

LC-MS/MS based comparative proteomics of floral nectars reveal different mechanisms involved in floral defense of *Nicotiana* spp., *Petunia hybrida* and *Datura stramonium*

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ABSTRACT

Tobacco floral nectar (FN) is a biological fluid produced by nectaries composed of sugars, amino acids and proteins called nectarins, involved in the floral defense. FN provides an ideal source of nutrients for microorganisms. Understanding the role of nectar proteins is essential to predict impacts in microbial growth, composition and plants-pollinators interactions. Using LC-MS/MS-based comparative proteomic analysis we identified 22 proteins from *P. hybrida*, 35 proteins from *D. stramonium*, and 144 proteins from 23 species of *Nicotiana*. The data are available at ProteomeXchange (PXD014760). GO analysis and secretory signal prediction demonstrated that defense/stress was the largest group of proteins in the genus *Nicotiana*. The *Nicotiana* spp. proteome consisted of 105 exclusive proteins such as lipid transfer proteins (LTPs), Nectar Redox Cycle proteins, protease inhibitors, and PR-proteins. Analysis by taxonomic sections demonstrated that LTPs were most abundant in *Undulatae* and *Noctiflora*, while nectarins were more abundant in *Rusticae*, *Suaveolens*, *Polydicliae*, and *Alata* sections. Peroxidases (Pox) and chitinases (Chit) were exclusive to *P. hybrida*, while *D. stramonium* had only seven unique proteins. Biochemical analysis confirmed these differences. These findings support the hypothesis that, although conserved, there is differential abundance of proteins related to defense/stress which may impact the mechanisms of floral defense.

Significance: This study represents a comparative proteomic analysis of floral nectars of the *Nicotiana* spp. with two correlated *Solanaceous* species. Significant differences were identified between the proteome of taxonomic sections providing relevant insights into the group of proteins related to defense/stress associated with Nectar Redox Cycle, antimicrobial proteins and signaling pathways. The activity of FNs proteins is suggested impact the microbial growth. The knowledge about these proteomes provides significant insights into the diversity of proteins secreted in the nectars and the array of mechanisms used by *Nicotiana* spp. in its floral defense.

1. Introduction

Pollination is one of the important drivers of gene flow and genetic diversity in the plant kingdom. Flowers of most angiosperms, including

Nicotiana spp., produce FN (floral nectar) which is an aqueous secretion produced by a unique floral organ, the nectary. FN is composed primarily of simple sugars, such as sucrose, fructose and glucose, ions and amino acids [1] as well as many other components [2] that pollinators

Abbreviations: FN, floral nectar; HSP, heat shock proteins; LTP, lipid transfer proteins; Nec I, nectarin I; Nec II, nectarin II; Nec III, nectarin III; Nec IV, nectarin IV; Nec V, nectarin V; PI, protease inhibitors; POX, peroxidases; PR, pathogenesis related; XEG, xyloglucanases; XEGIP, xyloglucan-specific fungal endoglucanase inhibitor protein

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feed upon. These solutions are rewards that improve the efficiency of the pollen transfer process.

For several *Nicotiana* species, the composition of sugars and amino acids of FN are related to the preferences of particular types of pollinators [3], (Silva et al. submitted). In addition to these small molecular weight compounds, flowers of *Nicotiana* secrete a variety of proteins in FN that have a role in floral defense against microorganisms [4]. The most abundant proteins are nectarins (Nec I through Nec V) [5,6], which function in coordinated manner, i.e. in the Nectar Redox Cycle [7,8], to produce high levels of antimicrobial compounds, such as hydrogen peroxide [4,7,9,10]. Nec I is a germin-like protein with manganese-superoxide dismutase activity [5,7]. In concert with Nec V, an enzyme in the class of flavin-containing berberine bridge enzymes (BBE), Nec I increases the levels of H₂O₂ in nectar [6,7]. Nec III, another important enzyme in the Nectar Redox Cycle has bifunctional activity as carbonic anhydrase as well as monodehydroascorbate reductase. Its role is to maintain the pH-balance of nectar and to recycle ascorbate [9]. Nec IV, a xyloglucan-specific endoglucanase inhibitor protein (XEGIP), prevents fungal invasion [11]. By interacting with fungal endoglucanases (XEGs), Nec IV binds to Nec V, which, in turn, increases the catalytic activity of Nec V, thereby regulating H₂O₂ levels [12]. In addition to the proteins involved in oxidation-reduction, there are other classes of proteins present in nectar, such as glucosidases, galactosidases, xylosidases, RNases, pathogen-related proteins (e.g. chitinases and thaumatin), and lipid transfer proteins [13].

Despite nectar's central importance in fueling the ecological role provided by pollinators, investigations of the classes of enzymes and proteins found in *Nicotiana* FN remains limited. The goals of such studies are not only to achieve a better understanding of the types of nectar proteins, but to provide new insights into the biochemistry of FN and to form a basis for future studies of plant-pollinator interactions. As an important first step, we have performed a comparative proteomics analysis of FN's from 23 different species of *Nicotiana*, a range of species that covers 10 different sections of the genus. Additionally, we made comparisons with proteomes of two related *Solanaceae* species, *P. hybrida* and *D. stramonium*.

2. Materials and methods

2.1. Chemicals

N α -Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA), chitinase, dimethyl sulfoxide (DMSO), guaiacol, 3-amino-9-ethylcarbazol, ferrous ammonium sulfate, 4-methylumbelliferyl- β -D-N,N',N''-triaceetylchitotrioside [4-MU- β -(GlcNAc)₃], sorbitol, superoxide dismutase, peroxidase type VI-A, triton X-100, nitroterazolum blue chloride (NBT), tetramethylethylenediamine (TEMED), and xylenol orange were obtained from Sigma Aldrich, USA (www.sigmaldrich.com).

2.2. Plant material and growth conditions

Nicotiana spp., were obtained from US Department of Crop Science, North Carolina State University, Raleigh, NC, USA (cals.ncsu.edu/crop-and-soil-sciences/). *Petunia hybrida* and *Datura stramonium* were obtained commercially. Seeds of species were germinated in 4-inch pots in a greenhouse. After two weeks, seedlings were transplanted to individual 30-cm pots containing potting mix. Plants were grown under a daylength of 16 h day/8 h night until they flowered. Standard stages of floral development were as previously described [14]. When flowers reached stage 12, FN was collected following a published protocol [5]. Nectar was stored at -80°C in 1.5 mL microcentrifuge tubes.

2.3. Sample preparation

The nectar proteins were resuspended in 20 μL of 0.05 M ammonium bicarbonate prior to trypsin digestion. This mixture was incubated

for 15 min and then centrifuged ($10,000 \times g$) for 15 s at room temperature ($22 \pm 2^{\circ}\text{C}$). Afterwards the samples were reduced with 5 μL of 0.1 M dithiothreitol, incubated at 37°C for 30 min, and alkylated with 5 μL of 0.3 M iodoacetamide at 37°C for 30 min in darkness. The samples were digested with 2 μg trypsin from Promega, Madison, WI, USA (<https://www.promega.com/>) in 0.05 M ammonium bicarbonate at 37°C for 16 h. At the end of the process the peptide fractions were desalted using a Speed Vac Thermo Fisher Scientific, Bremen, Germany (www.thermofisher.com), and then stored at -80°C until LC-MS/MS analysis.

2.4. Liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS)

The peptides were rehydrated in 100 μL with 2% acetonitrile: 2% formic acid in water. The final peptide mixture was separated on a reverse-phase chromatography system using a Thermo Scientific EASY-nLC II system with a reverse-phase pre-column Magic C-18AQ (100 μm internal diameter, 2 cm length, 5 μm , 100 $\text{\AA}$, Michrom BioResource Inc., Auburn, CA) pre-column and a reverse-phase nano-analytical column Magic C-18AQ (75 μm internal diameter, 15 cm length, 5 μm , 100 $\text{\AA}$, Michrom BioResource Inc.).

The chromatography system was coupled to an LTQ Orbitrap Velos mass spectrometer equipped with a Nanospray II source from Thermo Fisher Scientific (www.thermofisher.com). The mobile phases were as follows: Solvent A - 0.1% formic acid in 2% acetonitrile; Solvent B - 0.1% formic acid in 90% acetonitrile. The equilibration of the 249 bar (5 μL) pre-column and 249 bar (8 μL) nano-column was performed. The samples were analyzed using a 90-min gradient (5% solvent B for 0 min, 45% solvent B for 80 min, 90% solvent B for 2 min, 90% solvent B for 8 min) at a flow rate of 300 nL min^{-1} . Other parameters on the LTQ Orbitrap Velos included: a spray voltage of 2.2 kV; capillary temperature 225°C ; survey MS1 scan range 400–2000 m/z profile mode; resolution 60,000 @ 400 m/z with AGC target 1E6; one micro-scan with maximum inject time 200 ms; Lock mass Siloxane 445.120024 for internal calibration with preview mode for FTMS scans: ON; injection waveforms: ON; monoisotopic precursor selection: ON; rejection of charge state 1. The five most intense ions (charge state 2–4) exceeding 5000 counts were selected for a 7500-resolution high collision dissociation (HCD) (FT MSMS fragmentation scans 2–6). Detection was in profile mode. Dynamic exclusion settings were: repeat count: 2; repeat duration: 15 s; exclusion list size: 500; exclusion duration: 60 s with a 10-ppm mass window. The HCD activation isolation window was: 2 Da; AGC target: 1E5; maximum inject time: 500 ms; activation time: 0.1 ms; activation Q: 0.250 HCD collision energy: 30%.

2.5. Raw data analysis parameters

Raw LC-MS/MS files were converted to Mascot files (MGF) using Proteome Discoverer 1.4, Thermo Fisher Scientific (www.thermofisher.com). Mascot files were processed with PEAKS 6 (Bioinformatics Software Inc.) with Peaks DB and Spider searches enabled against the SWISS-PROT database, the sequences downloaded from UniProtKB, and searches were performed using the *viridiplantae* organism group. The settings used were as follows: instrument type set as FT-ICR/Orbitrap; high energy CID as fragmentation mode; parent ion error tolerance 8 ppm; fragment ion error tolerance 0.03 Da; precursor mass search type: monoisotopic; trypsin as proteolytic enzyme; up to one missed cleavage allowed; carbamidomethylation as a fixed modification; deamidation and oxidation as variable modifications; max variable PTM per peptide: 3.

The identified proteins were loaded, validated, and filtered using the Scaffold Scaffold (v 4.6.0 Proteome Software, Portland, Inc., Portland, OR, United States). The parameters used to accept an identification were as follows: peptide spectrum match (PSM); false discovery rates (FDRs), peptides, and protein levels [15]. All results were

filtered with FDR of $\leq 1\%$, peptide threshold of 95% and a minimum of two peptides per protein identification. The proteins identified in mass spectrometry have been deposited to the ProteomeXchange Consortium via PRIDE [16] partner repository with the dataset identifier PXD014760 and <https://doi.org/10.6019/PXD014760>.

2.6. Protein identification

Basic Local Alignment Search Tool for proteins (BLASTp) was used to annotate the predicted proteins identified by mass spectrometry. The FASTA files generated from Scaffold (v 4.6.0 Proteome Software, Portland, Inc., Portland, OR, United States), containing the amino acid sequences, and all hits for each LC-MS/MS run were blast searched against the Universal Protein Resource (Uniprot) database accessed online (<https://www.uniprot.org/blast/>). The BLAST hits were filtered at a threshold *E*-value $< e-5$. Descriptive names, gene ontology (GO), and annotations were collected. The signal peptide prediction for each identified protein was analyzed using the SignalP 5.0 server (<http://www.cbs.dtu.dk/services/SignalP/>) [17].

2.7. Analysis of FN proteins of *Nicotiana* spp. by taxonomic sections

To perform the analysis by taxonomic section, the proteins were grouped into five different groups related to defense/stress. Protein distribution was performed based on the quantitative values normalized from the total spectrum count.

2.8. Biochemical assays

2.8.1. Protein quantification

Protein concentrations were determined by the dye-binding method [18], with bovine serum albumin (BSA) as the standard.

2.8.2. Hydrogen peroxide assay

Hydrogen peroxide (H_2O_2) in nectar was measured using the FOX reagent [19,20]. Ten microliters of nectar were used in the assay reaction with the Fox reagent which contained sulfuric acid 1.2 mM, xylenol orange 0.1 mM, ferrous ammonium sulfate 0.25 mM and sorbitol 0.1 mM. The H_2O_2 concentration in nectar was determined from a standard curve by measuring the absorbance at 560 nm.

2.8.3. Trypsin proteinase inhibitors assay

Trypsin inhibition activity was measured according method described by Erlanger et al. [21]. Ten microliters of trypsin $0.3 \text{ mg} \cdot \text{mL}^{-1}$ was dissolved in 780 μL Tris-HCL 50 mM, pH 7.5 containing 20 mM CaCl_2 and 10 μL of nectars for 10 min at 37°C . The reaction was started by addition of 500 μL of $1.5 \times 10^{-3} \text{ M}$ *N* α -Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) prepared in 1% (v/v) DMSO in assay buffer. After 15 min at 37°C , the reaction was stopped by addition of 120 μL of 30% (v/v) acetic acid. The enzymatic hydrolysis of the substrate was measured by recording the absorbance at 410 nm. One unit of inhibition (UI) was defined as a decrease of 0.01 absorbance unit compared to the reaction without the inhibitor.

2.8.4. 2Chitinase assay

The chitinase activity was measured as described by Brants and Earle [22] using 25 $\mu\text{g} \cdot \text{mL}^{-1}$ of 4-methylumbelliferyl- β -D-*N,N'*-triacetylchitotrioside [4-MU- β -(GlcNAc) $_3$] as substrate in 0.1 M sodium acetate buffer pH 5.2. Aliquots of 10 μL of nectar were added to 200 μL of substrate and incubated at 30°C for 30 min. Afterward, the reaction was stopped by the addition of 30 μL of the reaction mixture to 1.9 mL of Na_2CO_3 0.2 M buffer. The activity was measured on a Varian Cary Eclipse Fluorescence Spectrophotometer at 360 nm excitation and 450 nm emission wavelengths. One unit of enzyme activity (U) was defined as the amount of enzyme able to release 1 μmol of reducing sugar per minute per microliter of nectar.

2.8.5. Superoxide dismutase activity

For SOD activity in gel was performed according method described by Carter and Thornburg [7]. The proteins were separated on 10% polyacrylamide gels under native conditions. Aliquots 10 μL of nectar were used with application buffer, (25 mM Tris-HCL, 190 mM glycine, pH 8.3) in the ratio 1:1 (v/v) and subjected to electrophoresis (125 V, 20 mA, and 10 W per gel). Gels were developed for SOD activity according to Beauchamp and Fridovich [23]. Gels were incubated in 50 mL of 2 mM NBT for 15 min, washed, and incubated in the dark in 50 mL of 100 mM potassium phosphate buffer, pH 7.0, containing 28 mM TEMED and riboflavin 0.1 mg/mL, for 15 min. The gels were washed in distilled water and exposed in the light (30 μmol of photons $\text{m}^{-2} \text{ s}^{-1}$) for induction of the photochemical reaction.

2.8.6. Peroxidase assay

Peroxidase enzymatic activity was determined according to Caruso et al. [24]. After electrophoresis the gels were incubated in 0.05 M sodium acetate buffer, pH 5.2 for 30 min at 25°C . The peroxidase activity was revealed by incubating the gel in 0.05 M sodium acetate buffer, pH 5.2 containing 0.2% (v/v) guaiacol, 0.01% 3-amino-9-ethylcarbazole (m/v), and 0.03% (v/v) H_2O_2 . The reaction was stopped by washing the gels with distilled water.

2.9. Phylogenetic analysis

The amino acid sequences corresponding to mature LTPs proteins were aligned and phylogenetic tree constructed in MEGA 6 software using the Neighbor-Joining algorithm with bootstrap test of phylogeny performed with 1.000 repetitions [25].

2.10. Statistical analysis

The experiments were conducted using nectars sampled from multiple flowers from three different plants, i.e. three independent biological replicates. The data were evaluated using a one-way ANOVA test. The differences between species were analyzed using a Student's test at $p < .05$ significance level.

3. Results

3.1. Proteomic profile of floral nectar

We identified 144 nectar associated proteins in this study from 23 different species across 10 taxonomic sections of the *Nicotiana* (Table 1). The identified proteins are listed in Table 2, and Supplemental File 1. We were able to group them into six major functional classes as shown in Fig. 1A. These included cell structure, cellular homeostasis, defense/stress, metabolism, protein processing, and signaling. In addition, one *Nicotiana quadrivalvis* protein was not identified and therefore has an unknown function. In addition to the 144 *Nicotiana* spp. proteins identified, the proteomic analysis identified 22 different proteins in the nectar of *P. hybrida* and 35 proteins in the nectar of *D. stramonium* (Tables 3, 4 and Supplemental File 1).

3.2. Metabolism

The GO analysis demonstrated that the largest group of proteomic biological functions with the nectar proteins was related to metabolism. In *Nicotiana* spp. these were 45.6% of all proteins were related to metabolism, while in *P. hybrida* 54.5%, and in *D. stramonium* 65.7% of proteins had metabolic functions (Fig. 1A). These proteins operate in many different pathways inside the cell. The dominant group of metabolic enzymes were those in the glycolytic pathway. $> 1/3$ of FN proteins from *Nicotiana* spp. (23 proteins) were glycolysis related. In *P. hybrida* and *D. stramonium*, there were 12 (56.2%) and 5 (41.7%) glycolytic pathway-related proteins, respectively (Fig. 1B-C; Supplemental

Table 1List of *Nicotiana* and others *Solanaceous* species used in the proteomic study.

Number	Section ^a	Species	Pollination syndrome	Reference
1	<i>Undulataea</i>	<i>N. glutinosa</i>	Moth, butterflies	(Anon 1972–2017) [26]
2	<i>Noctiflorae</i>	<i>N. glauca</i>	Birds	(Ollerton et al., [27])
3		<i>N. noctiflora</i>	Moth, butterflies	(Anon 1972–2017) [28]
4	<i>Paniculatae</i>	<i>N. benavidesii</i>	Bee, butterflies	(Anon 1972–2017) [29]
5		<i>N. paniculata</i>	Hummingbird	(Anon 1972–2017) [30]
6	<i>Rusticae</i>	<i>N. rustica</i>	Moth, bee	(Raguso et al.,; Tiedge and Luhaus, [31,3])
7	<i>Repandae</i>	<i>N. repanda</i>	Moth, butterflies	(Anon 1972–2017) [32]
8	<i>Sylvestres</i>	<i>N. sylvestris</i>	Hawkmoth	(Mahr [33])
9	<i>Suaveolentes</i>	<i>N. maritima</i>	Moth, bee	(Anon 1972–2017) [34]
10		<i>N. gossei</i>	Moth, butterflies	(Anon 1972–2017) [35]
11		<i>N. africana</i>	Sunbird	(Marlin et al., [36])
12		<i>N. benthamiana</i>	Autogamus	(Adler et al., [37])
13	<i>Nicotianae</i>	<i>N. tabacum (Xan)</i>	Hummingbird	(Tiedge and Lohaus 2017) [3]
14		<i>N. tabacum (Sam)</i>	Hummingbird	(Tiedge and Lohaus [3])
15	<i>Polydichiae</i>	<i>N. quadrivalvis</i>	Moth, butterflies	(Anon 1972–2017) [38]
16		<i>N. clelandii</i>	Moth, bee, other	(Anon 1972–2017) [39]
17	<i>Alatae</i>	<i>N. longiflora</i>	Hawkmoth	(Raguso et al., [31])
18		<i>N. forgetiana</i>	Hummingbird	(Anon 1972–2017) [40]
19		<i>N. langsdorffii</i>	Hummingbird, bee	(Kaczorowski et al., [41])
20		<i>N. bonariensis</i>	Small moth	(Kaczorowski et al., [41])
21		<i>N. plumbaginifolia</i>	Hawkmoth, autogamus	(Kaczorowski et al., [41])
22		<i>N. alata</i>	Hawkmoth	(Kaczorowski et al., [41])
23		<i>N. sanderae</i>	Moths, butterflies	(Anon 1972–2017) [42]
24	<i>Petunia^b</i>	<i>P. hybrida</i>	Hawkmoth, bees	(Stuurman et al., [43])
25	<i>Datura^c</i>	<i>D. stramonium</i>	Hawkmoth	(Raguso et al., [31])

^a Taxonomic section of genus *Nicotiana*.^b Genus *Petunia* (not *Nicotiana*).^c Genus *Datura* (not *Nicotiana*).

File 2). Additional proteins were also identified from the citric acid cycle, as well as from the pentose phosphate pathway. The presence of so many proteins from these central metabolic pathways is consistent with the hypothesis [10] that cell death and nectary cell disruption is a vital process occurring during nectar secretion in *Nicotiana* spp.

Other metabolic processes that were represented include amino acid metabolism and lipid metabolism. Energy metabolism was represented by a significant number of proteins in *Nicotiana* (19.4%), *P. hybrida* (33.3%) and *D. stramonium* (13.0%) (Fig. 1B-C; Supplemental File 2). These proteins were mostly mitochondrial ATP synthase subunits and chloroplastic RUBISCO subunits. We also found one enzyme involved in single-carbon metabolism: S-adenosylhomocysteinase in nectar of *Nicotiana* spp. and *D. stramonium*.

3.3. Defense/Stress

The proteins related to Nectar Redox Cycle, a biochemical pathway that generates high levels of hydrogen peroxide in tobacco FN and serves to maintain nectar in an axenic state, represented 8.3% total of proteins identified in *Nicotiana* spp. nectars [8]. Overall, the defense/stress proteins represented 34% of proteins, of these, 22% were lipid transfer proteins (LTPs). A large number of proteinase inhibitor proteins were also identified including multi-domain versions of proteinase inhibitor II and at least two Kunitz-type proteinase inhibitors. In addition, an RNase was also identified. As previously observed [20], *Petunia hybrida* had low numbers of Nectar Redox Cycle-associated proteins. Instead, there was an abundance (66.7%) of PR-proteins, including acid chitinases, osmotins and peroxidases (Table 3; Supplemental File 2). *Datura stramonium* nectar lacked both Nectar Redox Cycle-associated proteins and PR-proteins.

3.4. Other

A diversity of other proteins were found. Several signaling proteins – 14-3-3 proteins, calreticulin, calmodulin, GPI-anchored protein were detected in nectar of solanaceous species. Two different types of

proteins involved in cellular homeostasis were identified in nectars of *Nicotiana* spp. The first group were actins and profilins that are involved in vesicle transport (Table 2; Fig. 1B-C). The second group was a pair of V-type ATPases related to vacuolar pH maintenance. Two isoforms of actins and one V-type ATPase were found in FN of *D. stramonium* (Table 4). *Petunia hybrida* nectar lacked actins and profilins. Plant cell wall proteins, such as expansins and extensins, were identified exclusively in the nectar of *Nicotiana* spp. (Table 2). Finally, we identified a few proteins involved in protein translation (elongation factors and an RNA binding glycine-rich protein), as well as a number of chaperone proteins [44], including HSP-60 (3 proteins), HSP-70 (4 proteins) and HSP-90 (1 protein) (Table 2). Chaperone proteins were also identified in nectar of *P. hybrida* (HSP-90) and *D. stramonium* (HSP-60), (HSP-70) and (HSP-90) (Tables 3, 4).

3.5. Prediction of proteins secreted in the nectars

This proteomic analysis identified several proteins with primary roles in cellular metabolism that are normally localized in different subcellular locations. The analysis of secretory signal peptide predictions showed that 57 *Nicotiana* spp. nectar proteins were targeted to the secretory pathway via secretory signal peptides (Fig. 1D-E; Supplemental File. 2, 3). In *P. hybrida* and *D. stramonium* the signal peptides were predicted only to seven proteins (Fig. 1D). The secretory signal peptide prediction showed that when analyzed by biological function, about 90% or 43 of the proteins secreted into the nectars of *Nicotiana* spp. were related to defense/stress (Fig. 1E). Similar results were found for the nectar of *P. hybrida* in which 7 proteins were defense/stress-related (~ 83%), while most of the secreted proteins in the nectar of *D. stramonium* were related to the metabolism. A number of *Nicotiana* spp. nectar proteins appear to lack predicted signal peptides, we make two observations on this fact. First, we have observed that cell death and cellular disruption is an important process during nectar secretion [10]. Second, it is clear from the study of plant secretomes [45] that not all secreted proteins have signal peptides; therefore, proteins that lack signal peptides can still appear in the plant secretomes via a number of

Table 2
Summary of total identified proteins in floral nectar of *Nicotiana spp.*

Biological function	N°	Protein identified	Access number (Uniprot)	Organism
Cell structure	1	Annexin	O24132_TOBAC	<i>Nicotiana tabacum</i>
	2	Beta-expansin-like	Q9AXE4_TOBAC	<i>Nicotiana tabacum</i>
	3	Pistil extensin-like protein	Q40385_NICAL	<i>Nicotiana attenuata</i>
	4	Proline-rich protein	Q07894_NICAL	<i>Nicotiana attenuata</i>
Cellular homeostasis	5	V-type proton ATPase subunit B2	A0A0V0IFF9_SOLCH	<i>Solanum chacoense</i>
	6	V-type ATPase, V1 domain	A0A059BXG9_EUCGR	<i>Eucalyptus grandis</i>
	7	Actin	A0A0A0KJ21_CUCSA	<i>Cucumis sativus</i>
	8	Actin	Q7DMB2_PEA	<i>Pisum sativum</i>
	9	Actin 1	ACT1_SORBI	<i>Sorgo bicolor</i>
	10	Profilin-2	PROF2_TOBAC	<i>Nicotiana tabacum</i>
	11	Profilin	A0A103Y2A9_CYNCS	<i>Cynara cardunculus</i>
Defense/Stress	12	Lipid-transfer protein	Q6E0V0_NICGL	<i>Nicotiana glauca</i>
	13	Lipid-transfer protein	Q42952_NLTP1_TOBAC	<i>Nicotiana tabacum</i>
	14	Lipid-transfer protein	M1AZ37_SOLTU	<i>Solanum tuberosum</i>
	15	Lipid-transfer protein	A0A0K0K6K1_NICAT	<i>Nicotiana attenuata</i>
	16	Non-specific lipid-transfer protein	F2ZAM1_TOBAC	<i>Nicotiana tabacum</i>
	17	Non-specific lipid-transfer protein	B3A0N2_NLTP_LYCBA	<i>Lycium barbarum</i>
	18	Non-specific lipid-transfer protein	A0A0V0HEK2_SOLCH	<i>Solanum chacoense</i>
	19	Non-specific lipid-transfer protein	F2ZAM0_TOBAC	<i>Nicotiana tabacum</i>
	20	Non-specific lipid-transfer protein	Q2VT51_CAPAN	<i>Capsicum annuum</i>
	21	Non-specific lipid-transfer protein	Q8LK72_TOBAC	<i>Nicotiana tabacum</i>
	22	Non-specific lipid-transfer protein	Q5QJ48_NICAT	<i>Nicotiana attenuata</i>
	23	Berberine bridge enzyme-like 12	BBE12_ARATH	<i>Arabidopsis thaliana</i>
	24	Nectarin 1b	A0A0K0K6J6_NICAT	<i>Nicotiana attenuata</i>
	25	Nectarin 5	Q84N20_NICLS	<i>LxS</i>
	26	Nectarin 5	Q84N21_NICLS	<i>LxS</i>
	27	Nectarin 5b	A0A0K0K5B3_NICAT	<i>Nicotiana attenuata</i>
	28	Nectarin 5a	A0A0K0K5B2_NICAT	<i>Nicotiana attenuata</i>
	29	Nec5-like protein	A0A0K9QR43_SPIOL	<i>Spinacia oleracea</i>
	30	Nec5-like protein	A0A022PTH7_ERYGU	<i>Erythrante guttata</i>
	31	Nec5-like protein	A0A0K9RUR9_SPIOL	<i>Spinacia oleracea</i>
	32	Nec5-like protein	M5X476_PRUPE	<i>Prunus persica</i>
	33	Nec5 like protein	M1D3J3_SOLTU	<i>Solanum tuberosum</i>
	34	Nec5-like protein	A0A067G6J1_CITSI	<i>Citrus sinensis</i>
	35	Acidic endochitinase Q	CHIQ_TOBAC	<i>Nicotiana tabacum</i>
	36	Acidic endochitinase P	CHIP_TOBAC	<i>Nicotiana tabacum</i>
	37	Class IV chitinase	A1IJ67_TOBAC	<i>Nicotiana tabacum</i>
	38	Glucan endo-1,3-beta-glucosidase	E13C_TOBAC	<i>Nicotiana tabacum</i>
	39	Osmotin-like protein	A0A075E2S9_TOBAC	<i>Nicotiana tabacum</i>
	40	Osmotin/thaumatococin-like	B2CZJ9_CAPAN	<i>Capsicum annuum</i>
	41	Osmotin like protein	A0A068UL43_COFCA	<i>Coffea canephora</i>
	42	Miraculin-like protein	A0A097BU08_TOBAC	<i>Nicotiana tabacum</i>
	43	Subtilisin-like serine protease SBT1.5	A0A0A1CNP8_TOBAC	<i>Nicotiana tabacum</i>
	44	Aspartic proteinase like protein-1	A0A0V0IFS7_SOLCH	<i>Solanum chacoense</i>
	45	Carboxypeptidase	E0CJP2_TOBAC	<i>Nicotiana tabacum</i>
	46	Kunitz proteinase inhibitor	Q850R9_NICGU	<i>Nicotiana glutinosa</i>
	47	Kunitz-like inhibitor	K4BJT7_SOLLC	<i>Solanum lycopersicum</i>
	48	Proteinase inhibitor type II NGPI-2	Q9SDW7_NICGU	<i>Nicotiana glutinosa</i>
	49	Proteinase inhibitor II	B3F0C1_TOBAC	<i>Nicotiana tabacum</i>
	50	Six domain proteinase inhibitor	Q2XVY5_TOBAC	<i>Nicotiana tabacum</i>
	51	PI_II (5 domain inhibitor)	Q1WL41_9SOLA	<i>Nicotiana spegazzinii</i>
	52	PI_II (6 domain inhibitor)	Q1WL40_9SOLA	<i>Nicotiana miersii</i>
	53	PI_II (8 domain inhibitor)	Q1WL31_9SOLA	<i>Nicotiana obitusifolia</i>
	54	PI_II (6 domain inhibitor)	Q1WL45_9SOLA	<i>Nicotiana repanda</i>
	55	Catalase	A0A0V0IEQ7_SOLCH	<i>Solanum chacoense</i>
	56	Thioredoxin peroxidase 1	A0A077D9N5_TOBAC	<i>Nicotiana tabacum</i>
	57	SA2-RNase	Q40384_NICAL	<i>Nicotiana alata</i>
	58	Nectarin 4	Q3KU27_NICLS	<i>LxS</i>
	59	Xyloglucan-specific fungal endoglucanase inhibitor protein	A0A077DBL0_TOBAC	<i>Nicotiana tabacum</i>

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Table 2 (continued)

Biological function	N°	Protein identified	Access number (Uniprot)	Organism
Metabolism	60	Alanine aminotransferase	Q6VEJ5, CAPAN	<i>Capsicum annuum</i>
	61	Putative methionine synthase-like	A0A0V0ISV3, SOLCH	<i>Solanum cacheense</i>
	62	Threonine dehydratase	Q9AXU4, NICAT	<i>Nicotiana attenuata</i>
	63	Methionine synthase	A0A0L9VN25, PHAAN	<i>Phaseolus angularis</i>
	64	Pollen allergen MetE	E3VW74, AMARE	<i>Amaranthus retroflexus</i>
	65	Endoglucanase	Q9XF22, NICAL	<i>Nicotiana alata</i>
	66	Alpha-galactosidase	F5BFT0, TOBAC	<i>Nicotiana tabacum</i>
	67	Alpha-L-Arabinofuranosidase/beta-D-Xylopyranosidase	A0A0A8JBT3, TOBAC	<i>Nicotiana tabacum</i>
	68	Beta-D-glucan exohydrolase	O82151, TOBAC	<i>Nicotiana tabacum</i>
	69	Putative beta-xylosidase/alpha-L-arabinofuranosidase 2-like	A0A0V0IT87, SOLCH	<i>Solanum cacheense</i>
	70	Sucrose synthase	B3F8H6, NICLS	<i>LxS</i>
	71	UDP-glucose 6-dehydrogenase	Q6IVK6, TOBAC	<i>Nicotiana tabacum</i>
	72	Xyloglucan endotransglucosylase/hydrolase	C0IRG9, ACTDE	<i>Actinidia deliciosa</i>
	73	UTP-glucose-1-phosphate-uridylyltransferase	A0A103Y5R9, CYNCS	<i>Cynara cardunculus</i>
	74	Beta glucosidase	K4B3H0, SOLLC	<i>Solanum lycopersicum</i>
	75	UTP-glucose-1-phosphate-uridylyltransferase	K4C285, SOLLC	<i>Solanum lycopersicum</i>
	76	Fructokinase	A0A068JCD7, TOBAC	<i>Nicotiana tabacum</i>
	77	Malate dehydrogenase	A0A075F1V0, TOBAC	<i>Nicotiana tabacum</i>
	78	Malate dehydrogenase	A0A0V0HZC4, SOLCH	<i>Solanum cacheense</i>
	79	Aconitate hydratase	Q9FVE9, TOBAC	<i>Nicotiana tabacum</i>
	80	ATP synthase subunit alpha	Q5M9V4, TOBAC	<i>Nicotiana tabacum</i>
	81	ATP synthase subunit alpha	H8XXL6, SILVU	<i>Silene vulgaris</i>
	82	ATP synthase subunit alpha	A0A059Q6J8, HYONI	<i>Hyoscyamus niger</i>
	83	ATP synthase subunit beta	M0U2D0, MUSAM	<i>Musa acuminata</i>
	84	ATP synthase subunit beta	A0A0R4J4C8, SOYBN	<i>Glycine max</i>
	85	ATP synthase subunit beta	A0A023JGH0, 9LILI	<i>Amana edulis</i>
	86	ATP synthase subunit beta	A0A0X8HNP4, 9MAGN	<i>Sabia yunnanensis</i>
	87	ATP synthase subunit beta	A0A023HN67, 9MAGN	<i>Sabia yunnanensis</i>
	88	Ribulose biphosphate carboxylase large chain	RBL, NOLSP	<i>Nolana spathulata</i>
	89	Ribulose biphosphate carboxylase large chain	I3NTM6, 9SOLA	<i>Petunia axillares</i>
	90	Ribulose biphosphate carboxylase large chain	B8X871, 9SPER	<i>Araucaria biramulata</i>
	91	Ribulose biphosphate carboxylase large chain	Q06QN1, 9LAMI	<i>Picconia excelsa</i>
	92	Ribulose biphosphate carboxylase large chain	C7A616, 9ROSI	<i>Sclerolimon digynum</i>
	93	Enolase	C5J0G6, TOBAC	<i>Nicotiana tabacum</i>
	94	Enolase	Q9M434, LUPLU	<i>Lupinus luteus</i>
	95	Enolase	M0ZSD6, SOLTU	<i>Solanum tuberosum</i>
	96	Enolase	A0A164WJJ6, DAUCA	<i>Daucus carota</i>
	97	Fructose-bisphosphate aldolase	Q2PYX3, SOLTU	<i>Solanum tuberosum</i>
	98	Fructose-bisphosphate aldolase	K4D3E4, SOLLC	<i>Solanum lycopersicum</i>
	99	Fructose-bisphosphate aldolase	A0A022RI82, ERYGU	<i>Erythranthe guttata</i>
	100	Fructose-bisphosphate aldolase	G9MA91, 9ROSI	<i>Linum grandiflorum</i>
	101	Fructose-bisphosphate aldolase	Q9SXX4, NICPA	<i>Nicotiana paniculata</i>
	102	Glyceraldehyde-3-phosphate dehydrogenase	A0A0L9UB16, PHAAN	<i>Phaseolus angularis</i>
	103	Glyceraldehyde-3-phosphate dehydrogenase	A0A061FXI2, THECC	<i>Theobroma cacao</i>
	104	Glyceraldehyde-3-phosphate dehydrogenase	U3PLJ3, 9FABA	<i>Polygala fruticosa</i>
	105	Glyceraldehyde-3-phosphate dehydrogenase	Q9SSU3, CUCME	<i>Cucumis melo</i>
	106	Glyceraldehyde-3-phosphate dehydrogenase	A0A0F7JLU6, NICBE	<i>Nicotiana benthamiana</i>
	107	Glyceraldehyde-3-phosphate dehydrogenase	I0DG17, 9ASPA	<i>Phalaenopsis hybrid</i>
	108	Glyceraldehyde-3-phosphate dehydrogenase	W9R8N5, 9ROSA	<i>Morus notabilis</i>
	109	Glyceraldehyde-3-phosphate dehydrogenase	A0A0V0HU14, SOLCH	<i>Solanum cacheense</i>
	110	Phosphoglycerate kinase	PGKY, TOBAC	<i>Nicotiana tabacum</i>
	111	Phosphoglycerate kinase	M4SKS4, ATRBE	<i>Atropa belladonna</i>
	112	Phosphoglycerate kinase	K4CHY4, SOLLC	<i>Solanum lycopersicum</i>
	113	Phosphoglycerate kinase	A0A0R0IRH4, SOYBN	<i>Glycine max</i>
	114	Triosephosphate isomerase	A0A068JFR6, TOBAC	<i>Nicotiana tabacum</i>
	115	Triosephosphate isomerase	W1PJM9, AMBTC	<i>Amborella trichopoda</i>
	116	Putative lipoxygenase homology domain-containing protein 1-like	A0A0V0HLG7, SOLCH	<i>Solanum cacheense</i>
	117	epoxide hydrolase	A0A0V0GSE7, SOLCH	<i>Solanum cacheense</i>
	118	Adenosylhomocysteinase	B8LKF8, PICSI	<i>Picea sitchensis</i>
	119	Adenosylhomocysteinase	A1KYB0, TOBAC	<i>Nicotiana tabacum</i>
	120	Adenosylhomocysteinase	S8EJ11, 9LAMI	<i>Genlisea aurea</i>
	121	Serine hydroxymethyltransferase	A0A0V0I7X4, SOLCH	<i>Solanum cacheense</i>
	122	Serine hydroxymethyltransferase	A0A022QS28, ERYGU	<i>Erythranthe guttata</i>
	123	Transketolase	C3RXI5, TOBAC	<i>Nicotiana tabacum</i>
	124	Transketolase	A1BQW9, NICAT	<i>Nicotiana attenuata</i>
	125	Transaldolase	A0A068VDR8, COFCA	<i>Coffea canephora</i>
	126	Spermidine synthase	A8R0T1, NICBE	<i>Nicotiana benthamiana</i>

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Table 2 (continued)

Biological function	N°	Protein identified	Access number (Uniprot)	Organism
Protein process	127	chaperonin 60 subunit beta 2, chloroplastic	A0A078CKW7_BRANA	<i>Brassica napus</i>
	128	Heat shock protein 70	A4ZX75_9ERIC	<i>Cyclamen persicum</i>
	129	Heat shock cognate 70 kDa like protein	A0A0F7CS07_9ROSI	<i>Betula luminifera</i>
	130	Heat shock protein 70	C7EPE8_ULVPR	<i>Ulva prolifera</i>
	131	Heat shock protein 90-3	A0A0M3SBS3_NICBE	<i>Nicotiana benthamiana</i>
	132	Putative dnaK-type molecular chaperone hsc70.1	A7Y710_PRUDU	<i>Prunus dulcis</i>
	133	Cpn60-like protein	K4DAD5_SOLLC	<i>Solanum lycopersicum</i>
	134	Chaperoning 60-like protein	A0A059D5S8_EUCGR	<i>Eucalyptus grandis</i>
	135	Elongation factor 1-alpha	A0A0J8D0N7_BETVU	<i>Beta vulgaris</i>
	136	Elongation factor 1 alpha	M0VS48_HORVD	<i>Hordeum vulgare</i>
	137	RNA-binding glycine-rich protein-1c	Q40425_NICSY	<i>Nicotiana sylvestris</i>
	138	Putative calcium and calcium/calmodulin-dependent serine/threonine-protein kinase-like	A0A0V0IBF3_SOLCH	<i>Solanum cacheense</i>
	139	Putative calreticulin-like	A0A0V0IBE5_SOLCH	<i>Solanum cacheense</i>
Signaling	140	Putative lysM domain-containing GPI-anchored protein 1-like	A0A0V0I620_SOLCH	<i>Solanum cacheense</i>
	141	14-3-3-like protein C	1433C_TOBAC	<i>Nicotiana tabacum</i>
	142	14-3-3 h-1 protein H1	Q75ZD6_TOBAC	<i>Nicotiana tabacum</i>
	143	14-3-3 a-1 protein A1	Q75ZE5_TOBAC	<i>Nicotiana tabacum</i>
	144	Uncharacterized protein	B9GT92_POPTR	<i>Populus trichocarpa</i>

processes.

3.6. Identification of differentially expressed proteins in nectar of *Nicotiana* spp., *P. hybrida* and *D. stramonium*

To gain a better understanding of nectar proteins among the three solanaceous genera that we evaluated, we performed a Venn diagram analysis (Fig. 2). The analysis revealed that 105 proteins were exclusive to *Nicotiana* spp. proteome, including groups of proteins related to defense/stress such as LTPs, Nectar Redox Cycle and proteinase inhibitors (Fig. 2). Between *Nicotiana* spp. and *P. hybrida* nectars, 16 shared proteins were identified, and six were unique. These unique proteins were related to defense/stress (chitinases, osmotins and two peroxidases), metabolism (alpha-L-arabinofuranosidase) and signaling (14.3.3). A few proteins (28) were common in the nectar of *Nicotiana* spp. and *D. stramonium*. Seven proteins related to cellular homeostasis, defense/stress and metabolism were exclusive to the *D. stramonium* nectar. Five proteins from three different biological functions were common to the proteomes of *Nicotiana* spp., *P. hybrida* and *D. stramonium*. These included ATP synthase subunit beta, fructose biphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, HSP-90 and putative calcium calmodulin-dependent serine/threonine.

3.7. Distribution of proteins related defense/stress of *Nicotiana* spp. nectar by sections

Proteomic analysis of *Nicotiana* spp. nectars has identified numerous proteins related to defense/stress among the species (Supplemental File 1). The proteomic diversity showed section-specificity as well as a striking variation in the abundance of these proteins content between species (Supplemental File 4). Among the 10 sections of the genus that we sampled, we identified five different groups of species that displayed similar defense/stress proteins (Fig. 3A-D and 4). LTPs were one of the most abundant groups of proteins in the nectar of *Rusticae*, *Repandae*, *Suaveolentes*, *Polydicliae* and *Alatae* sections (Fig. 3A). To further examine the expression of LTPs in tobacco nectar, we conducted a phylogenetic analysis of LTPs among the genus. Using BLASTp analysis of the NCBI databases, we identified 274 LTP sequences. Following alignment, a phylogenetic tree was constructed using the NJ (Neighbor-Joining) algorithm as described in Materials and Methods. Subsequently the display of this tree (Supplemental File 5) showed that these proteins form a large, related phylogeny. Sequences that were identified by Mass Spectroscopy were highlighted in red on this phylogenetic tree. As can be seen, half of these identified proteins group together in one clade of this tree (the red circle), suggesting that other sequences among this highlighted group should perhaps be examined for nectar

expression.

Proteins associated with Nectar Redox Cycle were also prevalent in the nectar of four sections: *Rusticae*, *Suaveolentes*, *Polydicliae* and *Alatae* (Figs. 3B and 4). Nec IV was a particularly abundant protein in the nectar of the section *Suaveolens*. The aspartic proteinase-like protein 1 was widely identified in the nectars of *Noctiflorae*, *Paniculatae*, *Rusticae*, *Suaveolentes* and *Nicotianae* sections (Fig. 4). We also identified PR-proteins as other group of proteins with wide distribution in nectars. Among the PR-proteins, osmotins had high prevalence in nectars of *Paniculatae* section, whereas chitinases and protease inhibitors were abundant in the species of the *Nicotianae* and *Alatae* sections (Fig. 3C-D).

3.8. Enzymatic biochemistry of nectars

We also examined nectars for expression of several enzymatic activities (Supplemental File 6). Hydrogen peroxide was found to be important in several species, especially among the *Alatae*, but also including several unique species including *N. goseii* and *N. clevelandii*. Hydrogen peroxide was not found at significant levels in either *D. stramonium* or *P. hybrida*, confirming previous results [20,47]. Trypsin proteinase inhibitor (TPI) activity was found at moderate levels throughout all species examined. *N. repanda* and the species of section *Polydicliae*, *N. quadrivalvis*, and *N. clevelandii* showed the highest levels of TPI in their nectar. Chitinase was found to be widely expressed in many of the species examined. Highest levels were found in *N. bonariensis* and *N. langsdorffii* while lowest levels were found in *N. longiflora* and *N. sylvestris*, *N. glutinosa*, and *N. forgentiana*; however, specific patterns were not observed to correlate with various sections of the genus *Nicotiana*. The other genera, *Petunia* and *Datura*, showed moderate levels of Chitinase. Finally, using an in-gel assays, we examined nectars for the presence of superoxide dismutase (SOD) and peroxidase (POX). The Nectarin I protein, Nec I, was originally identified as a germin-like superoxide dismutase [7] so examination of nectars for SOD activity examines for the presence of Nec I. As can be seen in Fig. 5, SOD activity is widely expressed among the *Nicotianae*. These levels of SOD activity correlated well with the levels of hydrogen peroxide found (However, little activity was found in either *Datura stramonium* or *Petunia hybrida*). Further, we examined peroxidase activity in these same species. Although we did not identify peroxidase activity in the *Nicotianae* (with the exception of *N. tabacum*) we did identify peroxidase activity in *P. hybrida* nectar. *D. stramonium* nectar showed little peroxidase activity. Other proteins with high abundance included the anti-fungal protein, Nec IV, in nectar of *Suaveolentes* species and an aspartic proteinase (Asp1) in the nectar of species of *Alatae* section (Fig. 4).

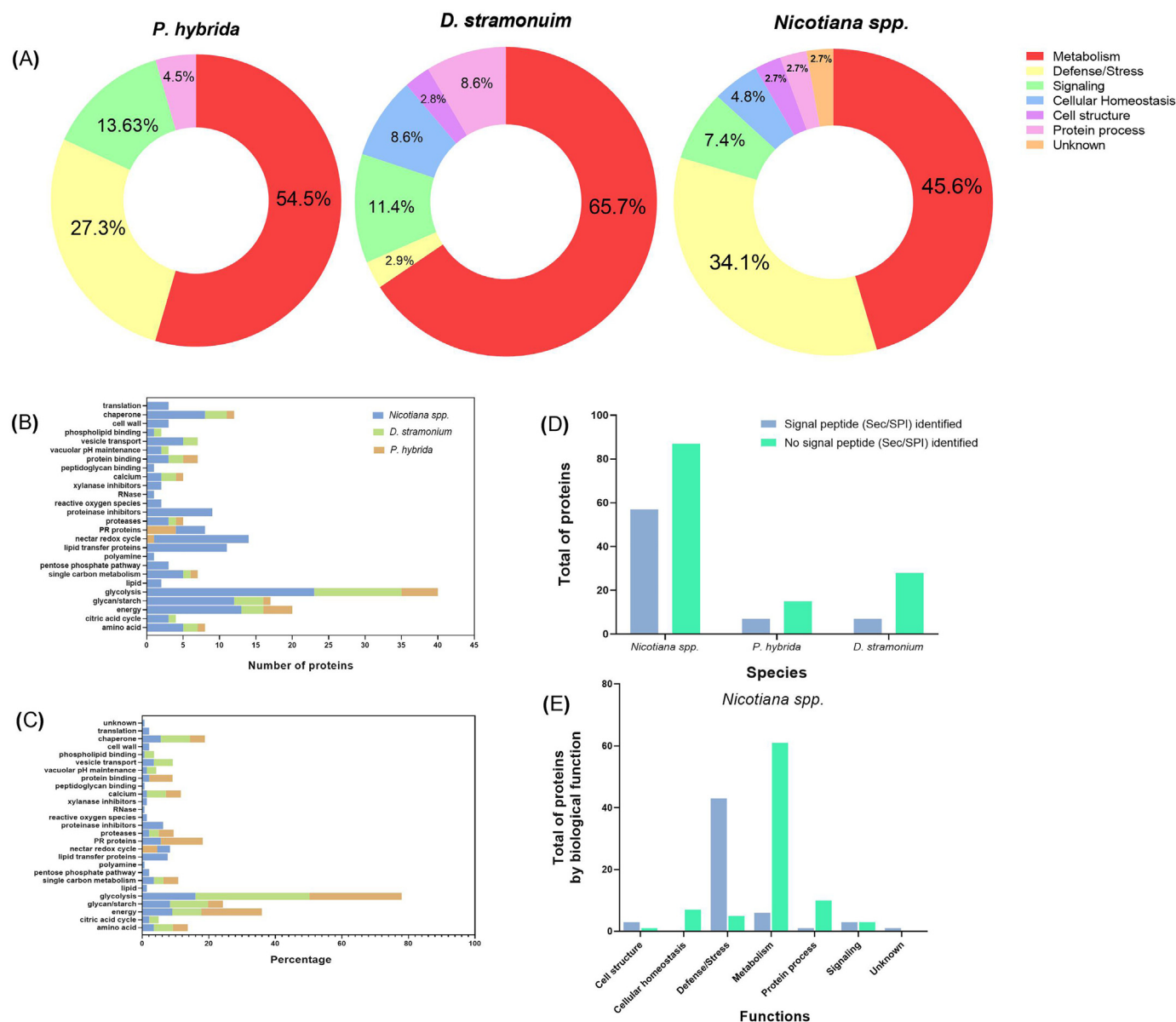


Fig. 1. Gene Ontology (GO) of proteins secreted in the nectars of *Nicotiana spp.*, *P. hybrida* and *D. stramonium* according to the biological functions, molecular process and secretion prediction. (A) The pie charts represent the classification of 22, 35, and 144 nectar proteins respectively of *P. hybrida*, *D. stramonium* and *Nicotiana spp.* floral nectars identified by LC-MS/MS. (B-C) Molecular function of floral nectar proteins of *Nicotiana spp.*, *P. hybrida* and *D. stramonium*. (B) Sum of total of proteins highlighting the contribution of each molecular function. (C) Percentage of proteins by molecular function. (D-E) Signal peptide secretion prediction of floral proteins identified by LC-MS/MS. (D) Secretion prediction of proteins from *Nicotiana spp.*, *P. hybrida* and *D. stramonium*. (E) Secretion prediction of proteins from *Nicotiana spp.* distributed by biological functions showing the large number of secreted proteins in floral nectar related to defense/stress. Software SignalP5.0 was used to analyze the different signal peptide sequences of identified proteins. Sec/SPI mean the standard secretory signal peptides transported by the Sec translocation and cleaved by Signal Peptidase I.

4. Discussion

4.1. 4.1.1. Floral nectar proteins

Floral nectar is a complex and nutritive fluid secreted by flower-associated nectaries to attract pollinators [43,46]. The presence of proteins in nectars [5,7] has led to a better understanding of the biochemistry of floral nectar [4,5,8,12,92]. Our study of the proteomes of 23 species of *Nicotiana*, as well as closely related genera, *Petunia* and *Datura* nectars revealed significant differences between the species. These plants secrete an array of nectar proteins that modify the biochemistry of floral nectar. Of the total of 144 proteins that we identified in floral nectar of the *Nicotiana spp.*, most were targeted for secretion and associated with defense/stress supporting the hypothesis that the

major function of proteins secreted into of *Nicotiana spp.* nectars play a role in floral defense [4,10].

In addition, several proteins identified from our proteomic analysis have primary roles in metabolism and are normally localized in plastids or cytoplasm. Nectar proteins, secreted from the nectaries, are expected to be targeted to the secretory pathway via secretory signal peptides [47]. The proteins identified in floral nectar related to primary metabolism were involved in glycolysis, citric acid cycle, or pentose phosphate pathways. The identification of several key proteins of central metabolism suggests that nectary cell death and spilling of cellular contents may be a significant event that occurs during the process of nectar secretion. Cell death as a natural process involved in nectar secretion was first identified in tobacco nectaries [4,10].

Among the other metabolism-associated proteins that we found in

Table 3
Summary of total identified proteins in floral nectar of *Petunia hybrida*.

Biological function	N°	Protein identified	Access number (Uniprot)	Organism
Defense/Stress	1	Nectarin 1b	A0A0K0K6J6_NICAT	<i>Nicotiana attenuata</i>
	2	Acid endochitinase T	CHIT_PETHY	<i>Petunia hybrida</i>
	3	Osmotin-like protein	A0A075E2S9_TOBAC	<i>Nicotiana tobacum</i>
	4	Aspartic proteinase like protein-1	A0A0V0IFS7_SOLCH	<i>Solanum chacoense</i>
	5	Peroxidase	A0A022PUU9_ERYGU	<i>Erythranthe guttata</i>
	6	Peroxidase	M1A9V8_SOLTU	<i>Solanum tuberosum</i>
Metabolism	7	Alpha-L-Arabinofuranosidase	B9GF88_POPTR	<i>Populus trichocarpa</i>
	8	Pollen allergen MetE	E3VW74_AMARE	<i>Amaranthus retroflexus</i>
	9	ATP synthase subunit alpha	Q5M9V4_TOBAC	<i>Nicotiana tobacum</i>
	10	ATP synthase subunit alpha	A0A059Q6J8_HYONI	<i>Hyoscyamus niger</i>
	11	ATP synthase subunit beta	M0U2D0_MUSAM	<i>Musa acuminata</i>
	12	Ribulose biphosphate carboxylase large chain	I3NTM6_9SOLA	<i>Petunia axillaris</i>
	13	Fructose-bisphosphate aldolase	K4D3E4_SOLLC	<i>Solanum lycopersicum</i>
	14	Fructose-bisphosphate aldolase	Q9SXX4_NICPA	<i>Nicotiana paniculata</i>
	15	Glyceraldehyde-3-phosphate dehydrogenase	A0A0L9UB16_PHAAN	<i>Phaseolus angularis</i>
	16	Glyceraldehyde-3-phosphate dehydrogenase	A0A061FXI2_THECC	<i>Theobroma cacao</i>
	17	Glyceraldehyde-3-phosphate dehydrogenase	U3PLJ3_9FABA	<i>Polygala fruticosa</i>
	18	Serine hydroxymethyltransferase	A0A0V0I7X4_SOLCH	<i>Solanum chacoense</i>
Protein process	19	Heat shock protein 90-3	A0A0M3SBS3_NICBE	<i>Nicotiana benthamiana</i>
Signaling	20	Putative calcium and calcium/calmodulin-dependent serine/threonine-protein kinase-like	A0A0V0IBF3_SOLCH	<i>Solanum chacoense</i>
	21	14-3-3 protein	M9WUV8_PETHY	<i>Petunia hybrida</i>
	22	14-3-3 protein	M9WMF1_PETHY	<i>Petunia hybrida</i>

Nicotiana spp. nectar were enolase and triosephosphate isomerase. Both of these enzymes have been previously described from FN of *Nicotiana attenuata* [13] as well as in the extrafloral nectaries of *Acacia cornigera* and *Ricinus communis* [48,49]. It would appear that production of energy is necessary to support processes prior to anthesis. This appears to be coupled with an increase in the number of mitochondria as the demand for energy rises during rapid starch breakdown and nectar

secretion [50]. Other proteins such as ATP synthase are essential for provision of energy for active transport of solutes into the apoplast, which is an important step in the creation of an osmotic gradient that subsequently drives nectar secretion [51].

Organic acids are important components of nectar that affect its attractiveness to pollinators [52]. Malate is one of the main organic acids in tobacco nectar [3]. This compound is produced during starch

Table 4
Total identified proteins in floral nectar of *D. stramonium*.

Biological function	N°	Protein identified	Access number (Uniprot)	Organism
Cell structure	1	Annexin	Q24132_TOBAC	<i>Nicotiana tobacum</i>
Cellular homeostase	2	V-type proton ATPase subunit B2	A0A0V0IFF9_SOLCH	<i>Solanum chacoense</i>
	3	Actin 66	A0A1D1YWF9_9ARAE	<i>Anthurium amnicola</i>
	4	Actin	Q7DMB2_PEA	<i>Pisum sativum</i>
Defense/stress	5	Cysteine protease	V5K5R5_CAPAN	<i>Capsicum annuum</i>
Metabolism	6	Arachin 6	A1DZF0_ARAHY	<i>Arachis hypogaea</i>
	7	Gly-1	Q9FZ11_ARAHY	<i>Arachis hypogaea</i>
	8	Alpha-galactosidase	F5BFT0_TOBAC	<i>Nicotiana tobacum</i>
	9	Alpha-L-Arabinofuranosidase/beta-D-Xylopyranosidase	A0A0A8JBT3_TOBAC	<i>Nicotiana tobacum</i>
	10	Putative beta-xylosidase/alpha-L-arabinofuranosidase 2-like	A0A0V0IT87_SOLCH	<i>Solanum chacoense</i>
	11	UTP-glucose-1-phosphate-uridylyl transferase	K4D601_SOLLC	<i>Solanum lycopersicum</i>
	12	Malate dehydrogenase	A0A0V0HZC4_SOLCH	<i>Solanum chacoense</i>
	13	ATP synthase subunit alpha	I1TKS4_9ASPA	<i>Tulbaghia alliacea</i>
	14	ATP synthase subunit beta	M0U2D0_MUSAM	<i>Musa acuminata</i>
	15	Ribulose biphosphate carboxylase large chain	B8X871_9SPER	<i>Araucaria biramulata</i>
	16	Fructose-bisphosphate aldolase	Q2PYX3_SOLTU	<i>Solanum tuberosum</i>
	17	Fructose-bisphosphate aldolase	K4D3E4_SOLLC	<i>Solanum lycopersicum</i>
	18	Fructose-bisphosphate aldolase	G9MA91_9ROSI	<i>Linum grandiflorum</i>
	19	Aldolase-type TIM barrel	A0A103YEQ4_CYNCS	<i>Cynara cardunculus</i>
	20	Glyceraldehyde-3-phosphate dehydrogenase	A0A0L9UB16_PHAAN	<i>Phaseolus angularis</i>
	21	Glyceraldehyde-3-phosphate dehydrogenase	A0A0F7JLU6_NICBE	<i>Nicotiana benthamiana</i>
	22	Glyceraldehyde-3-phosphate dehydrogenase	I0DG17_9ASPA	<i>Phalaenopsis hybrid cultivar</i>
	23	Phosphoglycerate kinase	PGKY_TOBAC	<i>Nicotiana tobacum</i>
	24	Phosphoglycerate kinase	M4SKS4_ATRBE	<i>Atropa belladonna</i>
	25	Phosphoglycerate kinase	A0A0R0IRH4_SOYBN	<i>Glycine max</i>
	26	Triosephosphate isomerase	W1PJM9_AMBTC	<i>Amborella trichopoda</i>
	27	Enolase	M0ZSD6_SOLTU	<i>Solanum tuberosum</i>
	28	Adenosylhomocysteinase	B8LKF8_PICSI	<i>Picea sitchensis</i>
Protein process	29	Heat shock protein 70	A4ZX75_9ERIC	<i>Cyclamen persicum</i>
	30	Heat shock protein 90-3	A0A0M3SBS3_NICBE	<i>Nicotiana benthamiana</i>
	31	Cpn60-like protein	K4DAD5_SOLLC	<i>Solanum lycopersicum</i>
Signaling	32	Putative calcium and calcium/calmodulin-dependent serine/threonine-protein kinase-like	A0A0V0IBF3_SOLCH	<i>Solanum chacoense</i>
	33	Putative calreticulin-like	A0A0V0IBE5_SOLCH	<i>Solanum chacoense</i>
	34	14-3-3-like protein C	1433C_TOBAC	<i>Nicotiana tobacum</i>
	35	14-3-3 h-1 protein H1	Q75ZD6_TOBAC	<i>Nicotiana tobacum</i>

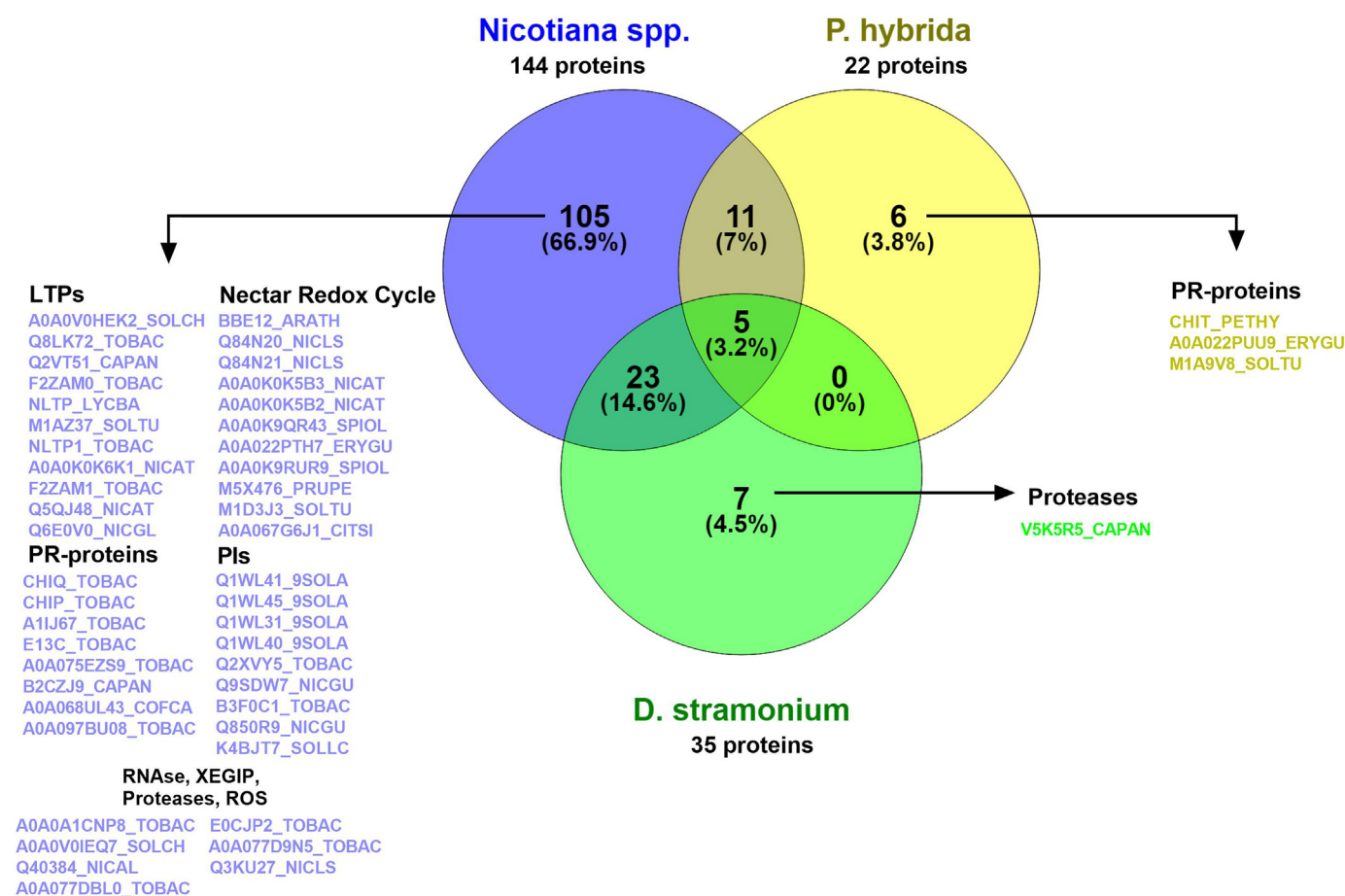


Fig. 2. Venn diagram of *Nicotiana* spp. nectar proteins showing identified proteins of *Nicotiana* spp. proteins 144, compared to 22 proteins of *P. hybrida* and 35 proteins of *D. stramonium*. Numbers into the Venn diagram refer to the number of *Nicotiana* spp. having hits to each nectar proteins identified in the organisms as labeled. The arrows indicate the access numbers of exclusive identified proteins related to defense/stress in each FN.

breakdown. Malate dehydrogenase that we identified in our study may act as a mechanism for regulating malate levels of nectar.

We identified several proteins with important roles in metabolic processes. These include alanine aminotransferase, S-adenosylmethionine transferase, S-adenosylhomocysteinase, the pollen allergen MetE, a putative methionine synthase-like protein, serine hydroxymethyltransferase, threonine dehydratase and spermidine synthase. These proteins are related to S-adenosyl methionine (SAM) cycle, which is important in regulating plant development. SAM cycle is a source of single-carbon biosynthetic units for methylation reactions, including key factors such as S-adenosylmethionine (SAM) or S-adenosylhomocysteine (SAH) for ethylene, nicotianamine and polyamine biosynthetic pathways [53]. SAM-cycle proteins have been previously described from nectaries [48,49], but their function in nectar remains elusive. Outside of nectaries, SAM-cycle proteins have a central role in biosynthesis of juvenile hormone (JH) in insect pollinators [54] and lack of S-adenosylhomocysteinase expression led to several disorders in larval development, metamorphosis and reproduction [55].

Carbohydrates are the most abundant compounds found in nectar and are the main components for attracting pollinators. Carbohydrate composition differed significantly between *Nicotiana* species [4,56]. We identified several enzymes in these nectars related to carbohydrate metabolism, such as, sucrose synthase, fructokinase and α -galactosidase, which have previously been described in nectar [47,57]. Both sucrose synthase and fructokinase are enzymes associated with sugar metabolism and the regulation of sucrose levels is also associated with invertases that control monosaccharide amounts and ratios in nectar [58–60]. Similar roles have been proposed for fructokinase, i.e. it may

operate in a manner similar to a hexokinase [47,61]. Alpha-galactosidase catalyzes the conversion of raffinose to monosaccharide (α -D-galactose) and a disaccharide (sucrose), thereby adding complexity to the main carbohydrate mix of glucose and fructose that is typically found in nectar [62]. Raffinose has never been identified from *Nicotiana* nectar, which implies that the enzyme may have no function in post-secretion hydrolysis. It is possible that this enzyme may, instead, be involved in restructuring the cell walls during fruit development [57]. Heteroxylans are another class of polysaccharides found in flowers. During infection by pathogens, heteroxylans are degraded, which, in turn releases oligosaccharides into nectar. The catalytic activities of xylanosidase and α -L-arabinofuranosidase/ β -D-xylopyranosidase may function in the generation of oligosaccharides produced by microorganisms in the nectar. These released oligosaccharide elicitors trigger signaling pathways that reduces the virulence of the pathogens [63].

We also identified a β -D-glucan exohydrolase. This enzyme hydrolyzes β -D-glucans, a carbohydrate that is naturally found in cell walls of pathogens [64]. Together with other enzymes such as chitinases and endoglucanases the hydrolytic activity of β -D-glucan hydrolase may combine to hydrolyze cell wall polymers, thereby inhibiting the growth of microorganisms.

4.2. The main function of the proteins targeted to the nectar is related to floral defense

We have previously suggested that floral defense is one of the major roles of nectar [4,8,65]. The identification of numerous proteins with secretory signal peptide that are related to defense/stress substantiates

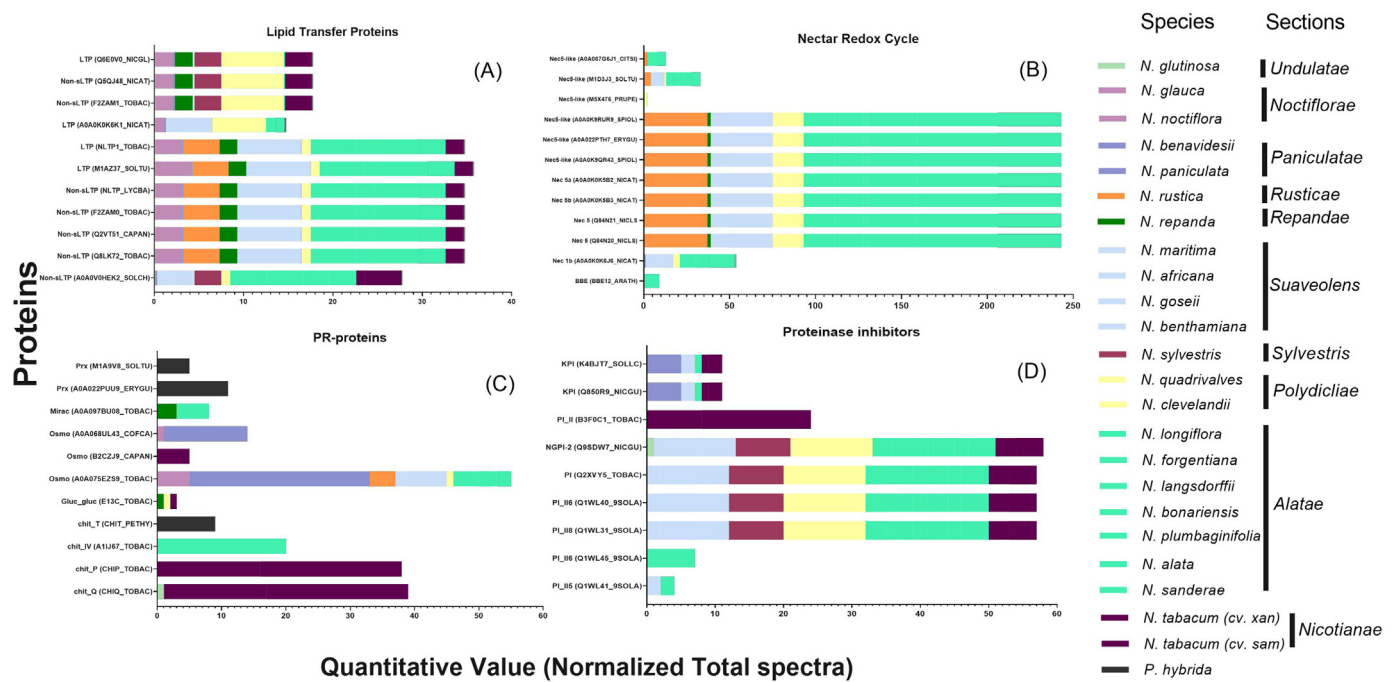


Fig. 3. Analysis by mass spectrometry (LC-MS/MS - normalized spectrum count) of proteins identified in nectar related to defense/stress through the sections of genus *Nicotiana*, *P. hybrida* and *D. stramonium*. The graph shown the four main class of proteins: (A) lipid transfer proteins (LTPs), (B) Nectar Redox Cycle, (C) PR-proteins and (D) proteinase inhibitors highlighting the contribution of each group of nectar proteins in floral defense by section. The *t*-test was applied to verify significant differences ($p \leq .05$) between means. Species coding is indicated at the right of this figure.

supports this observation. Chitinases, endoglucanases, LTPs and glucosidases were among the defense proteins that we identified in *Nicotiana* nectars. Many of these proteins have been reported previously in floral nectars of *Nicotiana tabacum* [66], *Nicotiana attenuata* [13] and *Liriodendron tulipifera* [67]. Chitinases and endoglucanases are glycosyl hydrolases that catalyze the hydrolysis of glycosidic bonds in complex sugars. These enzymes were found in *Acacia* extrafloral nectar in which are involved in defense against several phytopathogens through of the synergistic effects in the microbial cell wall [68]. These proteins have also been identified in other secreted fluids, such as gymnosperm

pollination drops [69–71] and extrafloral nectar of *Ricinus communis* [49]. We identified an aspartic proteinase-like protein-1 (Asp1) in many of the *Nicotiana* species and also in *P. hybrida* floral nectar. This aspartic proteinase had two active site motifs Asp-Thr-Gly and Asp-Ser-Gly that were also similar to ones found in apoplastic aspartic protease (CDR1) of *Arabidopsis* [72]. In those plants, aspartic protease is involved in constitutive resistance as well as signaling. The aspartic proteases nepentensin is also important for protein processing and nitrogen acquisition in the insectivorous plant *Nepenthes* [73]. These proteins have been reported in extrafloral and floral nectars of *Ricinus communis*

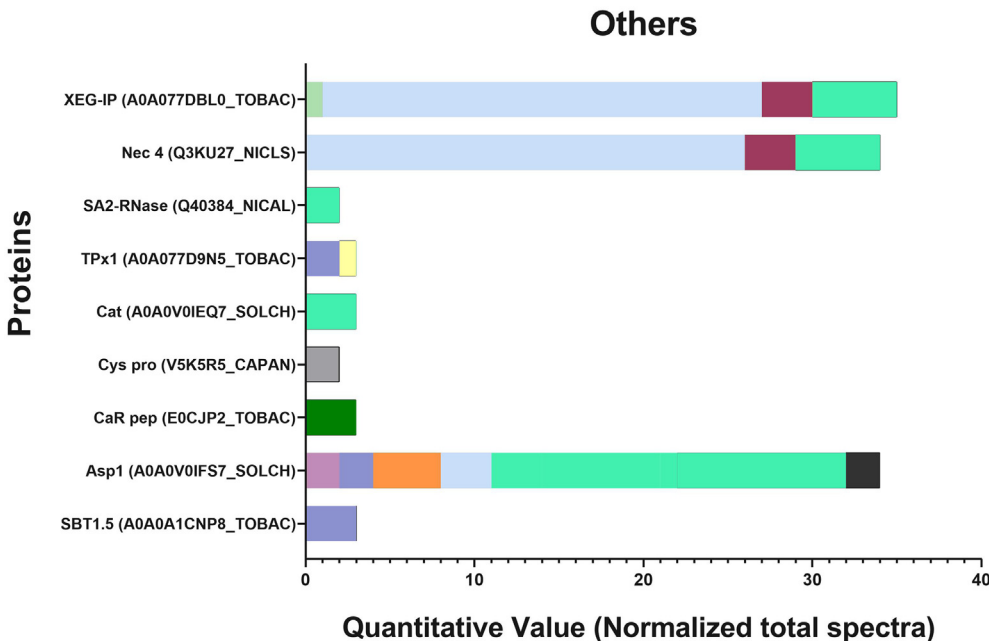


Fig. 4. Nectar proteins identified by mass spectrometry (LC-MS/MS) through the sections of genus *Nicotiana* spp., *P. hybrida* and *D. stramonium*. The proteins are related to proteases, reactive oxygen species, RNases and xylanase inhibitors. The *t*-test was applied to verify significant differences ($p \leq .05$) between means. Species coding is identical to that shown in Fig. 3.

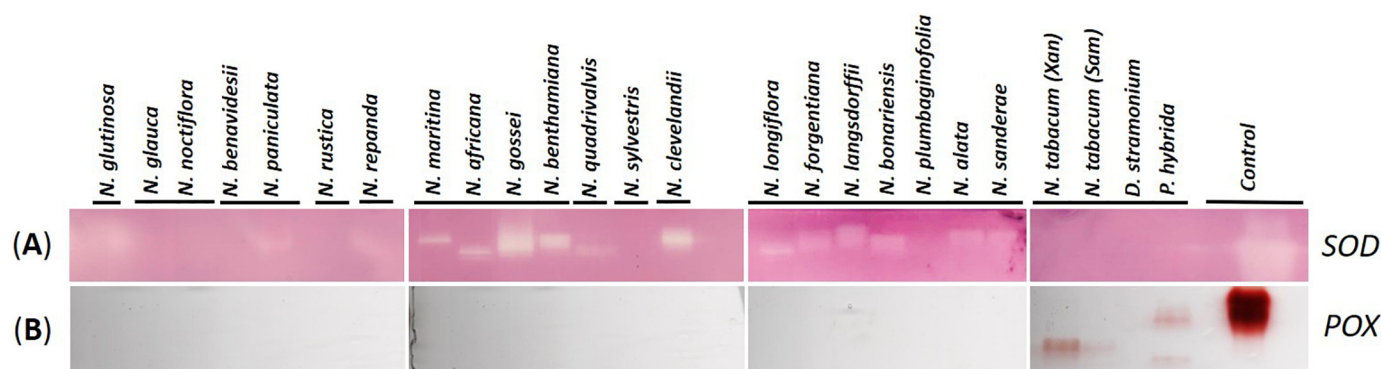


Fig. 5. Biochemical analysis of *Nicotiana* spp., *P. hybrida* and *D. stramonium* nectars. (A) Nectarin I SOD activity in gel on a 10% native PAGE. (B) Guaiacol peroxidase activity. 26 units of the MnSOD, FeSOD from *E. coli* and the Cu/ZnSOD from bovine erythrocytes were used as controls. Horseradish peroxidase type VI-A (64 units) was used as a control in peroxidase assay. Raw floral nectar (10 μ L) was applied to each lane.

and also in exudates from carnivorous plants [74], suggests that this protein may function in reabsorption of nectar.

The *Nicotiana* spp., nectar demonstrated the two classes of LTPs in floral nectars. LTPs are multigenic protein families with small molecular weights and show great diversity among plants [75,76]. LTPs were originally characterized as proteins that transfer lipids between cells or cellular organelles and across the membranes playing the role in wax or cutin deposition of cell wall in epidermis of secretory tissues [77,78]. However, a wide range of studies suggests that LTPs may also function directly in host defense [79–84]. LTPs have been identified in secretions of plant glandular trichomes and are related to resistance to insects [83]. Recently it was shown that lipid transfer protein 2.1 (BrLTP2.1) is able to prevent microbial growth in *Brassica rapa* nectar [85]. LTPs are induced by fungal elicitors [86] and participate in plant defense [87,88] contributing to the antimicrobial protection of floral nectar, lipid metabolism and seed development [83]. Our inability to identify LTPs in the proteomes of *P. hybrida* and *D. stramonium* suggests that these species may have other mechanisms of anti-microbial defense.

We also found osmotin-like proteins in nectars. The osmotin proteins belong to family 5 of PR-proteins which are produced in response to stress. Osmotins are involved in defense acting by inhibition of defensive cell wall barriers, cytotoxicity effects, cell wall binding and increasing membrane permeabilization of microorganisms. These proteins may be also be involved in inhibition of germination and growth of phytopathogens [89]. Taken together, such arrays of antimicrobial proteins constitute strong, complex mechanisms of defense against growth of microorganisms.

Several isoforms of trypsin proteinase inhibitors were also detected in nectars. These proteinase inhibitors are a type of PR-protein that protect plants by inhibiting digestive proteases of herbivorous insects [90]. The presence of these inhibitors is harmful to pollinators and reduces their visitation frequency. Multiple-domain versions of proteinase inhibitor II and at least two Kunitz-type proteinase inhibitors have been characterized as cell death markers [91]. Targeted cellular death within nectary tissue may be a normal process during floral maturation. Cell death may also explain the large number of general metabolic proteins from glycolysis, citric acid cycle, and pentose phosphate in nectar that we found in our proteomic analysis. In addition, protease inhibitors may have an endogenous role in *Nicotiana* nectar that includes regulation of protein concentrations, e.g. germin-like proteins, as well as regulation of H_2O_2 levels [92]. Identification of numerous PI isoforms in *Nicotiana* spp. proteomes suggest that these proteins may have alternative regulatory functions other than the simple inhibition of digestive proteases.

4.3. Nectarins are differentially distributed throughout *Nicotiana*

Within the genus *Nicotiana*, nectarins (Nec I, Nec II, Nec III, Nec IV, and Nec V) are the most abundant proteins found in nectar of *Nicotiana* spp. Within these species, H_2O_2 levels and nectarins are shown to vary through the genus [56]. These proteins regulate the production of H_2O_2 via the Nectar Redox Cycle, one of the main defense mechanisms against microbial growth in nectar [8,92]. This process involves a NADPH oxidase that produces superoxide anion [10]. Subsequently, Nec I converts the superoxide into H_2O_2 [7], the levels of which are further increased to 4 mM by Nec V [6,7]. Differences of Nec I activity between the sections of the *Nicotiana* regulate the levels of H_2O_2 impacting the pollinators visitation [92] and the growth of specialized and opportunistic microorganisms altering the mutualistic relationships. Another nectarin, Nec IV, is important for both pathogen recognition and signaling [7,11,12]. This nectarin were very abundant in species of *Suaveolentes* section. Pathogens secrete XEG proteins, responsible for digestion of polysaccharide cell walls, which is recognized by Nec IV, inhibiting XEG activity [11]. The complex NecIV:XEG may activate the NecV, in turn catalyzing the H_2O_2 production and increasing the content levels in floral nectars [12].

4.4. Nucleotide binding and translational factors

Several proteins related to nucleotide binding and to transcription factors, such as RNA-binding proteins, glycine-rich RNA-binding proteins and elongation factor 1-alpha were previously identified from both FN [13,20,47] and extrafloral nectar [48,49]. Although these proteins play a role in regulation of nucleotide metabolism, they are essential to antimicrobial defense. For example, SA2-RNases have defensive functions in nectar of *P. hybrida* [20,47]. Glycine-rich RNA-binding proteins are implicated in plant immunity by virtue of an RNA-recognition motif (RRM) that induces defenses against pathogens, including tobacco mosaic virus [93]. Elongation factor 1-alpha has been found in the latex of *Hevea brasiliensis*. EF1 α is considered to be a human allergenic component due its high hydrophobicity and its ability to bind lipids in cell membranes [94].

4.5. Chaperones

The high levels of H_2O_2 produced in nectar may be potentially harmful to molecules that can oxidize other non-target molecules. We identified three groups of stress-related heat shock proteins (HSPs), HSP-60s, HSP-70s and HSP-90s. HSP-60s is a mitochondrial chaperone involved in the mitochondrial transport process. HSP-70s, on the other hand, are thought to protect cellular proteins from thermal or oxidative stress. Because of the highly oxidative stress caused by the Nectar Redox Cycle, it makes great sense that HSP-70s are also present in

nectar. The most common chaperones found in nectars, HSP-90s, are more selective than other chaperones, but also bind proteins and protect them from cellular stresses [95–97]. The presence of HSP-70s and HSP-90s may act as a mechanism to prevent oxidative damage of non-target proteins against high level of H₂O₂.

4.6. Signaling

We identified a number of different signaling proteins in the floral nectars of *Nicotiana spp.*, *P. hybrida* and *D. stramonium*. A Calcium calmodulin-dependent Ser/Thr-protein kinase (CDPK) plays a role in signaling of immune responses, in particular hypersensitive responses that occur after infection [98]. These act to increase resistance to phytopathogens [99]. Ser/Thr-protein kinases proteins may recognize effector molecules that interact with protein kinases and trigger programmed cell death. This process is itself controlled by 14–3–3 proteins via phosphopeptide binding proteins [100,101]. We also found a GPI-anchored protein that is related to a peptidoglycan binding protein and may bind to bacterial peptidoglycan fragments. The presence of proteins involved in bacterial recognition [102] suggests that sophisticated defenses are present in nectar.

4.7. Cellular homeostasis

Two different groups of proteins related to cellular homeostasis were identified in our studies. The first includes actins and profilins. These proteins are involved in vesicle transport, an active process that occurs during the nectar secretion [103]. Actin acts in the dynamic restructuring of cytoskeleton and is associated with remodeling cell walls during floral nectary development [104]. Profilin is a protein found in mature pollen and elongating pollen tubes [105]. Profilins can bind to actin to prevent actin polymerization [106]. The second class of homeostatic proteins were V-type ATPases. These proteins are membrane-bound proton pumps that frequently occur in the secretory pathway of plants. V-type ATPases have a primary role during development and stress adaptation [67], as is they occur in the floral nectary both prior to and after anthesis.

4.8. Cell structure

Several proteins related to cell structure that we found, such as annexins, have been previously reported in nectar [13]. For example, Ca²⁺ dependent proteins associate with membrane phospholipids and are thought to coordinate the organization of membrane proteins during secretion [107]. Expansin and extensin, both of which are plant cell wall proteins, are involved in extension of cell walls. In the context of rapid growth during floral development, which includes cell wall loosening of the floral nectary gland during its development and maturation, thus, it is not surprising that proteins, such as expansins and extensins, accumulate in nectar [107,108].

5. Conclusion

We have demonstrated that floral nectars of *Nicotiana spp.*, *P. hybrida* and *D. stramonium* have rich and diverse proteomes. Most of identified proteins targeted to secretion had molecular functions related to defense. Nectar Redox Cycle proteins, LTPs, PR-proteins and proteinase inhibitors were the most important groups related to floral defense. These proteins were differentially distributed among the taxonomic sections of *Nicotiana* and differed in its solanaceous relatives, *P. hybrida* and *D. stramonium*. The similarities and differences in nectar proteins, and underlying biochemistry, between these species suggest that many similar mechanisms of defense exist among these closely related plant species. However, the differences, especially related to Nectar Redox Cycle proteins lead us to conclude that the global mechanisms of nectar defense differ among these species, especially as

related to those species outside of the *Nicotiana* clade. These findings enhance our understanding of *Nicotiana* nectar protein composition and provide information useful that will allow us to predict or to test their impacts on interactions between plant and microorganism, as well as plant and pollinator.

Authors contributions

RT and PvA conceived the ideas and designed methodology;
AG and CB collected the data;
PvA and FS analyzed the data;
RT and FS led the writing of the manuscript.

Data accessibility

These proteomic data are available via ProteomeXchange with identifier PXD014760.

Declaration of Competing Interest

All authors contributed critically to the draft and gave final approval for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jprot.2019.103618>.

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