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CHEMOECOLOGICAL STUDY OF TROPHIC INTERACTION BETWEEN *Pseudosphinx tetrio* L. LARVAE AND *Allamanda cathartica* L.

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Abstract– The larval caterpillar *Pseudosphinx tetrio* L. is a species that feeds specifically on plants belonging to the family Apocynaceae. The genus *Allamanda* includes many shrubs or flowering trees which are grown throughout the tropical regions and are well known due their several biological properties. This work explores the interaction between the caterpillar and this plant using several analytical techniques such as thin layer chromatography, nuclear magnetic resonance spectroscopy, high performance liquid chromatography coupled with mass spectrometry and cyclic voltammetry. Further, a multivariate data analysis was performed in attempt to obtain better insights regard the samples.

The results point out the similar profile between the health and herbivore *Allamanda cathartica* L.'s leaves and the caterpillars' faeces. The similar analytical profile between the leaves of *A. cathartica* and the faeces of *P. tetrio*, as well as the difference with the caterpillar's bodies suggest a selective excretion of compounds by the caterpillar. These compounds found selectively in the faeces (and not in the body) can explain that *P. tetrio* can feed this toxic Apocynaceae species.

Key Words – *Pseudosphinx tetrio*, Apocynaceae, *Allamanda cathartica*, Chemical ecology, Phytochemistry, Electrochemical ecology, PCA analysis.

INTRODUCTION

Commonly known as “glutton caterpillar”, *Pseudosphinx tetrio* L. (Linnaeus 1771) is a velvety black caterpillar (Lepidopterae) about 12 cm long, with bright yellow stripes along the body and a bright orange-red head (Haxaire and Herbin 2000; Haxaire and Rasplus 1986). *P. tetrio* has the ability to eat up to twice its weight in food during a day this fact explains the common name of “glutton caterpillar”. According to aposematic hypothesis, it becomes toxic for its predators upon to absorb toxic compounds present in the latex of some plants (Warrell 2009). The larva of *P. tetrio* feeds preferentially on the leaves of plants as *Allamanda cathartica* L. (Apocynaceae), which are rich in toxic latex (Rajamanickam and Sudha 2013).

Allamanda cathartica L. (Petricevich and Abarca-Vargas 2019), also known as yellow *Allamanda* in the West Indies, is a widely grown shrub and frequently recognized as golden trumpet vine (Khanam *et al.* 2018). It is a native plant from Brazil (Pessoa *et al.* 2006) used in traditional medicine to treat jaundice (Nayak *et al.* 2006), presenting several biological activities as antioxidant (Hammeed *et al.* 2014), antimicrobial (Sharmin *et al.* 2015), antifungal (Souza *et al.* 2020), antimalarial (Conrad *et al.* 2013), anti-inflammatory (Boeing *et al.* 2018) and anticancer (Wong *et al.* 2011).

In the context of tropical chemical ecology, many different plant-herbivore interactions are highly specialized (Forister *et al.* 2015). In a self-defence process, plants can biosynthesize secondary metabolites to reduce herbivory. In turn, herbivores can react to these compounds in attempt to metabolize, excrete selectively or to use them for own defence (upon incorporation into the body) (Eisner and Meinwald 1995; Opitz and Muller 2009). Herein, we will explore the chemical interaction between *P. tetrio* and *A. cathartica* by several analytical methods, including an innovative electrochemical approach (named electrochemical ecology) and multivariate analysis in order to obtain better insights regard their specific interaction.

MATERIAL AND METHODS

Sample's preparation. Healthy and herbivored leaves of *A. cathartica*, as well as *P. tetrio* caterpillars have been collected in Guadeloupe Island (French West Indies), specifically in “Le Gosier” city (16°13'00.5"N 61°31'09.9"W). Leaves were cleaned (with distilled water) and lyophilized. The caterpillars have been kept in a cage for 24 hours, to collect their faeces, and starved for 48 hours to use their corpse in the evaluation of biomolecules incorporation to the caterpillar bodies excluding the digestive material. Finally, the dried leaves, the caterpillars' bodies and faeces were lyophilized and powdered. Afterwards, 50 g of each sample was extracted by maceration extraction for 48 hours. The maceration was carried out in a ternary mixture of dichloromethane/methanol/distilled water (Yu Lin *et al.* 2007; Mushtaq *et al.* 2014) (1:1:1 v/v, 200 mL). Upon the complete extraction, the solutions were filtered and extracted by liquid-liquid extraction and two phases were obtained for each manipulation (Table 1). Finally, these phases have been dried by rotary evaporation.

Table 1 Phases obtained for each manipulation after liquid-liquid extractions

	Organic Phase	Aqueous Phase
Healthy <i>A. cathartica</i> leaves	1.2	2.2
Herbivored <i>A. cathartica</i> leaves	1.3	2.3
Caterpillars	1.4	2.4
Caterpillar's faeces	1.5	2.5

Analysis and quantification. Analysis were performed using several techniques such as thin layer chromatography (TLC), nuclear magnetic resonance spectroscopy ($^1\text{H-NMR}$), high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) and cyclic voltammetry (CV).

Thin layer chromatography (TLC). The thin layer chromatography of all the organic phases were carried out with a gradient of two different mixtures of elution solvents composed by $\text{CH}_2\text{Cl}_2/\text{MeOH}$ or *n*-hexane/AcOEt with a volume of 10 mL in different proportions (80/20, 50/50, 90/10, 95/5, 99/1, 50/50). Further TLCs of all aqueous phases were made on cellulose TLC with a mixture of $\text{H}_2\text{O}/\text{MeOH}$ elution solvent in different proportions (50/50 – 100) as mobile phase.

Nuclear magnetic resonance spectroscopy ($^1\text{H-NMR}$). 15 mg of each extract was solubilized in 650 μL of DMSO and transferred to NMR tubes. 1D ^1H nuclear magnetic resonance spectra were recorded with a BRUKER Avance 300 MHz spectrometer equipped with a BBO probe and automatic tube changer. Chemical shifts (δ) are expressed in ppm relative to tetramethylsilane (TMS) taken as external reference and internal calibration is performed on the solvent signal. All spectra were processed using Topspin 2.1 software. The classical 1D proton with a 90° pulse width was performed. The spectra were acquired using 256 scans and 2 dummy scans of 32 K data points with a spectral width of 5411.255 Hz.

High performance liquid chromatography coupled with mass spectrometry (HPLC-MS). Triplicates of each extract were made and dissolved in 750 μL of methanol (LCMS grade) and 750 μL of ultra-pure water (ElgaPurelab Classic). The mixtures were sonicated in the ultrasonic bath then filtered on 13 mm and 0.45 μm PTFE filters and placed in amber vials. The analysis is performed on a Waters (Milford, MA, USA) Alliance e2695 liquid chromatographic system, equipped with a Waters 2996 photodiode array detector (PDA), coupled with the orthogonal quadrupole mass spectrometer (Micromass ZQ, Manchester, UK). The systems were controlled by MassLynx v.4.1 software (Micromass, Manchester, UK). The mass is equipped with an electrospray ionization ESI (Waters) source, the ionization is performed in positive mode. A double detection is carried out by mass spectrometry in ESI (range 71 to 1200 Da) and by a PDA diode array detector (UV detection between 210-400 nm). The analytical column was XTERRA MS C18 column (2.1 x 100 mm, 3,5 μm) (Waters). The elution is performed with a mobile phase flow rate of 0.2 mL/min consisting of a mixture of ultrapure water containing 0.1% formic acid (A) and methanol containing 0.1% formic acid (B). The program starts at $t = 0$ min ratio (A: B) 95:5, at $t = 2$ min (95:5), at $t = 5$ min (70:30), at $t = 15$ min (60:40), at $t = 40$ min (50:50), at $t = 55$ min (45:55), at $t = 60$ min (0:100), at $t = 65$ min (0:100), at $t = 75$ min (95:5) and at $t = 80$ min (95:5). The injection volume was 10 μL and detection was at 280 nm and at 320 nm. The ESI-MS parameters were as follows: desolvation gas (N_2) flow rate: 650 L/h; cone gas flow rate: 40 L/h; drying gas temperature: 450°C ; source temperature: 120°C ; capillary voltage: 3 kV; cone voltage: 85 V; RF lens voltage: 0.1 V.

Cyclic Voltammetry (CV). Cyclic voltammograms were obtained at 298 ± 1 K in a conventional three-electrode cell using a platinum wire auxiliary electrode and an Ag/AgCl (3M NaCl) reference electrode. Measurements were carried out with CH I660 equipment using 0.10 M potassium phosphate buffer at pH 7.0 as a supporting electrolyte. The working electrode was prepared by evaporating 50 μ L of an ethanol (EtOH) suspension of extract of interest, ground leaves or insect samples under air on a glassy carbon electrode (GCE, BAS MF 2012, geometrical area 0.071 cm²). In order to mimic the natural environment, no degasification of the electrolyte was performed.

Principal Component Analysis (PCA). The raw data from HPLC-MS was exported as netCDF files, using DataBridge software (Waters, USA), and pre-processed using XCMS Online (Mahieu *et al* 2016; Tautenhahn *et al* 2012) for feature detection, retention time correction and alignment of metabolites detected on HPLC-MS analysis. The dataset was created with 12 samples from each organic and aqueous extract fraction (3 samples from each manipulation (see Table 1)). Peak detection was performed using cent Wave peak detection ($\Delta m/z = 10$ ppm; minimum peak width, 5 s; maximum peak width, 20 s) and $mzwid = 0.015$, $minfrac = 0.5$, $bw = 5$ were used for retention time alignment. The processed data (csv file) was further exported to MetaboAnalyst 4.0 (Chong *et al* 2018). All data variables were scaled by pareto method prior to PCA. PCA is an unsupervised method commonly used to identify patterns between multivariate samples (Wold *et al* 1987; Jolliffe, 2005) and was recently employed on chemical variability of *Allamanda cathartica* extracts (Rodrigues *et al* 2020).

RESULTS AND DISCUSSION

TLC analysis. First, a TLC study has been conducted. The different results of this approach were exposed in the Table 2. In our first hypothesis (Hyp. 1), we propose that the caterpillars ingest and store certain metabolites (for example the metabolite with a R_f of 0.16 on TLC 3 (orange color)). The second hypothesis proposed (Hyp. 2), also illustrated in Table 2, refers to a selective excretion of an unidentified molecule by the caterpillars (for example the metabolite with a R_f of 0.95 on TLCs 2 and 3 (blue color)). Finally, the third hypothesis (Hyp. 3), is related with the response of the healthy plant to the herbivory (for example the metabolite with a R_f of 0.28 on TLC 1 (pink color), as this metabolite is present in the healthy leaves and not in the herbivored ones. The opposite case can be observed with the metabolite with a R_f of 0.18 on TLC 4 (green color) that is only seen in healthy leaves and thus could be considered as a metabolic marker for herbivory.

Table 2 Organic phases obtained with TLCs results. (A) healthy *A. cathartica* leaves, (B) herbivored *A. cathartica* leaves, (C) caterpillar's bodies and (D) caterpillars's faces. Solvent percentages are presented as highest polarity in solution (MeOH and EtOAc)*

Elution solvent	%*	TLC	R _f	A (1.2)	B (1.3)	C (1.4)	D (1.5)
CH ₂ Cl ₂ / MeOH	100	1	0.16				
			0.28	Hyp. 1,3		Hyp. 1	
			0.58				
	5	2	0.05				
			0.19				
			0.22				
			0.60				
			0.91				
			0.95	Hyp. 2	Hyp. 2		Hyp. 2
	10	3	0.16	Hyp. 1	Hyp. 1	Hyp. 1	
			0.20				
			0.57				
			0.90				
			0.95	Hyp. 2	Hyp. 2		Hyp. 2
			0.18		Hyp. 1,3	Hyp. 1	
CH ₂ Cl ₂ / MeOH	1	4	0.30	Hyp. 1,3		Hyp. 1	
			0.79				
			0.93				
			0.94		Hyp. 2,3		Hyp. 2
			0.08				
			0.17				
	100	5	0.37				
			0.62				
			0.93				
C ₆ H ₁₄ / C ₄ H ₈ O ₂	100	6	0.03				
			0.37	Hyp. 1	Hyp. 1	Hyp. 1	
			0.17				
	20	7	0.30				
			0.34	Hyp. 1	Hyp. 1	Hyp. 1	
			0.64				
			0.94		Hyp. 2,3		Hyp. 2
			0.97				
			0.98				
	50	8	1				
			0.48		Hyp. 1,3	Hyp. 1	
			0.95				
			0.97				
	80	9	0.50				
			0.91				
			0.94				
C ₄ H ₈ O ₂	100	10	0.19				
			0.89				

* Similarly, metabolite between two extracts or more are described in colors: orange: A, B and C; blue: A, B and D; pink: A and C; yellow: B and D. Hypothesis that can be illustrated by each compound are cited.

¹H NMR. The ¹H NMR data were obtained only from plants and caterpillar organic extracts, using DMSO-d₆ as solvent. Solvents signals from deuterated DMSO and residual water at 2.5 and 3.3 ppm, respectively, were removed (Fig. 1). The analysis indicates minor differences between spectra of health and herbivored *Allamanda* leaves. This result is contradictory with regards our Hypothesis. 3 (were a reaction of the plant to aggression was attended).

The spectra derived from caterpillars' bodies and faeces present big differences between the samples, and the chemical profile of healthy *Allamanda* and caterpillar's faeces are very similar. This result confirms our Hypothesis 2, showing the similarity between the chemical profile of *A. cathartica* leaves and caterpillars' faeces when compared to the caterpillars' bodies. These similarities and dissimilarities confirm our hypothesis (Hyp. 3) of the selective excretion of toxic compounds.

Fig. 1 ^1H NMR of organic extracts in DMSO-d_6 . (2_1) Healthy *Allamanda* leaves; (3_1) Herbivored *Allamanda* leaves; (4_1) Caterpillar's bodies and (5_1) Caterpillar's faeces.

HPLC-MS. In this study, the samples containing the healthy and herbivored leaves of *Allamanda cathartica*, as well the caterpillars and their faeces were investigated using chromatographic techniques (Barny *et al* 2021; Marques *et al* 2018; Prabhadevi *et al* 2012; Khanam *et al* 2018) (Fig.2). The similar profile observed on these spectra, suggests the presence of the same main compounds in the healthy (A) and herbivored (B) *A. cathartica* leaves, with similar retention times (RT) of 20.00 min and 20.50 min, respectively. A second compound can be identified in these spectra with comparable retention times (RTs: 25.52 min and 26.02 min). These results are opposed to our hypothesis 3 (as we can see in the TLCs and ^1H NMR profiles). The analysis indicates a presence of this compound in the caterpillar's faeces (D, RT: 25.73 min) but not in their body (C), confirming as well our hypothesis 2 (in agreement with the ^1H NMR and TLC results). Finally, the results show that the compounds found in *P. tetrio*'s bodies (C, RT: 20.43 min and 25.66 min) are significantly different than other compounds found in the faeces (confirming as well our hypothesis 2).

Fig. 2 HPLC-MS profiles of (A) healthy and (B) herbivored leaves of *A. cathartica* and (C) caterpillar's body and (D) caterpillar's faeces

Electrochemical investigation. Cyclic voltammetry studies have been employed in attempt to investigate possible interactions between different compounds and were previously used for monitoring plant defence against external stressors (Cebrian-Torrejon *et al* 2015; Palmeira-Mello *et al* 2021; Doménech-Carbó *et al* 2015; Rozoy *et al* 2013). Here, the predation of *A. cathartica* by *Pseudosphinx tetrio* has been electrochemically monitored via cyclic voltammetry (CV). We investigate the voltammetric response of films (at the surface of a glassy carbon electrode) of organic and aqueous extracts of healthy and predated leaves of the plant and samples of body and excrements of *P. tetrio* in 0.10 mol L⁻¹ phosphate solution buffer at pH 7.0. The reported methodology is ultimately based on the voltammetry of microparticles (VMP) technique (Domenech-Carbó *et al* 2013).

The behaviour of films on glassy carbon electrodes of healthy and predated leaves of *A. cathartica* are showed in Fig.3. It was observed a set of three chemically irreversible oxidation processes A₁, A₂ and A₃ (0.28 V, 0.65 V and an undefined shoulder near to 1.00 V respectively). Where as A₁ and A₂ peaks decrease upon cycling until a totally passivated and stable state is attained approximately at the second cycle, an increasing of A₃ peak was observed. Thus, the variations in the ratio of peaks A₁ and A₃, can infer differences in the nature of the redox species in *A. cathartica* healthy and herbivored leaves. These oxidation processes represent a profile observed in several vegetable species and are attributed to the oxidation of polyphenolic organic compounds. In fact, the voltammogram of the predated leaves exhibits the same signals than healthy leaves, but the peak A₁, is depleted while the signal A₃ is enhanced. Finally, a reduction process approximately at -1,0 V can be observed when the potential sweep is reversed

towards less positive potentials. Although these processes are slightly more negative for the healthy than for herbivore leaves, both correspond to the typical behaviour of the oxygen reduction in water. This result is basically opposed to our hypothesis 3 about the chemical response to the herbivory, but by this technique we can identify slight differences between healthy and herbivore leaves maybe related with polyphenols (reaction against oxidative stress) (Singh *et al* 2021).

Fig. 3 Cyclic voltammograms of films from a) healthy and b) herbivored or predated leaves of *A. cathartica* on glassy carbon electrode in contact with air-saturated 0.10 M phosphate buffer at pH 7.0. scan rate 10 mV s⁻¹

The cyclic voltammetry of films on glassy carbon electrodes of *P. tetrion*'s bodies and its excrements after predation on leaves of *P. tetrion* are presented on Fig.4. The cyclic voltammogram does not show clear oxidation process in the initial anodic scan for the body of *P. tetrion*, but revealed three oxidation processes at 0.22, 0.45 and 0.78 V, for its faeces, namely A₁, A₂ and A₃, respectively, which are similar to those found for *A. cathartica* analysis. Once again, a typical oxygen reduction process in water was observed in cathodic region for both analysis. Further, a characteristic signal C1 appears in both samples suggesting the presence of oxidized metabolite of *P. tetrion*. This result confirms the hypothesis 2 in agreement with the other analytical approaches.

Fig. 4 Cyclic voltammograms of films from a) *Pseudosphinx tetrio*'s bodies and b) *Pseudosphinx tetrio*'s excrements after predation on leaves of *A. cathartica* on glassy carbon electrode in contact with air-saturated 0.10M phosphate buffer at pH 7.0. Potential scan rate 10 mVs⁻¹.

A comparison between the voltammograms of films from the water extracts of healthy and predated leaves of *A. cathartica* in contact with air-saturated 0.10 M phosphate buffer at pH 7.0 was performed (Fig. 5). Here, the signal A1 appears clearly recorded in the healthy leaves and is accompanied by a well-defined cathodic peak at -1.25 V (C₂). This signal C₂ disappears in the voltammograms of the predated leaves while the signal A₁ decreases and the signal A₃ at 0.78 V becomes clearly marked. This result confirms the hypothesis 3, showing a reaction of the leaves against the herbivory, the disparition of signal C₂ and the apparition of signal A₃ as result of the herbivory.

This voltammetry is to some extent reproduced in the aqueous extracts of *P. tetrio* and its excrements, reproduced in Fig. 6, thus suggesting the existence of common electroactive compounds.

Fig. 5 Cyclic voltammograms of films from the water extracts of a) healthy and b) predated leaves of *A. cathartica* on glassy carbon electrode in contact with air-saturated 0.10 M phosphate buffer at pH 7.0. Potential scan rate 10 mV s⁻¹

Fig. 6 Cyclic voltammograms of films from water extracts of a) *P. tetrico*'s bodies and b) its excrements after predation on leaves of *A. cathartica* on glassy carbon electrode in contact with air-saturated 0.10 M phosphate buffer at pH 7.0. Potential scan rate 10 mV s⁻¹.

PCA Analysis. A total of 24 chromatograms were acquired using HPLC-EI-MS, 12 from aqueous fraction and 12 from organic fraction (3 biological replicates for each species). To identify differences in the metabolites or even in their concentration in the samples, the set of 24 samples was analyzed obtained from XCMS and used for data analysis. Two spreadsheets were obtained after data processing of HPLC-MS analysis, with 168 and 412 variables from organic and aqueous fractions, respectively (retention time m/z). In the PCA analysis, two plots were generated, namely score and loading plots. The first one shows the samples grouping, whereas the second one indicates the contribution of each variable to these samples. The PCA analysis were focused on the detection of any inherent pattern within the data. As observed in Fig.7, samples of healthy and

herbivored *Allamanda* leaves are close related, with small difference each other, when compared with the caterpillars' bodies and faeces. Further, when all samples are compared, the predated and not predated leaves from *Allamanda* are very similar to each other, and different from the other samples. In addition, the Caterpillars' bodies extracts present a great difference. In Fig. 8 (loadings plot) are presented the variables that influences the grouping observed.

Fig. 7 Score plot of 12 samples of organic and aqueous extracts of all manipulation, obtained from 168 (left) and 412 (right) variables, respectively. (2_1 and 2_2) red circle, healthy *Allamanda* leaves; (3_1 and 3_2) green circle, herbivored *Allamanda* leaves; (4_1 and 4_2) blue circle, caterpillar's bodies and (5_1 and 5_2) light blue circle, caterpillar's faeces.

Fig. 8 Score plots of 12 samples of organic and aqueous extracts of all manipulation, obtained from 168 (left) and 412 (right) variables, respectively. Each dot represents one variable (retention time m/z).

In conclusion, we investigated the plant-herbivore interaction between caterpillar *Pseudosphinx tetrio* and the flowering plant *Allamanda cathartica*. In this approach, several techniques were employed in attempt to gain better insights regarding this trophic relationship, such as TLC, ^1H NMR, HPLC-MS (analyzed by a

multivariate PCA) and an innovative approach by electrochemical methods (electrochemical ecology). The results point out the similar profile between the health and herbivored *A. cathartica*'s leaves and the caterpillars' faeces. The similar analytical profile between the leaves of *A. cathartica* and the faeces of *P. tetrio*, as well as the difference with the caterpillar's bodies suggest a selective excretion of compounds by the caterpillar (proposed hypothesis 2). These compounds found selectively in the faeces (and not in the body) can explain that *P. tetrio* can feed this toxic Apocynaceae species.

DECLARATIONS.

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