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Sustainable production of bioactive compounds from *Persea indica*

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Abstract: In this work we have investigated the accumulation of ryanoids in different plant parts (leaves, stems roots) of aeroponically grown *Persea indica* cloned trees (one year old cloned individuals) and a selected mature wild tree. We have tested the insect antifeedant (against *Spodoptera littoralis, Myzus persicae* and *Rhopalosiphum padi*) and nematicidal (against *Meloidogyne javanica*) effects of ethanolic extracts from these different plant parts. The HPLC-MS analysis of *P. indica* extracts showed that the mature tree (wild) leaves had two times more chemical diversity than the stems. The aeroponic plants showed lower differences in chemical diversity between leaves and stems, with the lowest diversity found in the roots. The ryanodane epiryanodol (**1**) was present in all the plant parts, with the mature stems (wild) having the highest amount. The aeroponic stems also accumulated ryanoids including **1**, cinnzeylanol **2** and cinnzeylanone **4**. The insect *Spodoptera littoralis* was strongly affected by the stem extracts while the leaf ones were moderately active. Based on the predicted vs. the real antifeedant values we conclude that the ryanoid content (**1** or a combination of **2**, **4** and **1**) explained the antifeedant effects of the stem extracts while additional components contributed to the activity of the leaf ones. Therefore, careful individual selection of *P. indica* seedlings should be carried out prior to proceed with its aeroponic cultivation in order to obtain ryanodane-rich stem or leaf extracts with strong antifeedant effects on *S. littoralis.*

Keywords: *Persea indica*; aeroponic; ryanodane

1. Introduction

The Macaronesian laurel forest, with a unique and endemic species composition, is a plant community comparable to the evergreen humid forests that were abundant in the Mediterranean during the Paleogene and Neogene. These forests have been considered a relict vegetation from the Tertiary. However, they represent remnants of the Macaronesian Pliocene/Pleistocene forests that modified their distribution areas due to temperature oscillations during the Pleistocene and consist of plant communities recruited from European/Mediterranean and tropical regions [1].

One of the dominant species of this forest is *Persea indica* (L.) Spreng., a perennial tree belonging to the Lauraceae family that probably originated from American tropical lineages [1]. *P. indica* has a characteristic defoliated appearance resulting from the seasonal action of the wild rat (*Rattus*). *P. indica* aerial parts contain ryanodane and isoryanodane

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diterpenes [2-5], alkene-γ-lactones [6] and avocadofuranes [6-8], while the fruits only contain avocadofuranes [6].

The ryanodane diterpenes isolated from *P. indica* were toxic to mice [2 and had strong insect antifeedant effects on *Spodoptera litura* and *S. littoralis* [4,9] and moderate to *Leptinotarsa decemlineata* [5, 10]. The insecticidal activity of these ryanoids is selective [10], acting on a nonneuronal target [11]. Given the potential of these ryanodanes as bioinsecticides, supercritical and supercritical antisolvent CO_2 (SC or SAS / CO_2) selective extraction methods have been developed to separate polar ryanodanes (epiryanodol and related) from alkyl-γ-lactones and related components of low polarity [12].

However, the fact that this plant is a unique endemic species from the Laurel Forest represents a bottle neck to produce ryanodane-based bioinsecticides. Therefore, we have cultivated *P. indica* in an aeroponic system [13] as a method for the sustainable production of its biomass. Aeroponic cultivation is a soilless production system independent of environmental conditions. It is carried out in a controlled environment, while spraying the roots intermittently with nutrients of defined chemical composition. It offers complete access to the aerial parts and roots throughout production time and provides opportunities to optimize the yield of natural products of interest, thereby facilitating commercial-scale production of bioactive compounds.

The aeroponic production of food crops is well known but there are only a few examples of aeroponic plant production for the isolation of secondary metabolites such as whitanolides [14]. The aeroponic cultivation of *P. indica* allowed for the detailed chemical study of its roots, characterized by alkane-γ-lactones, alkyne-γ-lactones, avocadofurane precursors, cis- and trans-p-coumarate esters of (−)-borneol, and ryanoid diterpenes previously described in the aerial parts [13]. Furthermore, among the compounds only found in the roots, (−)-borneol cis-p-coumarate and (+)-majorenolide were insecticidal / antifeedant and (+)-majorynolide insecticidal and nematicidal [13].

A preliminary study on the natural production of ryanoids in wild *P. indica* indicated that the foliar epiryanodol content of mature naturally growing trees did not show seasonal variations and did not correlate with their nitrogen, water or phenolic content. This diterpene varied among the individual trees and was found to accumulate in the stems suggesting a genetic-based control [15]. In this work we have investigated the accumulation of ryanoids in different plant parts (leaves, stems roots) of aeroponically grown *P. indica* cloned trees (one year old cloned individuals) in contrast with a selected mature wild tree. We have tested the insect antifeedant (against *Spodoptera littoralis, Myzus persicae* and *Rhopalosiphum padi*) and nematicidal (against *Meloidogyne javanica*) effects of extracts from these different plant parts.

2. Results and Discussion

The HPLC-MS analysis of *P. indica* extracts showed that in the mature tree (wild) the leaves had 2 times more chemical diversity than the stems (18 vs. 9 compounds detected). The immature plants (aeroponic) showed lower differences in chemical diversity between leaves (12 compounds) and stems (11 compounds) with the lowest diversity found in the roots (9 compounds) (Table 1).

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Retention	Wild		Aeroponic			m/z			Identification
time (min)	Stems	Leaves	Stems	Leaves	Roots				
3.00-5.00	19.61	18.41	39.75	17.83	20.7				Polar compounds
7.30		5.18	6.40	4.33		449	353	266	
7.70		3.36	1.34	7.31		421			
7.73					2.86	153			
7.91					1.48	325	289	417	
7.94	2.53	1.89				397	433		
8.05	3.54	3.63	2.66	2.93		417	381		
8.18	9.59	3.98				399			
8.24			6.12			419	421		Cinnzeylanol **2**
8.35		6.97		14.80	6.96	137			
8.49			2.36			417	483	335	
8.87			4.34			504	505	419	
8.82	1.15	5.57				441	477		
8.94		1.87				431	467	397	
9.00				1.56		433	397		Cinnzeylanine **3**
9.11		1.16		2.65		167			
9.34	6.17	10.70		11.72	7.14	401			
9.53		5.18				195			
9.85		0.44	11.90	3.39		417	381		Cinnzeylanone **4**
10.22		2.11				432	459		
10.31				13.84		486			
10.92				2.21		301	424		
11.03		10.49				179	137		
12.37	5.72	2.69				241	181	113	
21.29					17.16	327	363	443	Majorenolide
22.64		1.38	4.44	2.02	28.63	265			
22.93					3.69	299	147		
23.40	4.02		2.23	8.51	4.95	249	147		
23.69			9.49			461	524	249	
25.43	38.91	13.79	5.25		6.40	339			Epiryanodol **1**
26.31	5.38	3.79				666			

Table 1. Chemical composition (HPLC-MS analysis) of *Persea indica* extracts.

The distribution of the identified ryanoids is shown in Figure 1. The molecules are shown in Figure 2 and include epiryanodol **1** (39% in wild stems, 13.8% in wild leaves, 5.2% in aeroponic stems and 6.4% in aeroponic roots), cinnzeylanone **4** (12% in aeroponic stems), cinnzeylanol **2** (6.1% in aeroponic stems) and cinnzeylanine **3** (1.6% in aeroponic leaves). These results indicate that epiryanodol (**1**) was present in all the plant parts, with the mature stems (wild) having the highest amount. The inmature stems (aeroponic) also accumulated ryanoids but with higher molecular diversity.

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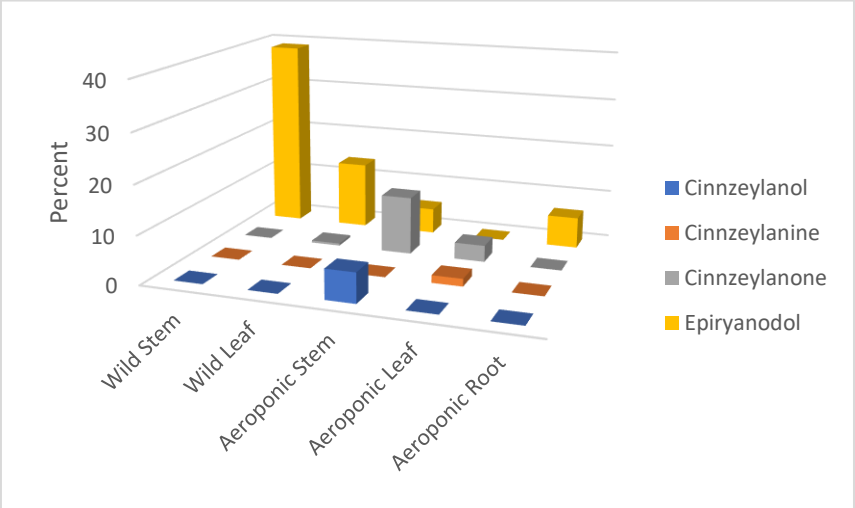
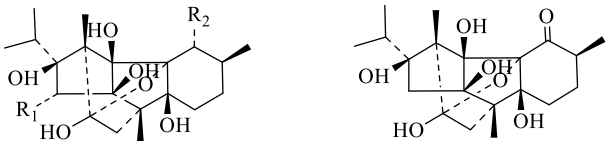
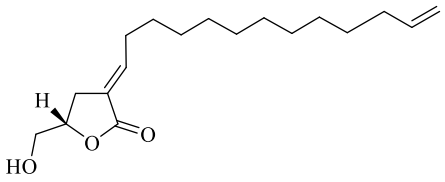


Figure 1. Percent identified ryanodanes **1-4** in *Persea indica* extracts



1 Epiryanodol R1, R2 = OH
2 Cinnzeylanol R1 = H, R2 = OH
3 Cinnzeylanine R1 = H, R2 = OAc

4 Cinnzeylanone



Majorenolide

Figure 2. Molecular structures of ryanoids **1-4** and majorenolide

The major compound detected in the root (28,63%, rt 22.6 min) was not identified. This compound was also present in mature leaf (1.4%), aeroponic stem (4.4%) and aeroponic leaf (2.0%) extracts in smaller amounts (Table 1). Additionally, molecular ions compatible with majorenolide (17%, rt 21.23 min, M+280, m/z M+2Na+H) were only present in the root extract (Table 1). Majorenolide has been previously isolated from the aeroponic roots of *P.* indica, was moderately antifeedant against the aphids *R. padi* and *M. persicae* (EC_{50} values of 17.6 and 15.8 μg/cm²) and cytotoxic to Sf9 insect cells [13].

These extracts were bioassayed for antifeedant effects against several insect species (*S. littoralis, R. padi* and *M. persicae*) and the nematode *M. javanica*. *S. littoralis* was affected by these extracts, with the stems being the most active (mature tree, EC_{50} = 8.5 μg/cm2; inmature tree, EC_{50} = 12.1 μg/cm2). The leaf extracts were moderately active (mature tree, EC_{50} = 36.8 μg/cm2; inmature tree, EC_{50} = 52.6 μg/cm2) while the aeroponic root extract was not active (Table 2).

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Table 2. Insect antifeedant (against *Spodoptera littoralis*) and nematicidal (against *Meloidogyne javanica*) effects of *Persea indica* extracts

Origin	Part	*S. littoralis*	*R. padi*	*M. persicae*	*M. javanica*
		%FI[a]	%SI[a]		% Mortality[b]
Aeroponic	Leaf	62.30±5.88*	48.8±10.5	17.1±7.2	4.45 ± 0.60
		52.6 (31.2-88.0)[c]			
	Stem	94.85±2.85*	44.9±8.4	21.7±6.5	7.86 ± 3.09
		12.1 (7.8-18.7)[c]			
	Root	39.38±7.73	28.5±7.2	22.1±7.2	5.01 ±0.51
		>100			
Wild	Leaf	89.67±10.13*	43.7 ± 8.3	42.6 ± 8.7	0.09±2.07
		36.8 (28.0-48.0)[c]			
	Stem	98.46±1.49*	44.8± 7.3	41.5 ± 8.8	0.08±0.98
		8.5 (2.7-23.9)[c]			

[a]Percent feeding (FI) or setting (SI) inhibition at a dose of 100 µg/cm2. Values are means of 10 or twenty replicates respevctively. Values with asterisk (*) are significantly different according to Wilcoxon paired rank test ($P < 0.05$).
[b]Percent mortality at a dose of 1.0 mg/mL Values (%) are means of four replicates (corrected according to Schneider-Orelli's formula [20].
[c]EC_{50} (95% lower-upper confidence limits), concentration needed to produce 50% feeding / setting inhibition

Based on the relative ryanoid content and the efficient antifeedant doses calculated for each compound identified, we calculated the predicted antifeedant effect (%FI/cm^2) for each extract (Table 3). The predicted vs. the real antifeedant (% FI) values were similar for both stem extracts (mature and inmature) but not for the leaves (2.6 times different). Therefore, the ryanoid content (epiryanodol **1** or a combination of cinnzeylanol **2**, cinnzeylanone **4** and epiryanodol **1**) explained the antifeedant effects of the stem extracts while additional components contributed to the activity of the leaf ones.

Table 3. Ryanoid-based (compounds **1**-**4**) predicted antifeedant effects of *Persea indica* extracts on *Spodoctera littoralis.*

[a]Predicted %FI (100 µg/cm^2) = [(%Compound /100) X 50]/EC_{50}
[b]From Table 2.

Compound	*S. littoralis*	Predicted %FI [a]			
	EC_{50} µg/cm^2 (95%	Wild		Aeroponic	
	Confidence Limits)	Stem	Leaf	Stem	Leaf
1	0.16 (0.07-0.35)	95.28	32.85	12.5	
2	1.26 (0.73-2.20)			2.5	
3	0.02 (0.07-0.34)				37.1
4	0.04 (0.02-0.09)		5.5	148	29.9
Total predicted %FI		95*	38[ns]	100*	67*
Calculated %FI[b]		98*	89*	95*	62*

*Significant antifeedant effect. [ns]No significant effect.

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Previous phytochemical studies of aerial parts from mature *P. indica* trees showed the presence of ryanodanes other than **1**-**4**. Epi-cinnzeylanol, and epiryanodol monoacetate with antifeedant effects against *S. litura* [9] and *S. littoralis* [10], the isoryanodanes vignaticol and perseanol with antifeedant effects against *S. litura* and *S. littoralis*, along with the inactive indicol [4,10]. Additionally, minor ryanoids such as anhydrocinnzeylanone, garajonone, 2,3-didehydrocinnzeylanone and anhydrocinnzeylanine with antifeedant effects against *S. littoralis* [6] have been isolated from the aerial parts. Therefore, the presence of these minor components in the leaves could explain the difference between the predicted (based on the identified ryanoids) and real antifeedant effects of the extracts.

Compounds previously isolated from the roots included inactive alkene-, alkyne and alkane-γ-lactones, avocadofurane precursors and *cis* / *trans*-p-coumarates of borneol as the major components, along with minor amounts of ryanodanes such as cinnzeylanone, anhydrocinnzeylanone, cinnzeylanine, cinnzeylanol, perseanol, cincassiol E, perseaindicol, secoperseanol and epiryanodol [13]. Among these root components, majorenolide was not antifeedant to *S. littoralis*, moderately-low antifeedant against the aphids *R. padi* and *M. persicae* (EC_{50} values of 17.6 and 15.8 μg/cm^2) and cytotoxic to Sf9 insect cells [13].

A previous study demonstrated that the variations in ryanodol and cinnzeylanol content among *P. indica* mature trees was not seasonal and that depended on the individual tree sampled [15]. Therefore, careful individual selection of *P. indica* seedlings should be carried out prior to proceed with its aeroponic cultivation in order to obtain ryanodane-rich stem or leaf extracts and/or majorynolide-rich root extracts. These extracts can be enriched in bioactive compounds by selective SC or SAS / CO_2 extraction of the biomass (leaves or stems) as previously demonstrated [12].

3. Conclusion

The HPLC-MS analysis of *Persea indica* extracts showed that the mature tree (wild) leaves had 2 times more chemical diversity than the stems. The aeroponic inmature plants showed lower differences in chemical diversity between leaves and stems, with the lowest diversity found in the roots. Epiryanodol (**1**) was present in all the plant parts, with the mature stems (wild) having the highest amount. The inmature stems (aeroponic) also accumulated ryanoids with higher molecular diversity. The insect *Spodoptera littoralis* was strongly affected by the stem extracts while the leaf ones were moderately active. Based on the predicted vs. the real antifeedant values we conclude that the ryanoid content (epiryanodol **1** or a combination of cinnzeylanol **2**, cinnzeylanone **4** and epiryanodol **1**) explained the antifeedant effects of the stem extracts while additional components contributed to the activity of the leaf ones. Therefore, careful individual selection of *P. indica* seedlings should be carried out prior to proceed with its aeroponic cultivation in order to obtain ryanodane-rich stem or leaf extracts with strong antifeedant effects on *S. littoralis.*

4. Materials and Methods

4.1 Plant material

Persea indica (L.) Spreng branches were collected from a mature tree located in "Monte de las Mercedes, Tenerife (28°28′10″N 16°17′30″W, 719 m) using a 3 m long guillotine cutter. Additionally, *P. indica* seedlings, donated by Garajonay National Park, Gomera-Tenerife (28°07′34″N 17°14′14″W, 1023 m) were multiplied by stem cutting in perlite-vermiculite with indole-3-acetic acid (IAA). The plants were watered three days/week with a nutrient solution (Nutrichem 20:20:20 N, P, K of Miller Chemical & Fertilizer Corp.; 3 g/l) and maintained a growth chamber (25 °C, 70% rh, 16:8 L:D) until their transfer to the aeroponic chamber.

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4.2. Aeroponic cultivation

Rt (min)	M^+	m/z			Compound	Reference
8.8	384	419	383		Perseanol	[4]
8.9	384	419	421	383	Cinnzeylanol (**2**)	[2]
9.5	408	461	425		Anhydrocinnzeylanine	[6]
9.6	426	397	433		Cinnzeylanine (**3**)	[9]
10.1	382	417	381		Cinnzeylanone (**4**)	[9]
21.2	280	327	363	443	Majorenolide	[13]
22.7	300	121			(−)-Borneol-cis-*p*-coumarate	[13]
25.4	336	487	181		Indicol	[4]
25.4	400	339			Epiryanodol (**1**)	[2, 13]

The chamber for aeroponic cultivation of plants (90 l, 1800×1520×820 mm) was located in an environmentally controlled greenhouse (20–30 °C) supplemented with artificial light (16:8, L:D). Vegetative *P. indica* plant replicates (4) were inserted into the plant holders of the aeroponic system and cultivated for twelve months. The roots were sprayed under constant pulverization (every 12 s) with a nutritive solution of 0.2 g/L Nutrichem and 0.03% H2O2 (33% w/v) in water at 26 °C and recollected periodically, when the length was between 20 and 30 cm.

4.3 Extraction and isolation

The mature plant parts were separated, air dried and grounded prior to their extraction in a Sohxlet with ethanol (EtOH) (leaves, 20.7% yield; stems, 10.6 % yield). The aeroponically grown plants were oven dried (40 °C, 48 h) and extracted with ETOH at room temperature (leaves, 21.8% yield; stems, 5.2% yield and roots, 16.5% yield). The cold extracts were filtered and concentrated in vacuo**.**

4.4 HPLC-MS analysis

The separation and identification of compounds was performed with LC-MS/MS instrument (Agilent 1200 LC system with G1322A degasser, G1311A binary pump and Agilent 6410 triplequad MS/MS system), employing electrospray ionization (ESI). For separation, a reversed-phased RP-ODS (Agilent Zorbax 150 x 4.6 mm, 5µm) analytical column fitted with an ODS (5µm) precolumn was used. Separation was performed with a gradient elution binary system composed by acetonitrile (ACN) and 10 mM ammonium acetate at a flow rate of 0.6 mL/min (0 min, 0% B; 1–5 min, 0%–50% B; 5–10 min, 50%–52% B; 10–20 min, 52%–100%; 20–35 min, 100%–65%) and 20 µL of pre-filtered at 0.45 µm sample were injected. A 10 mM ammonium acetate solution was prepared fresh and filtered through Whatman nylon filters (0.45 µm) using a vacuum system and degassed by ultrasound (60 Hz) during 30 min. Separation was performed at room temperature and mass spectra were scanned over the m/z range of 100 to 1000 in the ESI positive ion mode and analysis of all analytes was carried out in MRM mode. The other operating parameters were as follows: nebulizer gas flow, 8 L/min; drying gas flow, 15 L/min; desolvation line (DL) temperature, 330°C; and heat block temperature, 400°C. All chromatographic data were processed using MassHunter software (v 1.10).

Pure compounds previously isolated from *P. indica* were injected at the conditions described above as external standards for identification purposes (see Table 4)**.**

Table 4. Molecular weight and LC-MS m/z adducts of compounds isolated from *P. indica*.

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4.5 Antifeedant Activity

S. littoralis, *M. persicae* and *R. padi* colonies are maintained at ICA-CSIC, reared on artificial diet, bell pepper (*Capsicum annuum*) and barley (*Hordeum vulgare*) plants, respectively, and kept at 22 ± 1 °C and >70% RH, with a photoperiod of 16:8 h (L:D) in a custom-made walk-in growth chamber.

The bioassays were conducted as described [17]. The upper surface of *C. annuum* and *H. vulgare* leaf disks or fragments (1.0 cm2) were treated with 10 µL of the test substance. The extracts and products were tested at an initial dose of 10 or 5 µg/µL (100 or 50 µg/cm2) respectively. A total of 5 to 7 Petri dishes or 20 ventilated plastic boxes (2 × 2 cm) with 2 sixth-instar *S. littoralis* larvae (>24 h after molting) or 10 apterous aphid adults (24–48 h old) each were allowed to feed in a growth chamber (until 75% larval consumption of control disks or 24 h for aphids, environmental conditions as above). Each experiment was repeated 2–3 times. Feeding inhibition or aphid settling was calculated by measuring the disk surface consumption (digitalized with https://imagej.nih.gov/ij/ [18] or by counting the number of aphids on each leaf fragment. Feeding/Settling inhibition (%FI or %SI) was calculated as % FI/SI = [1 − (T/C) × 100], where T and C represent feeding/settling on treated and control leaf disks, respectively. The antifeedant effects (% FI/SI) were analyzed for significance by the nonparametric Wilcoxon paired signed-rank test comparing the consumption/settling between the treatment and control leaf disks. Extracts and compounds with an SI > 70% were further tested in a dose-response experiment (1:2 serial dilutions to cover a range of activities between 100 and <50% feeding inhibition with a minimum of 3 doses) to calculate their effective dose EC_{50} (dose to give a 50% settling reduction) from linear regression analysis (% FI/SI on Log-dose, STATGRAPHICS Centurion XVI, version 16.1.02).

4.6 Nematicidal Bioassay

A *Meloidogyne javanica* population maintained on *Lycopersicon esculentum* plants (var. Marmande) in pot cultures at 25 ± 1 °C, 70% relative humidity has been used in this work. Egg masses of *M. javanica* were hand-picked from infected tomato roots. Second-stage juveniles (J2) were obtained from hatched eggs by incubating egg masses in a water suspension at 25 °C for 24 h. Bio-assays were performed in 96-well plates (BD Falcon, San Jose, CA, USA) as described by Andrés et al. [19]. Extracts and compounds were dissolved in water with 5% of DMSO-Tween solution (0.5% Tween 20 in DMSO), 5 µL of this solution was added to 95 µL of water containing 90–100 nematodes to obtain an initial concentration of 1 mg/mL per well. Treatments were replicated 4 times. As a control, 4 wells were filled with 95 µL of solvent. The plates were covered to prevent evaporation and were maintained in the dark at 25 °C. After 72 h, the dead J2 were counted under a binocular microscope. The nematicidal activity data were presented as percent dead J2s corrected according to Schneider-Orelli's formula [20].

5. Conclusions

The HPLC-MS analysis of *P. indica* extracts showed that the mature tree (wild) leaves had 2 times more chemical diversity than the stems. The aeroponic inmature plants showed lower differences in chemical diversity between leaves and stems, with the lowest diversity found in the roots. Epiryanodol (**1**) was present in all the plant parts, with the mature stems (wild) having the highest amount. The inmature stems (aeroponic) also accumulated ryanoids with higher molecular diversity. The insect *Spodoptera littoralis* was strongly affected by the stem extracts while the leaf ones were moderately active. Based on the predicted vs. the real antifeedant values we conclude that the ryanoid content (epiryanodol **1** or a combination of cinnzeylanol **2**, cinnzeylanone **4** and epiryanodol **1**) explained the antifeedant effects of the stem extracts while additional components contributed to the activity of the leaf ones. Therefore, careful individual selection of *P. indica*

seedlings should be carried out prior to proceed with its aeroponic cultivation in order to obtain ryanodane-rich stem or leaf extracts with strong antifeedant effects on *S. littoralis*.

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