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Plant antiherbivore defenses in Fabaceae species of the Chaco

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Abstract

The establishment and maintenance of plant species in the Chaco, one of the widest continuous areas of forests in the South American with sharp climatic variations, are possibly related to biological features favoring plants with particular defenses. This study assesses the physical and chemical defenses mechanisms against herbivores of vegetative and reproductive organs. Its analyses of 12 species of Fabaceae (Leguminosae) collected in remnants of Brazilian Chaco shows that 75% present structural defense characters and 50% have chemical defense – defense proteins in their seeds, like protease inhibitors and lectins. Physical defenses occur mainly on branches (78% of the species), leaves (67%), and reproductive organs (56%). The most common physical characters are trichomes and thorns, whose color represents a cryptic character since it does not contrast with the other plant structures. Defense proteins occur in different concentrations and molecular weight classes in the seeds of most species. Protease inhibitors are reported for the first time in seeds of: *Albizia niopoides*, *Anadenanthera colubrina*, *Mimosa glutinosa*, *Prosopis rubriflora*, and *Poincianella pluviosa*. The occurrence of physical and chemical defenses in members of Fabaceae indicate no associations between defense characters in these plant species of the Chaco.

Keywords: structural characters, protease inhibitors, lectins, defense mechanisms.

Defesas das plantas anti-herbivoria em espécies de Fabaceae do Chaco

Resumo

O estabelecimento e a manutenção de espécies no Chaco, uma planície semi-árida da América do Sul com variações climáticas importantes, possivelmente estão relacionados a características biológicas que favorecem as plantas detentoras de defesas particulares. Este estudo teve como objetivos avaliar os mecanismos de defesa física e química anti-herbivoria em órgãos vegetativos e reprodutivos. Analisamos 12 espécies da família Fabaceae (Leguminosae) obtidas em remanescentes de Chaco brasileiro. Observamos que 75% das espécies estudadas apresentam atributo de defesa física e 50% possuem defesa química – proteínas de defesa nas sementes, como inibidores de protease e lectinas. As defesas físicas ocorrem principalmente nos ramos (78% das espécies), nos órgãos reprodutivos (56% das espécies) e nas folhas (67%). Os atributos físicos mais frequentes são tricomas e espinhos, cuja coloração não contrastante com as demais estruturas das plantas representa um caráter críptico. Proteínas de defesa ocorrem nas sementes da maioria das espécies, com diferentes concentrações e classes de pesos moleculares. Inibidores de protease nas sementes estão sendo relatados pela primeira vez em: *Albizia niopoides*, *Anadenanthera colubrina*, *Mimosa glutinosa*, *Prosopis rubriflora* e *Poincianella pluviosa*. A ocorrência de defesas física e química entre os membros de Fabaceae indica que não há associações entre as características de defesa das espécies de plantas avaliadas no Chaco.

Palavras-chave: características estruturais, inibidores de protease, lectinas, mecanismos de defesa.

1. Introduction

When present in plants, physical and chemical defenses affect the development and survival of their attackers – herbivores (Hanley et al., 2007). Physical defenses act as barriers to herbivory through rigid protuberances and structures as thorns and/or spines, trichomes, leaf rigidity, formation of minerals - raphides and druses - in vegetal tissues, and seeds protected by hard testae (Dickison, 2000; Valverde et al., 2001). Spinescence, raphides, and druses are mainly associated with protection from mammals, while pubescence and sclerophylly essentially thwart the access of insects (Wagner, 1991; Hanley et al., 2007).

Chemical defenses against herbivores are characterized by the synthesis of primary metabolites, as defense proteins, and secondary metabolites, as terpenoids and nitrogen-containing and phenolic compounds, resulting from the production of compounds that are essential to plants (Chen, 2008). Direct defenses thwart herbivores by producing secondary metabolites or defense enzymes that act directly against attackers, while indirect defenses involve producing volatile compounds that attract natural enemies of herbivores (Pieterse et al., 2012). Among plant defense proteins are protease inhibitors (PIs) and lectins, which are found in vegetal tissues, mainly in reserve organs, and usually act against insects, bacteria and fungi (Peumans and Van Damme, 1995; Dunaevsky et al., 2005). Lectins and PIs affect the digestive process of insects by reducing the breakdown of the ingested proteins into amino acids (Murdock and Shade, 2002; Macedo et al., 2004; Vandenborre et al., 2011).

The preponderance of legumes (Fabaceae) in different plant formations worldwide suggests that some biological features favor their establishment. In these species, the main physical characters related to herbivory are thorns, spines, tector trichomes, secretory trichomes, and seeds with hard testae. Fabaceae species also produce chemical compounds of different classes that have distinct functions against herbivores (Levin, 1976; Kortt and Jermyn, 1981). In this family, defense proteins have been studied in species as *Adenanthera pavonina* L., *Bauhinia bauhinioides* (Mart.) J.F.Macbr., *Dimorphandra mollis* Benth., *Inga laurina* (Sw.) Willd., *Plathymenia foliolosa* Benth., among others (Macedo et al., 2002, 2004, 2011; Ramos et al., 2008; Sumikawa et al., 2010).

Only found in South America, the Chaco is a semi-arid grassland with extreme climatic conditions that encompasses parts of Argentina, Bolivia, Paraguay, and of the southwestern region of the state of Mato Grosso do Sul, Brazil. In the Brazilian Chaco, Fabaceae stands out by the richness and diversity of its species, many of which present physical defenses as thorns and trichomes (Alves and Sartori, 2009; Noguchi et al., 2009). Yet no investigations have ever considered the chemical characters of legumes growing in the Brazilian Chaco.

The establishment and maintenance of species in this region must require biological features favoring plants with particular characters. The occurrence of legumes with thorns, spines, and seeds with hard testae and the report of defense proteins in seeds of some species of this family suggest a possible relation between these defense mechanisms, an aspect not yet studied. This work thus assesses the physical defenses of legumes and the presence of defense proteins in their seeds.

2. Methods

Botanical material was collected in remnants of wooded steppic savanna (Wooded Chaco), forested steppic savanna (Forested Chaco), and transition areas between Chaco and Cerrado, Porto Murtinho municipality, western part of the state of Mato Grosso do Sul, Brazil (21°40'S, 57°52'W), from April to September 2011. Seeds of tree and shrub legume were collected simultaneously from at least three individuals sampled for each species, according to their availability in the environment. Were assessed 12 species, six Mimosoideae and six Caesalpinioideae (Table 1).

Physical defenses were assessed on vegetative (leaves and stem) and reproductive (flowers and fruits) organs. They include the following characters: spinescence, trichome types, and the color of thorns/spines compared to that of branches (Ronel and Lev-Yadun, 2012). Physical characters like spinescence and color of thorns were analyzed on field specimens, while trichome types on herbarium and field specimens. Botanical material was deposited in the Herbarium of *Universidade Federal de Mato Grosso do Sul* (CGMS Herbarium). Family nomenclature follows Lewis et al. (2005).

As for chemical defenses in seeds, the presence or not of protease inhibitors (PI) and lectin was assessed. The collected seeds of each species were macerated into fine granulated flours that were subjected to delipidation and protein extraction. Protein were extracted with (0.1M, pH 7.6) potassium phosphate buffer and (0.15 N) NaCl buffer in PI and lectin assays, respectively. Protein concentration was then estimated according to the method of Bradford (1976), with absorbance measured at 595 nm.

The presence of PIs was observed through the method of Erlanger (Erlanger et al., 1961). For each species, 5 µg of proteins were used per µL of crude extract and assays were carried out with Tris(hydroxymethyl) aminomethane buffer, N-benzoyl-DL-arginine-p-nitroanilide (BApNA) substrate, and bovine trypsin, and absorbance was read at 410 nm. Inhibitory activity was defined by the following formula: $IU = (T - A) / (0.250 \times V_{assay})$, where: IU = inhibition unit; T = trypsin reading; A = sample reading; and V_{assay} = volume of sample used in the assay. The occurrence of PIs was considered as a chemical defense mechanism when concentration exceeded 100 IU g^{-1}.

The presence of lectin was observed through hemagglutinating activity (HA) using microtitration plates. For each species, a 100 µL sample was assayed in triplicate serial dilutions and homogenized. Then, 100 µL of a 2%, suspension of red blood cells prepared with human blood (type A Rh positive) were added and red blood cell agglutination was observed 60 minutes later. Results were expressed in hemagglutination units (HU), defined as the reciprocal of the highest dilution in which hemagglutination was observed.

To estimate the apparent molecular weight, the proteins extracted from each species were analyzed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), according to Laemmli (1970). A molecular mass marker with six proteins: lysozyme (14 kDa), β-lactoglobulin (18 kDa), trypsinogen (24 kDa), pepsin (34 kDa), and albumin (66 kDa) (SIGMA) was used. All chemical analyses were performed at the *Laboratório*

Table 1. Fabaceae species from the Brazilian Chaco classified in subfamilies showing data on spinescence, trichome types, thorn color, protease inhibitors, and lectins.

Plant Species	Spinescence and trichome types			Spinescent stipule	Thorn color	Lectin (HU)	Protease inhibitor (IU g^{-1})
	Leaves	Stems	Flowers and fruits				
MIMOSOIDEAE							
Albizia niopoides	-	-	-	-	-	4	108
Anadenanthera colubrina	-	-	-	-	-	-	193
Microlobius foetidus	-	-	TT	-	-	-	0
Mimosa glutinosa	-	S	-	-	Vinaceous	-	188
M. hexandra	TT	S / TT	-	+	Brown	-	1
Prosopis rubriflora	TT	S / TT	TT	+	Brown	-	190
CAESALPINIOIDEAE							
Libidibia paraguariensis	-	-	-	-	-	-	10
Poincianella pluviosa	TT	GT	GT / TT	-	-	-	102
Parkinsonia praecox	TT	S / TT	-	+	Brown	-	0
Peltophorum dubium	-	GT / TT	TT	-	-	-	129
Pterogyne nitens	TT	-	-	-	-	-	1
Senna occidentalis	TT	TT	TT	-	-	-	0

S: spinescence; GT: Glandular trichomes; TT: Tector trichomes; HU: Hemagglutination units; IU: Inhibition units. +: Present; –: Absent.

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3. Results

Among the 12 species assessed, nine (75%) presented physical defenses and six (50%), chemical defenses (Table 1). In most species (67%) different types of physical defenses occurred on more than one plant structure, predominantly on vegetative organs. The most frequent were tector trichomes (67%) and spinescence (33%). Stems and branches presented most defenses (78%), followed by leaves (67%) and reproductive organs (56%).

Leaves only presented tector trichomes, while reproductive organs bore tector and glandular trichomes, and branches had spinescence and both tector and glandular trichomes (Table 1). Spinescence was observed in four species, three of which had spinescent stipules. Thorns were mainly brown and only presented vinaceous ends in *Mimosa glutinosa* Malme.

All the species of Mimosoideae assessed presented some kind of physical or chemical defense. *Mimosa glutinosa* and *Prosopis rubriflora* Hassl. had both, *Mimosa hexandra* Micheli and *Microlobius foetidus* (Jacq.) M.Sousa & G.Andrade only showed physical defenses, and *Albizia niopoides* (Spruce ex Benth.) Burkart and *Anadenanthera colubrina* (Vell.) Brenan only presented chemical defenses.

Of the six species of Caesalpinioideae studied, only *Libidibia paraguariensis* (D. Parodi) G.P.Lewis had none of the defenses assessed. *Poincianella pluviosa* (DC.) L.P.Queiroz and *Peltophorum dubium* (Spreng.) Taub. presented both types. *Parkinsonia praecox* (Ruiz and Pav. ex Hook.) J. A. Hawkins, *Pterogyne nitens* Tul., and *Senna occidentalis* (L.) Link, only presented physical defenses.

Lectin, although in low concentration (4 HU), was only found in the seeds of *Albizia niopoides*. Six species presented high concentrations of PIs (above 100 IU g^{-1}) and three very low concentrations (1-10 IU g^{-1}), as shown in Table 1. The molecular weights of the soluble proteins found in the seeds varied from 10 to 66 kDa. In *Albizia niopoides* and *Anadenanthera colubrina*, protease inhibitors ranged 18-24 kDa; *Prosopis rubriflora* and *Poincianella pluviosa*, 10-24 kDa; *Mimosa glutinosa*, 10-18 kDa; while in *Peltophorum dubium* 66 kDa.

4. Discussion

Trichomes on leaves, branches, and reproductive organs protect plants against herbivores, pathogens, excess of heat, and water loss (Wagner, 1991; Agrawal and Fishbein, 2006). Physical defenses, as spinescence, play a more important role to protect stems and branches than reproductive organs (Ronel and Lev-Yadun, 2012). Among the features analyzed, it is worth highlighting that in spinescent species thorns color tends to be a cryptic character, i.e. it is the same as that of the structure that bears it.

In addition to constituting a physical barrier to herbivory, in some species, thorns and spines may have warning coloration to mammals herbivores and may be associated with pathogenic microorganisms (Lev-Yadun, 2001). Chemical and physical defense mechanisms can act together to potentiate defenses against insects-plagues in plants of economic interest, like PIs expression in leaf trichomes of transgenic plants, which increases density and ramification of trichomes, resulting in extra resistance mechanism (Liu et al., 2006; Luo et al., 2009).

Plants respond to herbivory through several strategies, which lead to different interpretations for the evolution of plant defense (Agrawal, 2006; Moreira et al., 2016). There are species that invest in given defenses according to resource availability (Almeida-Cortez et al., 2004; Hanley et al., 2007). Moreover defense mechanisms antiherbivore may complement each other, favoring the presence of mixed defense (Carmona and Fornoni, 2013).

Plant species growing in similar environments converge on suites of co-varying defense characters, according to theory of plant defense syndromes (Agrawal and Fishbein, 2006). We verified that in the Brazilian Chaco there is no pattern in the occurrence of physical and chemical defense characters between members of Fabaceae. This suggests that in Chaco, plants must maximize their resources since they are subject to weather extremes as severe variations in temperature and water availability. Therefore, the preponderance of a physical or chemical defense mechanism is possibly not viable.

About defense proteins, molecular weight prevailing suggests inhibitors of the Kunitz type, except *Mimosa glutinosa*, that requires further investigations. Among legumes, the most studied families of proteinase inhibitors are of the Kunitz (20 kDa) and Bowman-Birk (8-10 kDa) types, which are frequently found in their seeds (Oliva et al., 2010; Macedo and Freire, 2011; Macedo et al., 2011). High concentrations of protease inhibitors in the seeds in five legumes: *Albizia niopoides*, *Anadenanthera colubrina*, *Mimosa glutinosa*, *Prosopis rubriflora* and *Poincianella pluviosa* are data published for the first time.

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