Cyanogenesis prospection in galled and non-galled tissues of *Microgramma squamulosa* (Polypodiaceae)

Prospecção da cianogênese em tecidos galhados e não galhados de *Microgramma squamulosa* (Polypodiaceae)

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ABSTRACT

Cyanogenic glycosides are defense substances that can produce hydrocyanic acid when they undergo hydrolysis as a result of herbivory, a process called cyanogenesis. Galls are neoformed structures of plant tissues induced by species-specific interactions between an inducer organism and a host plant. Earlier studies in *Microgramma* species have demonstrated cyanogenic polymorphism. *Microgramma squamulosa* is an epiphytic fern that may contain stem galls induced by *Tortrimosaica polypodivora* (Lepidoptera: Tortricidae). Thus, the objective of present study was to conduct a cyanogenesis prospection in the tissues of the stem, leaves and galls of *Microgramma squamulosa*. The study was conducted in populations located in the Rio de Janeiro state, Brazil. Cyanogenesis was assessed using the Feigl-Anger paper test. A total of 260 galled and non-galled tissues were analyzed, 45 gall samples, 67 sterile leaves, 43 fertile leaves, 103 stems and 2 croziers. Cyanogenesis was detected in only three sterile leaf samples (4.5%). In none of the samples were the stems or galls cyanogenic. Cyanogenesis frequency did not differ significantly between sterile leaves, fertile leaves, stems, galls and croziers.

Keywords — Chemical ecology; insect-fern interaction; gall; fern; cyanogenic glycosides.

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RESUMO

Glicosídeos cianogênicos são substâncias de defesa que são capazes de produzir ácido cianídrico quando sofrem hidrólise em consequência da herbivoria, processo chamado de cianogênese. Galhas são estruturas neoformadas de tecidos vegetais geradas por interações espécie-específica entre um organismo indutor e uma planta hospedeira. Estudos realizados demonstraram que há um polimorfismo cianogênico em espécies de *Microgramma*. *Microgramma squamulosa* é uma samambaia epífita que pode conter galhas caulinares induzidas por *Tortrimosaica polypodivora* (Lepidoptera: Tortricidae). Desse modo, o objetivo do presente trabalho foi realizar uma prospecção da cianogênese nos tecidos do caule, folhas e galhas de *Microgramma squamulosa*. O estudo foi realizado em populações de *M. squamulosa* localizadas no estado do Rio de Janeiro, Brasil. A cianogênese foi avaliada pelo teste de papel Feigl-Anger. Foram analisadas 260 amostras de tecidos galhados e não galhados, sendo 45 amostras de galhas, 67 de folhas estéreis, 43 de folhas férteis, 103 de caules e 2 de báculos. A cianogênese foi detectada em apenas três amostras de folhas estéreis (4,5%). Em nenhuma das amostras o caule ou as galhas foram cianogênicas. A frequência da cianogênese não diferiu significativamente entre as folhas estéreis, folhas férteis, caules, galhas e báculos.

Palavras-chave — Ecologia química; interação inseto-samambaia; galha; samambaias; glicosídeos cianogênicos.

INTRODUCTION

Plants can reduce the impacts of herbivory by acquiring defense or resistance mechanisms via the presence of chemical and/or physical attributes. Chemical defense is achieved through numerous substances and may be constitutive or induced (Boege & Marquis, 2005). Among these, we underscore cyanogenic glycosides. When these substances undergo hydrolysis, they release hydrocyanic acid (HCN), a process known as cyanogenesis that occurs in plants when their tissues are damaged by herbivory (Hegnauer, 1977; Vetter, 2000). The cyanogenesis is considered a constitutive defense due to the damage-independent production of preformed cyanogenic precursors (Kautz, Trisel, Ballhorn, 2014). Hydrocyanic acid is extremely toxic to various organisms, inhibiting, for example, the oxygen reduction in the cytochromes present in the respiratory electron transport chain (Francisco & Pinotti, 2000). Cyanogenesis has been detected in several species of fungi, lichens, lycophytes, ferns, gymnosperms, angiosperms, insects and a number of microorganisms (Buhrmester, Ebinger, Seigler, 2000; Gleadow, Bjarnholt, Jørgensen, Fox, Miller, 2011). Studies focusing on cyanogenesis in lycophytes and ferns are rare, the most noteworthy being those by Cooper-Driver & Swain (1976), Harper, Cooper-Driver, Swain (1976), Cooper-Driver, Finch, Swain, Bernays (1977), Schreiner, Nafus, Pimentel (1984), Hadfield & Dyer (1988) and Santos *et al.* (2005).

Galls are neoformed structures of plant tissues caused by cell hyperplasia and hypertrophy. They are generated by species-specific interactions between an inducer organism and a host plant. The inducer is a parasite that manipulates the metabolism of the host plant (Isaias, Oliveira, Carneiro, Kraus, 2014).

Microgramma C. Presl is a fern genus belonging to the Polypodiaceae family. Galls have been recorded on four species: *Microgramma percussa* (Cav.) de la Sota, *M. squamulosa* (Kaulf.) de la Sota, *M. vacciniifolia* (Langsd. & Fisch.) Copel. and *M. mortoniana* de la Sota (Santos, Hanson, Maia, Mehltreter, 2019; Lehn, Arana, Müller, Bianchini, 2020). *M. lycopodioides* (L.) Copel. (Hegnauer, 1977) and *M. vacciniifolia* (Santos *et al.,* 2005) are the only species that have been evaluated for cyanogenesis. The latter species was analyzed monthly for one year, and the results showed a variation over time in cyanogenesis between individuals and the organs of the same individual, that is, cyanogenic polymorphism. The frequency of cyanogenesis in *M. vacciniifolia* was measured in the stem (10%), fertile leaves (40%) and sterile leaves (47%) (Santos *et al.,* 2005).

Oliveira & Santos (2017) conducted cyanogenesis prospection in non-galled stems and two morphotypes stem galls of *M. vacciniifolia,* one induced by a microlepidoptera [*Tortrimosaica polypodivora* (Brown, Baixeras, Solorzano-Filho, Kraus, 2004)], and the other by a mosquito [*Primadiplosis microgrammae* (Maia & Santos, 2011) Cecidomyiidae-Diptera]. These authors reported cyanogenesis only in midge-induced galls and leaves, the latter with higher frequency.

The Neotropical fern *Microgramma squamulosa* (Kaulf.) de la Sota is a host plant of insects that promotes galls on its stem and leaves (Santos *et al.*, 2019). The stem gall is fusiform and induced by the microlepidoptera *Tortrimosaica polypodivora* (Maia & Santos, 2015) (Fig. 1).

The following are the main questions of this study: (1) Are *Microgramma squamulosa* cyanogenic? (2) Which organs are cyanogenic and what are their frequency? (3) Are galls cyanogenic? Thus, the objective of the present study was to conduct a cyanogenesis prospection in the tissues of the stem, leaves and galls of *Microgramma squamulosa*.

MATERIAL AND METHODS

The data were obtained from collections carried out in a forest area of the Nova Friburgo Country Club (Site 1) and Praça Suspiro (Site 2), both located in the municipality of Nova Friburgo, Rio de Janeiro state, in October 2017 and February 2018 (Rainy season) and June 2017 (Dry season), according Barbieri (2005).

Microgramma squamulosa is a rhizomatous epiphyte. In each tree, all the patches of *M. squamulosa* were considered a population. Only the populations with galls were sampled. Due to the difficulty of defining the individuals within each population, each apex of the rhizomes with galls was considered a sample, as well as, the adjacent non-galled stem, and the fertile and sterile leaves.

A total of 260 samples (Table 1) of *M. squamulosa* from 20 trees were analyzed. They were collected, placed in plastic bags and taken to the Biodiversity Laboratory

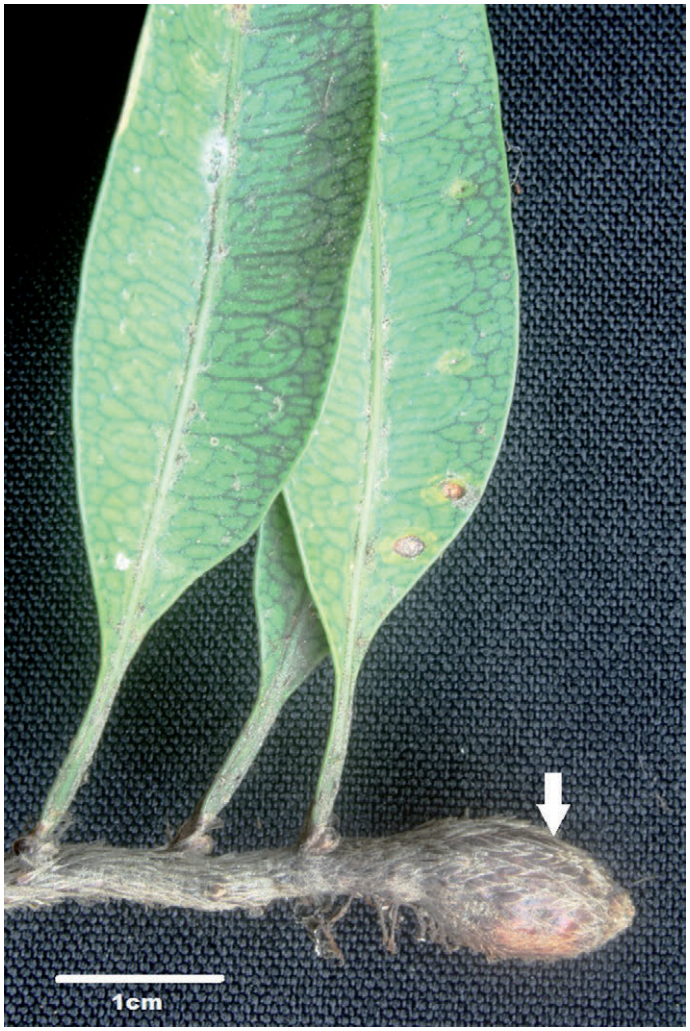

Fig. 1. *Microgramma squamulosa* (Kaulf.) de la Sota exhibiting a gall (arrow).

of the Faculdade de Formação de Professores (FFP), Universidade do Estado do Rio de Janeiro (UERJ). Part of the botanical material collected was herborized and deposited in the Faculdade de Formação de Professores da Universidade do Estado do Rio de Janeiro Herbarium (RFFP).

Cyanogenesis was detected using the Feigl-Anger test (Gleadow *et al.*, 2011). Sterile and fertile leaves were used, as well as galls and non-galled stems adjacent to the galls and croziers (young leaves). A total of 200 mg of fresh material was used from each plant sample. After being weighed, the material was ground in a test tube added with 3 drops of water. Next, a chemical indicator solution was dripped onto a strip of filter paper, folding one of the ends over the rim of the tube, which was sealed with a stopper. It is important to seal the tube quickly after grinding, since, when cyanogenic, the cyanogenic glycosides in the ground tissue simulating insect herbivory start to hydrolyze, releasing hydrocyanic acid. The tubes were examined immediately after grinding and after 30 min, 2h and 24 h. The absence of color on the paper strip indicates a negative result for cyanogenesis, while a blue-purple color shows a positive result.

The chi-squared test (X^2) was carried out to demonstrate the significant difference of cyanogenesis in different organs of *M. squamulosa*. PAST (PAleontological STatistics) software version 3.10 was used for data analysis.

RESULTS AND DISCUSSION

Cyanogenesis frequency did not differ significantly between sterile leaves, fertile leaves, stems, galls and croziers ($X^2=8.36$; $p=0.079$; DF=4) (Table 1). In relation to the galls, 21 were empty (because the inducing insect had already left the gall or died), 16 contained microlepidoptera larvae, 2 parasitoids and 6 ants. In none of the samples were the stems or galls cyanogenic (Table 1). Our results agree with those reported by Oliveira & Santos (2017) for galls induced by *Tortrimosaica polypodivora* in *Microgramma vacciniifolia*, a fern species with polymorphism in cyanogenesis (Santos *et al.*, 2005).

Cyanogenesis was detected in only three samples of sterile leaves (4.5%). According to Koricheva & Barton (2012), both the composition and secondary metabolite concentration of plants may vary, not only between different species or individuals from the same species, but also in a single plant over time. These authors describe a phenomenon known as heteroblasty, that is, since plants are modular organisms and these modules are semiautonomous units, each module may undergo different changes during development and maturation. For example, leaves formed during a specific phase of plant development exhibit morphological and physiological patterns specific to that phase, which may explain the positive results for cyanogenesis in so few samples of *M. squamulosa* leaves.

In addition to endogenous factors, cyanogenesis may also be influenced by exogenous aspects, such as nitrogen concentration, light intensity and water availability (Gershenzon, 1983; Miller, Gleadow, Woodrow, 2004).

In sterile leaves that displayed cyanogenesis, reaction time was immediate (0h) for one sample and occurred after 2h in the others. The speed and intensity of color change in the Feigl-Anger test are an indicator of cyanogenic glycoside concentration (Gleadow *et al.*, 2011). The positive result for cyanogenesis within 2h suggests the presence of both cyanogenic glycosides and hydrolytic enzymes, and the plants are

Table 1. Cyanogenesis in *Microgramma squamulosa* (Kaulf.) de la Sota. (C/T) = cyanogenic samples/total number of samples analyzed; n=total number of samples analyzed by plant tissues. (N=260 samples). $X^2=8.36$; $p=0.079$; DF=4.

	C/T Observation time (hours)				
Plant tissues	**0**	**1/2**	**2**	**24**	**n**
Sterile leaves	1/67	1/67	3/67	3/67	67
Fertile leaves	0/43	0/43	0/43	0/43	43
Galls	0/45	0/45	0/45	0/45	45
Stems	0/103	0/103	0/103	0/103	103
Croziers	0/2	0/2	0/2	0/2	2

considered cyanogenic in the field. After this period, the positive result indicates that cyanogenic glycosides spontaneously release HCN without enzymatic activity (Francisco & Pinotti, 2000).

CONCLUSION

The stem of *Microgramma squamulosa* and the gall induced by *Tortrimosaica polypodivora* showed no cyanogenesis in the samples analyzed. Some sterile leaf samples were positive for cyanogenesis but their frequency was low.

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