaves in flooding and field capacity showed medium strength intensity, and the rest presented weak intensity or absence.

Rodríguez (2015) found different results concerning the presence of allelochemicals when evaluating the phytochemical compounds of *Pennisetum purpureum* (also a grass*).* No saponins, triterpenes and steroids were found in their chemical composition (both in the aqueous extract and in the ethanolic extract). In the present study, these compounds were found in weak or medium strength intensity in most samples of *P. virgatum*. Differences comparing both studies were also found concerning the intensity of flavonoids presence; the referred authors found weak intensity and absence in *P. purpureum* when using the aqueous and the ethanolic extract, respectively. Weak intensity presence of alakaloids was a similar result found in both studies.

Martins et al. (2010) also verified the presence of saponins in the leaves of *Hedychium coronarium* through phytochemical prospecting. However, this species did not present tannins, alkaloids or flavonoids in its chemical composition, diverging from the results verified in this study.

Alkaloids, steroids and terpenes

The analysis of indolic alkaloids, performed by the Otto test, was the most effective for the identification of these secondary compounds. It presented positive results in all parts of *P. virgatum*, in all treatments. The alkaloids form a large family, with the presence of more than 15,000 nitrogenous secondary metabolites. Although these compounds are known for their pharmacological effects on vertebrates, almost all of them are toxic to human consumption. They are also responsible for a great part of livestock deaths (Taiz and Zeiger, 2009).

Through the Liberman-Bouchard test (Supplementary Figure 5), a strong intensity of steroid and terpenes was found in roots of plants in a flooded environment. A weak intensity of steroid was found in all parts of the plant under field capacity, and also in roots and leaves of plants under water deficit. When using the steriodal core test (Supplementary Figure 6), we did not found the presence of steroids and terpenes in the roots (plants under field capacity and water deficit) and leaves (plants under water deficit). A weak intensity was found in the other parts of the plant. The B extract (Supplementary Figure 7) was the most effective one. Through it, we noticed a strong intensity with shades of green in the leaves (both flooded and water stress plants), a strong intensity with brown and green color in stems (plants under water deficit) and a medium strength intensity with brown color in roots and leaves (plants under field capacity).

Some terpenes are responsible for plant growth or development. They also may repel herbivores through its toxicity, and sometimes can also be considered as primary metabolites rather than secondary (Taiz and Zeiger, 2009).

Lima et al. (2009) verified the presence of phenolic compounds, tannins and flavonoids when evaluating *Sonchus oleraceus* through phytochemical prospecting and extraction with water or ethanol as solvents. The species also showed the presence of saponins, triterpenes and steroids, when extracted with dichloromethane as a solvent.

In the general alkaloids identification test the method considered as the most efficient was the one using the Bertrand reagent, extraction A, portion I (Supplementary Figure 8). By using this method, a strong intensity of this class of metabolite was found in all parts of the plant in all treatments. While using extraction B, none of the reagents identified the presence of the secondary metabolite (Supplementary Figure 9).

The observed results are similar to those reported by Hartmann (2015), in a phytochemical study of *Brachiaria spp*. The latter authors pointed to the presence of alkaloids, steroids and triterpenoids, flavonoids, foamy saponins and tannins in this species.

When assessing the presence of general alkaloids, the Bertrand test, in the "A" extraction portion I, was the one with the strongest intensity (+++) in all treatments regardless of the water treatment (Table 2).

These results are similar to those found by Rodrigues et al. (2009), in a phytochemical study of *Senna alata*. The latter authors identified alkaloids in all parts of the plant: stem, flower, leaf, root, seeds and pods. Saponins and tannins were also identified in the evaluated parts, except in flowers and leaves, respectively. Flavonoids, steroids and triterpenoids were not identified.

According to Matos (1988), plants show a variation in chemical dynamism due to influences of seasonal changes, climate, soil and even due to solar radiation availability. This may explain the differences found in *P. virgatum* in each evaluated treatment. There was a subtle influence of water stress on the amount of allelochemicals found in each part of the plant. Silva et al. (2014) stated that the environmental changes that cause stress to the plant are capable of causing changes in the production levels of secondary compounds, whether due to abiotic or biotic factors.

We concluded that it is possible to verify the presence of several allelochemicals in plant parts (root, stem, leaf) of *P. virgatum* through phytochemical prospecting. The strongest intensity of secondary compounds was found in the aerial part of the plant. Phenolic and flavonoid compounds were found in higher concentration when compared to the other secondary metabolites. This is possibly the main cause of the plant's allelopathic potential, as well as the causative agent of its aggressiveness in agroecosystems. Flavonoids were found in stronger intensity in the stems and leaves of plants under flooded conditions, and in leaves of plants under field capacity. Tannins occurred in medium strength intensity only in stems and leaves of plants under field capacity conditions. Concerning saponin, in the leaves of *P. virgatum* we found a strong intensity (plants under both flooded and water deficit conditions). A medium strength intensity of the compound was found in stems (plants under flooded conditions) and in

Table 1. Phytochemical analysis of root, stem and leaf of *Paspalum virgatum*, submitted to three water availability (flooding, field capacity and water deficit).

Class of secondary metabolites	Test	Flooding			Field capacity			Water Deficit		
		Root	Stalk	Leaf	Root	Stalk	Leaf	Root	Stalk	Leaf
Indolic alkaloids	Otto	+	+	+	+	+	+	+	+	+
	Cacotelins	-	-	-	+	-	-	-	-	-
	Isoquinolytic alkaloids	-	-	+	-	-	-	-	-	-
Phenolic compounds	Ferric Chloride	+ +	+ + +	+ + +	+ +	+ + +	+ + +	+ +	++ +	+ + +
	NaOH 5%	+ + +	+	+ +	+ +	+ + +	+ + +	+ +	+ + +	+ + +
Steroids and terpenes	Bouchard	+ + +	-	-	+	+	+	+	-	+
	Esterioda core	+	+	+	-	+	+	-	+	-
	Extraction B	-	-	+++v	++ v	+ v + + c	++ v	+ v	++ v +++c	+++v
Flavonoids	Ferric Chloride	+	+ +	+ +	+	+ +	+ + +	+	+ +	+ +
	NaOH 5%	+	+ + +	+ + +	+	+ +	+ + +	+	+ +	+ +
Saponins	2	-	-	+	-	-	-	-	-	+
	5	-	+	+ +	-	-	+ +	-	+	+ +
	10	-	+ +	+ + +	-	+	+ +	+	+	+ + +
Tannins	Ferric Chloride	-	-	+	-	+ +	+ +	-	+	+
	Gelatine	+	-	+	+	+ +	+	+ +	+ +	+ +
	Copper Acetate	-	+	+ +	-	+ +	+ +	-	+	+

(-) absence; (+) weak intensity; (++) mean; (+++) strong; (c) brown coloration; (v) green coloration.

Table 2. Phytochemical analysis for the identification of general alkaloids of the root, stem and leaf of the *Paspalum virgatum*, submitted to three water availability (flooded, field capacity and water deficit).

General alkaloids	Test	Flooding			Field capacity			Water Deficit		
		Root	Stalk	Leaf	Root	Stalk	Leaf	Root	Stalk	Leaf
Extraction A Portion I	Mayer	+	+	+	+	+	+	+	+	+
	Drargendorff	+	+	+	+	+	+	+	+	+
	Bertrand	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Bouchadart	+	+	-	+	-	-	+	+	+
Extraction A Portion II	Mayer	+	+	++	+	+	+++	+	+	+
	Drargendorff	-	+	-	+	+	-	+	+	+++
	Bertrand	++	-	+++	-	-	+	-	-	-
	Bouchadart	-	-	-	-	-	-	-	-	-
Extraction B	Mayer	-	-	-	-	-	-	-	-	-
	Drargendorff	-	-	-	-	-	-	-	-	-
	Bertrand	-	-	-	-	-	-	-	-	-
	Bouchadart	-	-	-	-	-	-	-	-	-

(-) absence; (+) weak intensity; (++) mean; (+++) strong.

leaves (plants under field capacity). In terms of steroids, a strong intensity presence was found in the roots (plants under flooded conditions). A weak intensity was found in all plant parts (under field capacity) and also in the roots and leaves (plants under water deficit). Alkaloids were found with weaker intensity in the roots, regardless of the water treatment to which the plant is submitted.

Materials and methods

Plant Collection and Preparation

The seeds of *P. virgatum* were collected in the municipality of Alta Floresta - MT, in natural infestation areas around the urban center. For plant reproduction, the seeds were subjected to dormancy break by soaking in sulfuric acid P.A (H_2SO_4), for 5 minutes and then washing in running water for 10 minutes (Silva et al., 2017).

After breaking dormancy, seeds were taken to a greenhouse and initially sown in polystyrene trays with 128 cells, previously filled with Techness® commercial substrate.

Plant cultivation

When plants reached approximately 10 cm in height, they were transplanted to plastic vessels with 10 dm^3 capacity. All vessels were maintained in controlled temperature and humidity by using an automated greenhouse system.

The soil used in the experiment was collected in a 0 - 0.20 m depth layer and classified as Yellow Red Latosol (LVa), based on the classification proposed by Embrapa (2013). After collecting it, physical and chemical analyzes were performed. By using its characteristics, 5.0 g dm^{-3} of dolomitic limestone filler, 2.54 g dm^{-3} of single superphosphate, 0.089 g dm^{-3} of urea and 0.3 g of potassium chloride were used to correct and fertilize the soil.

Experimental design

The experiment was conducted under three water supply treatments (water deficit (D), field capacity (C) and flooded (A) conditions) and ten experimental units per treatment. The water deficit treatment was conducted to verify the capacity of the weed to develop under conditions of extreme lack of water. This is a major characteristic of weed rusticity, as found in some grasses that grow in the Amazon region. Treatments with excess of water were used to represent a common condition of areas with partial flooding for a few months during the Amazon rainy periods.

The water treatments were started when plants reached 15 cm of shoot length. The experimental units that were submitted to water deficit were irrigated only three times, by using a minimum amount for plant survival (100 mL per vessel). Field capacity treatments were irrigated on a daily basis, aiming to keep the experimental units always under the same condition. Experimental units that were submitted to flooding conditions were kept in plastic trays with a sufficient volume of water. The plants could develop under conditions of daily excess of water by saturation.

Variables collected and phytochemical screening

Phytochemical screening was performed as follows: plants of all treatments were carefully removed from the containers after 120 days. Subsequently, they were separated into three distinct parts: roots, stems and leaves. Afterwards, they were separately packed in identified paper bags and submitted to drying in a forced air circulation oven, at 40 ºC for 120 hours. After drying, samples were ground in a Willey knife mill and then packed in paper bags, considering equal and identified volume. This material was used for the determinations described as follows, and in some cases in more than one procedure. For the quantification of secondary metabolites, signs of (-) were used to indicate absence. To indicate the presence they were identified as (+) to weak, (++) to medium and (+++) to strong, according to its intensity. Regarding the identification of steroids and terpenes, the colorings, cloudy, (c) brown and (v) green indicated the presence of the compounds. The entire methodology for phytochemical screening processes followed the protocols of Royo et al. (2015). The identification of general alkaloids, indole alkaloids, phenolic compounds, steroids and terpenes, flavonoids, saponins and tannins followed the methodological procedure of Royo et al. (2015).

Conclusions

In *P. virgatum,* steroids were found with high intensity in roots under flooded conditions, and with a weak intensity in all parts of plant (under field capacity) and in roots and leaves (under water deficit). Alkaloids occurred with lower intensity in the roots, regardless of the water treatment in which the plant was submitted.

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