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REVIEW

Exploring floral scent in wild tobacco: comparison of volatile compounds across pollinator functional groups and *Nicotiana* sections

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Abstract

Plant-pollinator relationships drive floral diversity. Among many floral signals, floral scent attracts and ensures efficient pollination, like benzyl acetone and its derivatives are important for hawkmoth species to detect flowers. *Nicotiana* is a diverse genus in terms of floral phenotypes. Its evolution was driven by the close relationship with pollinators through floral specialization and hybridization, causing the emergence of new species. *Nicotiana* has a wide variety of floral shapes and colors. Hybridization is prevalent in the group, resulting in transgressive phenotypes impacting pollination-related visual traits. This study investigated the diversity of floral Volatile Organic Compounds (VOCs) of *Nicotiana* species and discussed the data according to pollinator functional groups and the known phylogenetic relationships of the species. A bibliographic review was carried out, including 30 years of floral volatile emissions studies. The results of 18 studies investigating floral volatiles in 19 species of *Nicotiana* were revised and organized by temporal emissions, day and night emissions. We retrieved information concerning methodology, volatile compounds, day/night emissions, and pollinator functional groups for each study and species. In total, 179 compounds were found, including mono- and sesquiterpenoids, nitrogenous compounds, and benzenoids. Most allopolyploid species lack floral emissions data, and total temporal emissions in *Nicotiana* are still underrepresented. The results suggest that night-pollinated species of *Nicotiana* have high emission blends' similarity to each other, compound-wise and class-wise. However, phylogeny still plays an important role in scent emission for species with different pollinators functional groups, considering the known phylogenetic relationship in the genus. Species known to be closely related by current phylogeny, like *N. forgetiana* and *N. alata*, share similar blends even though their pollinator functional groups are entirely different and opposite, suggesting a phylogenetic relationship in this phenotype and/or other processes, such as hybridization.

Keywords Evolution · Floral VOCs · Pollination · *Nicotiana*

Extended author information available on the last page of the article

Introduction

Pollinators commonly drive floral diversity through floral specialization, providing an effective barrier to gene flow and/or acting as an agent of hybridization (Darwin 1862; Stebbins 1970; Rezende et al. 2020; Turchetto et al. 2022). Both processes, floral specialization and hybridization, contributed to angiosperm diversification (van der Niet and Johnson 2012; Chase et al. 2010), making clear the dual role of pollinators as a primary driver of plant diversity involving microevolution and macroevolution processes. The extent of ecological interaction between plant and animal pollinators has long been investigated (van der Niet and Johnson 2012), with insects as pollinators of most angiosperms (Ollerton et al. 2011; Asar et al. 2022) and are recognized pointed out as key pollinators in angiosperm history (van der Kooi and Ollerton 2020).

As visual and olfactory cues, flowers' color, shape, and scent vary, attracting different groups of animal pollinators (Fenster et al. 2004; Armbruster 2014). The channel of communication between plants and pollinators through floral scent is complex. Floral volatile organic compounds (hereafter called floral VOCs) can help pollinators find their resource, which can respond to one or more VOCs influencing their behavior, regardless of the floral specialization level (Raguso 2008a). Hybridization can occur in pairs of species with different visual appearances and no shared pollinator (Turchetto et al. 2022), showing that pollinators are likely to be attracted not by visual cues but instead by learning and associating a flower VOC blend with a potential floral reward (Wright and Schiestl 2009). For instance, bees and hawkmoths can learn odors and thus forage flowers not presenting the visual cues of their preference (Daly and Smith 2000; Kunze and Gumbert 2001; Wright and Schiestl 2009; Lawson et al. 2018; Cook et al. 2020), showing that floral scent may have a more significant impact both on attracting a secondary or occasional pollinator, especially bees, and moths. Moreover, the floral scent may also be a repellent of ineffective pollinators (Raguso 2008b; Junker and Blüthgen 2010), or in some cases, floral perfumes could be considered mimetic (Lunau 1992).

As sessile organisms, plants have evolved a wide range of specialized metabolites, such as VOCs. Floral VOCs are a complex mixture of chemical compounds derived from different biosynthetic pathways, including mono and sesquiterpenes, phenylpropanoids, benzenoids, fatty acids, and nitrogen-containing compounds, that contribute to the attraction of pollinators to flowers (Muhlemann et al. 2014). Usually, the levels of emitted VOCs from flowers emit odors in daily rhythms varying according to the visitors, drawing out distinct behavioral responses in pollinators (Balao et al. 2011). For instance, VOCs emissions can contribute to attracting pollinators over long distances, and anthropologic pollutants can threaten this ecological interaction (Farré-Armengol et al. 2016). On the other hand, short-distance scent emissions determine choice at a short distance (Dötterl et al. 2006; Klahre et al. 2011).

The floral blend may structure the plant-pollinator interaction in a complex community across spatial and temporal scales (Burkle and Runyon 2017), thus being a crucial vector to floral plant diversification (Wester and Lunau 2017; Wang et al. 2019). The relationship between pollinators and floral cues is remarkable in the Solanaceae Juss., a family originating in the Neotropical region (Knapp 2010; Dupin et al. 2016). Floral specialization is well documented in this family; for example, many transitions of floral visual cues are seen throughout the phylogeny (Ng and Smith 2016). Moreover, the chemical composition of the floral scent was also documented to differ among clades and species associated with pollination syndromes (Moré et al. 2021). Taking *Alatae* section as an example, four distinct pollinator functional groups were differentiated within closely related species. *N. alata*, *N. plumbaginifolia*, and *N. longiflora* are hawkmoth pollinated, and the latter two also perform self-pollination as well (Kaczorowski et al. 2005). Closely related to them, *N. forgetiana*, *N. langsdorffii*, *N. mutabilis*, and the putative new species "Rastroensis" are all hummingbird pollinated (Kaczorowski et al. 2005). The only species in the section that does not follow those lines is *N. bonariensis*, a small white-flowered species that is hypothesized to be pollinated by small moths; however, so far, there is no visual record of it (Kaczorowski et al. 2005; Raguso et al. 2006). Hybridization is a crucial driver of species formation in *Nicotiana*, with homoploid and polyploid hybrid species of different ages documented in this genus (Kelly et al. 2009; Clarkson et al. 2017). On the other hand, floral specialization is the main driver of diversification in some *Nicotiana* clades. For example, some species pairs (Kaczorowski et al. 2005), such as *N. alata* Link & Otto and *N. forgetiana* Hemsl. (*Alatae* section; Knapp et al. 2004; Knapp 2020), have specialized pollination systems and have also been reported to hybridize in nature. Nonetheless, this process and its outcomes in this group remain largely unknown (Teixeira et al. 2022).

Nicotiana is a diverse genus with ~ 90 naturally occurring species that is mainly known due to the crop species *N. tabacum*, which also has a natural hybrid origin (Goodspeed 1954; Chase et al. 2003, 2021; Clarkson et al. 2004; Knapp 2020; Chase and Christenhusz 2021; Augsten et al. 2022). *Nicotiana* species are distributed throughout the Americas, Australia, and one species in Africa, with the center of origin pointed to the Andean region (Clarkson et al. 2004). Flowers of the genus are characterized by extensive variation in color, shape, and size related to primary functional pollinator groups (Kaczorowski et al. 2012; Knapp 2020). Transgressive phenotypes related to floral form and color were documented in hybrid species across the genus, impacting pollination (García et al. 2020; McCarthy et al. 2017, 2019). In this sense, floral volatiles should be considered in the context of pollination and the evolutionary relationship of species (Raguso et al. 2003, 2006; Jassbi et al. 2017). A phenotypically variable genus, such as *Nicotiana*, provides an excellent opportunity for understanding the role of olfactory cues on pollinator-driven diversification.

Given the close relationship of specialization, hybridization, and diversification in *Nicotiana*, this genus should be an excellent model to investigate how the floral scent varies across species and its correlations with plant-pollinator interactions and diversification. To this end, we performed a literature survey on *Nicotiana* floral volatile compounds to build a dataset on the natural emissions of floral volatiles. Additionally, this data has been subjected to comparative analyses, considering pollination mode and phylogenetic relationship of species. Specifically, we intended to investigate the following questions: (1) What is the current state of floral VOCs knowledge in *Nicotiana*? (2) What is the influence of phylogenetic constraint and pollinator functional groups in floral VOC emissions across the genus? And (3) Is there any scenario of floral VOCs variation that may enhance sporadic visits of secondary pollinators, like bees? First, we describe the volatile floral classes found in the *Nicotiana* genus. Second, we explore the relationship between floral scent and pollination mode and the phylogenetic relationship of species. We also highlight some perspectives to be addressed in future studies.

Materials and methods

Literature Search and Data Extraction. We searched for articles on floral scent in the *Nicotiana* genus on three databases: Web of Science® (Institute of Scientific Information, Thomson Scientific -https://apps.webofknowledge.com/*)*, Science Direct (https://www.sciencedirect.com/*)*, and ProQuest (https://www.proquest.com/*)* accessed in May 2022. The literature survey followed the protocol by Zhi Foo et al. (2021) with strings adapted to each search system, as shown in Fig. 1A. The initial search recovered 663 results from Web of Science, 1395 from Science Direct, and 824 from ProQuest, a total of 2884 studies. The second phase consisted of an abstract screening of previous results, cutting down the results pool to 121, and the final phase consisted of a full-text screening of the remaining papers, reducing it to 17. One extra paper was found among references, not previously recovered in any of the databases, and it was added, reaching the final total of 18 papers to this survey. The decision trees used to include/exclude paper are presented in Fig. 1B and C. The abstract screening decision tree included works that focus on *Nicotiana*'s floral VOCs Fig. 1B. The full-text screening tree decision included works that focus on *Nicotiana*'s floral VOCs, had an experimental methodology and did not study genetically modified organisms Fig. 1C. Studies with genetically modified organisms was excluded since the objective of the present work was natural genetic expressions. Thus, our final database comprised 18 studies that provide information on floral scents on one or more species of *Nicotiana*.

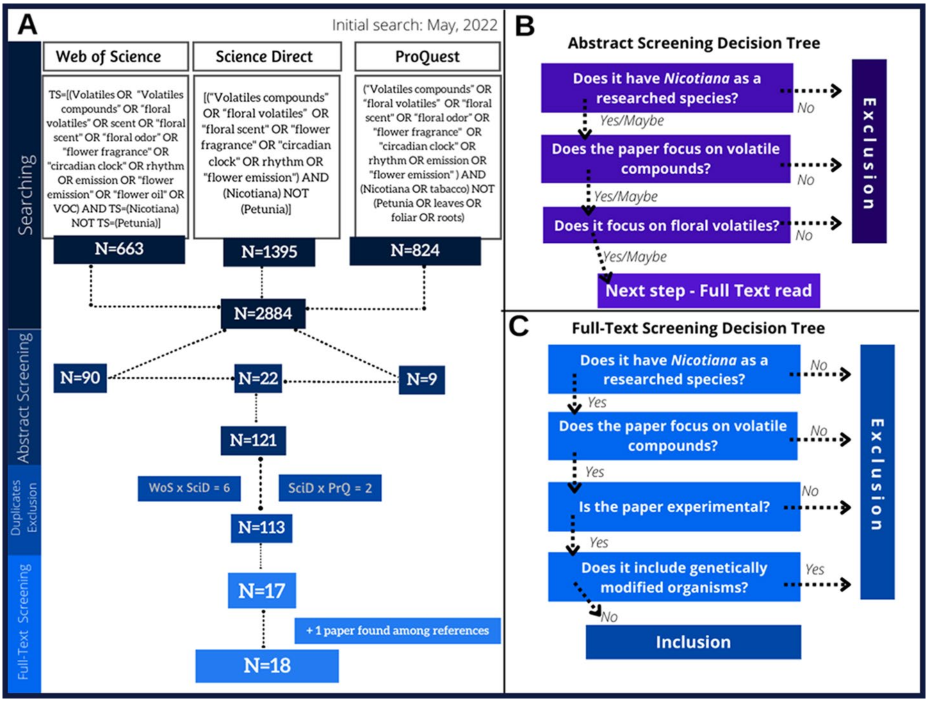

Fig. 1 Survey organization scheme of literature review. **A**: search strings and the number of papers retrieved after each step, **B**: Abstract screening decision tree, **C**: Full-text screening decision tree

The final screening recovered 30 years of literature on *Nicotiana* floral VOCs (1990–2020). For each study, we recorded the authors' names, titles, DOI, methodology details, and species classified by section and ploidy level (Knapp et al. 2004; Knapp 2020) (Table S1). All papers were classified into two categories based on the study's main objective: exploratory (ten) – where the plant compounds were studied extensively, and focused (eight) – where the research had one compound or class as the primary objective. We compiled a list of floral VOCs by species and information regarding functional pollination groups that visited each species retrieved from the published studies (Table S2). The primary pollinator of each species was considered to classify them into main functional groups: bees, hummingbirds and sunbirds, hawkmoths, small moths, and bats (Fenster et al. 2004). It is important to highlight that this study considers the current knowledge about pollination in *Nicotiana*, so there are species with fewer pollination experimental studies. Most species have their pollinators with visual records, however, *N. bonariensis*, *N. rustica*, and *N. glutinosa* have their pollinators inferred by floral syndrome to be small moth pollinated (Raguso et al. 2003; Silva et al. 2020). Therefore, those pollinator functional groups will be used in this study.

Chemical Data Entry. For each revised study (Table S1), we extracted the entire list of compounds emitted by flowers to construct our VOCs database. The VOCs were classified into their major classes: Alkanes (ALK), Benzenoids (BENZ), Fatty Acid Derivatives (FADER), Nitrogenous Compounds (NITRO), and Terpenoids (TOTALTERP). The latter was subdivided into minor classes: Irregular Terpenoids (IRRETEP), Monoterpenes (MONO), Oxygenated Monoterpenes (OXYMONO), Sesquiterpenes (SESQ), and Oxygenated Sesquiterpenes (OXYSESQ) (Dudareva et al. 2004; Maffei 2010; Raguso et al. 2003). Emission rates were provided in various units (ng/g of flower, % of total compounds, µg/g fresh of weight, ng/h/corolla, dry floral mass/h, µg scent/dry mass/h), and the statistical inferences used different methods for each objective (Student's t-test, ANOVA, two-way ANOVA, Spearman's rank correlation coefficient, Wilcoxon-Mann-Whitney test). For that reason, we organized our main database on the floral scent of each species by individual compounds, only scoring in "presence/absence" (1/0) (Table S2).

As the time of VOC emissions is important for ecological interaction with pollinators (Hoballah et al. 2005; Fründ et al. 2011; Fenske et al. 2015), we subdivided our Main Database (Table S2) into a separate Temporal Database according to the variation in day/night emissions. We used the temporal emissions information when provided by the original studies (eight studies), and when not, the collection time was considered to fit the temporal dataset (five studies). Five studies did not mention the collection time; therefore, that information was not included in the temporal dataset. Thus, the temporal database comprises three different datasets: (DS1) Species with total day and night emissions reported in the revised studies; (DS2) Species with day emissions reported in the revised studies; and (DS3) species presenting nighttime emissions reported in the revised studies (Table S3).

Data Description and Analyses. For data visualization, we used the Main and Temporal databases to describe the total compounds in floral VOCs in *Nicotiana* by class in percentage and ubiquitous presence. Using the Temporal Database, we also described the temporal emissions by pollinator's functional group. We used One-way ANOVA to test differences in floral VOCs richness by classes between pollinators' functional groups. We performed pairwise comparisons with the same predictor using Tukey HSD correction. One-way ANOVA and the pairwise comparison test were performed using the JAMOVI v2.2 Statistical software (Jamovi 2021).

We employed Canonical Analysis of Principal Coordinates (CAP) to investigate scent differences among groups, such as pollinators and phylogenetic groups (*Nicotiana* sections, Knapp et al. 2004). The analyses were run considering the total floral blend and the time of emissions, day emissions and night emissions. The CAP analysis was performed using Bray-Curtis distances (Legendre and Anderson 1999) calculated by the function vegdist as implemented in "capscale" function of the R package (R Core Team 2021) vegan v. 2.3-6 (Oksanen et al. 2022). The CAP is a constrained ordination approach that enables the variation analysis to be carried out among the given groups, and not taking the entire data set without supervision (Anderson and Willis 2003; Eisen et al. 2022). In addition, the CAP results were subjected to an ANOVA-like permutation test using the vegan function "anova.cca" for a significance test of the groups (pollinators and *Nicotiana* sections). The CAP results were plotted using the R package ggplot2 v. 3.4.0 (Wickham 2016).

We used hierarchical cluster analysis to investigate how the VOC's temporal emissions correlate *Nicotiana*'s species with each other. Hierarchical cluster analysis was run by the average linkage algorithm (cophenetic correlation for Fig. S1A: 0.96441270; Fig. S1B: 0.91926010; Fig. S1C: 0.92679320) was performed using "hclust" function from the package stats v.4.3.0, based in the same Bray-Curtis matrix previously calculated (Oksanen et al. 2022).

Results

Data Description and Taxonomic Data. The study recovered data on floral VOCs from 19 *Nicotiana* species, including the putative new species "Rastroensis", representing ~ 17% of the genus, most of which are diploid (73%). *Nicotiana suaveolens* was the species most studied, followed by *N. sylvestris* and *N. alata*, with seven, six, and five studies, respectively (Table 1). Eight out of 13 sections had species cited (61.5%). The *Alatae* section was the most represented, with 22 citations in eight papers, followed by section *Suaveolentes* (11 citations in six papers). Aside from sections composed of only one species (*Nicotiana, Rusticae*, and *Sylvestre*), which had 100% representation, the *Alatae* section has the highest relative representation (87.5% species), followed by *Tomentosae* (40%) (Table 1).

The Temporal Database DS2 and DS3 comprise floral VOCs emissions from day and night, respectively. In total, 16 species had data from the daytime and nighttime emissions. However, some species reported only floral VOCs from daytime emissions, such as *N. glutinosa*, *N. tabacum, N. tomentosiformis*, or nighttime emission, *N. cavicola*, *N. ingulba*, and *N. plumbaginifolia.* In total, 13 species had floral VOCs studies for daytime and nighttime emissions (DS1 dataset; Table S3).

Many of the studied species have white corollas and are hawkmoth-pollinated, such as *Nicotiana alata*, *N. longiflora, N. plumbaginifolia, N. attenuata, N. sylvestris, N. ingulba, N. cavicola*, and *N. suaveolens.* On the other hand, some species have pink to red corollas and are hummingbird-pollinated, for example, the "Rastroensis", *N. forgetiana, N. mutabilis*, and *N. tabacum. N. africana* and *N. langsdorffii*, pollinated by sunbirds and hummingbirds, respectively, display green corollas. Other species are pollinated by small moths, such as *N. bonariensis* with white corolla, *N. rustica* with white to green corolla, and *N. glutinosa* with pink corolla. Bats are the reported pollinators of one species (*N. otophora*) with white to pink corolla. Only one species (*N. tomentosiformis*, with pink corolla) does not have a

Table 1 *Nicotiana* species recovered in this review and the number of studies on floral VOCs for each species. Classification by section follows Knapp et al. (2004). Information on pollinators was taken from the literature. A: Knapp (2020). B: Raguso et al. (2003) C: Macnish et al. (2010), D: McCarthy et al. (2015), D: Raguso et al. (2006), F: McCarthy et al. (2019), G: Kaczorowski et al. (2005), H: Tiedge and Lohaus (2017); I: Kessler and Baldwin (2006), J: Nattero et al. (2003), K: Marlin et al. (2016). L: Chase (2021); M: Silva et al. (2020)

Section	Species	Ploidy[A]	Corolla Color	Main Pollinator Group	Cited
Alatae Goodsp.	*Nicotiana alata* Link & Otto, 1828	9, diploid	White[B, F]	Hawkmoth[G]	5
	Nicotiana bonariensis Lehm., 1818	9 diploid	White[B]	Small Moth[G, D]	3
	Nicotiana forgetiana hort. Ex Hemsl., 1905	9, diploid	Red[B]	Hummingbird[G]	3
	Nicotiana langsdorffii Weinm. Ex Roem & Schult., 1819	9, diploid	Green[B, D,F], red[B]	Hummingbird[G]	3
	Nicotiana longiflora Cav., 1802	10, diploid	White[B, F]	Hawkmoth, self[G]	3
	Nicotiana mutabilis Stehmann & Semir, 2002	9, diploid	White[C, D], pink[C, D]	Hummingbird[G]	3
	Nicotiana plumbaginifolia Viv. 1802	10, diploid	White[B, D,F]	Hawkmoth, self[G]	1
	"Rastroensis"	9, diploid	Pink [D]	Hummingbird [D]	1
Nicotiana Goodsp.	*Nicotiana tabacum* L., 1753	24, polyploid	White[D], pink[D, F]	Hummingbird[H]	2
Petunioides G. Don	*Nicotiana attenuata* Torr. Ex S. Watson, 1871	12, diploid	White[D]	Hawkmoth[H, I]	2
Rusticae G. Don	*Nicotiana rustica* L., 1753	24, polyploid	White[B], green[B, D]	Small Moth[,,B]	4
Sylvestres S. Knapp.	*Nicotiana sylvestris* Speg., 1898	12, diploid	White[B, D]	Hawkmoth[J]	6
Suaveolentes Goodsp.	*Nicotiana africana* Merxm., 1975	23, polyploid	Green[E]	Sunbird[K]	1
	Nicotiana cavicola N. T. Burb., 1960	20, 23, polyploid	White[E]	Hawkmoth[D]	2
	Nicotiana ingulba J. M. Black, 1933	20, polyploid	White[E]	Hawkmoth[D], self[L]	1
	Nicotiana suaveolens Lehm. 1818	16, polyploid	White[D]	Hawkmoth[B]	7
Tomentosae Goodsp.	*Nicotiana otophora* Griseb., 1879	12, diploid	White[B, D], pink [D, F]	Bats[J]	1
	Nicotiana tomentosiformis Goodsp., 1933	12, diploid	UV-white [D], pink[F]	-	1
Undulatae Goodsp.	*Nicotiana glutinosa* L., 1753	12, diploid	Pink[D]	Small Moth[M]	1

known pollinator. Interestingly, secondary floral visitors were also reported for most species, especially bees (Table S2).

All studies were conducted with greenhouse-germinated plants. Thus, the floral blend recovered in those studies is less prone to natural environmental influences. Although plants were grown in greenhouse common gardens, their provenance (populations of origin) was not mentioned for most of them. Moreover, population-level variation is almost overlooked in the relevant literature. Both studies on *N. attenuata* were performed using seeds from the same population of plants from Utah, USA. The *N. bonariensis* and *N. forgetiana* seeds from two different studies (Raguso et al. 2003; Fähnrich et al. 2012) came from the same accessions. *Nicotiana mutabilis* seeds from both studies were from the same location (Quebra Cabo, Rio Grande do Sul, Brazil) but not necessarily from the same accession. The same could be observed for *N. alata* and *N. langsdorffii* from Raguso et al. (2003) and Fähnrich et al. (2011) and TW7 and TW74 accessions, respectively (Table S1).

Diversity of Floral VOCs. In total, we compiled 179 floral VOCs in *Nicotiana* species. Terpenoid class was the most common, representing 40% of the total floral VOCs in *Nicotiana*. Terpenes (mainly mono- and sesquiterpenes) are followed by benzenoids (26%), fatty acid derivatives (15%), alkanes (11%), and nitrogenous compounds (8%). The terpenoid class was represented by compounds in five minor categories: monoterpenes (15%), oxygenated monoterpenes (9%), sesquiterpenes (11%), oxygenated sesquiterpenes (2%), and irregular terpenoids (3%) (Fig. 2A). Terpenoids, benzenoids, and fatty acids derivatives were the classes with the highest richness; 59, 43, and 42 different compounds, respectively (Table S2).

Our database identified 148 and 108 VOCs for daytime and nighttime emissions, respectively. Some differences were observed when we compared the presence of compounds from different classes of VOCs emission in the temporal variation (day and night emissions). All

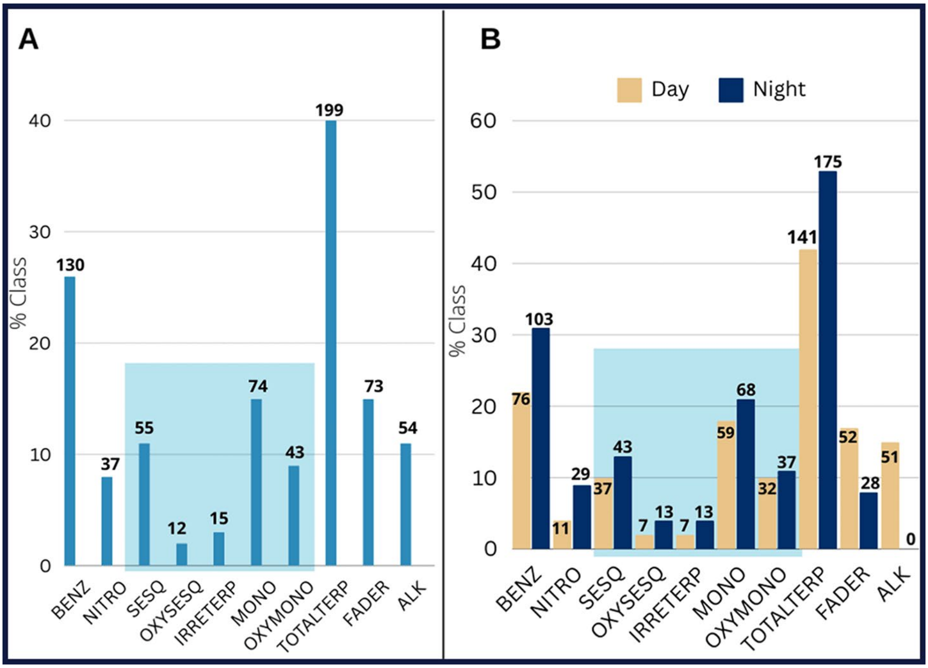

Fig. 2 Diversity of floral VOCs in *Nicotiana* from the revised studies. **A**: Total emissions of VOCs represented in % of compounds from the main database presented in Table S2; 19 species, 179 VOCs, and a total of citations - N_{total}=493. **B**: VOCs emissions for species with day and night emissions according to Table S3. Day emissions in yellow; 16 species, 148 VOCs, and a total of citations - N_{total}=331 (DS2). Night emissions in dark blue; 16 species 108 VOCs, and a total of citations - N_{total}=335(DS3). Alkanes ALK=Alkanes, BENZ=Benzenoids, FADER=Fatty Acid Derivatives, NITRO=Nitrogenous Compounds, TOTALTERP=Terpenoids, IRRETEP=Irregular Terpenoids, MONO=Monoterpenes, OXYMONO=Oxygenated Monoterpenes, SESQ=Sesquiterpenes, OXYSESQ=Oxygenated Sesquiterpenes

classes were present in daytime and nighttime emissions, except for the alkane class, which was present only in the day emissions. All monoterpene compounds were present in the day and night emissions. Day emission has the highest percentage of different VOCs for all classes, except for fatty acid derivatives. All classes presented differences in the presence/absence of individual VOCs (Fig. 2B; Table S3).

The Main Database of VOC emissions shows benzenoids and terpenoids as the most ubiquitous compounds in *Nicotiana* species (Fig. 3A; Table S2). Figure 3A shows the 12 most ubiquitous compounds when considering all the VOC compounds for daytime and nighttime emissions. In Fig. 3B, we display the proportion of the 13 compounds encompassing 2-phenyl-ethanol and methyl salicylate, which are most ubiquitous during nighttime and daytime. Benzyl alcohol, β-ocimene, benzaldehyde, and β-caryophyllene were present in the floral blends of most species (Fig. 3A). The Temporal Database (Table S3) shows the same classes as the most ubiquitous, however, with different percentages of compounds when compared to the Main Database. Benzyl alcohol, benzaldehyde, methyl salicylate, and 2-phenyl-ethanol were the benzenoids most common in temporal emissions, with differences among day/night emissions. (E)-β-ocimene, sabinene, limonene, β-myrcene, β-pinene, 1,8-cineole, α-pinene, linalool, and β-caryophyllene were the most common in Supplementary Data Table S3. Differences between day/night emissions occurred among (E)-β-ocimene, sabinene, 1,8-cineole, α-pinene, and β-caryophyllene (Fig. 3B).

When evaluated by primary pollinator's functional groups, the hawkmoth group shows a higher number of VOCs in all classes during the night period, except for the alkane and fatty

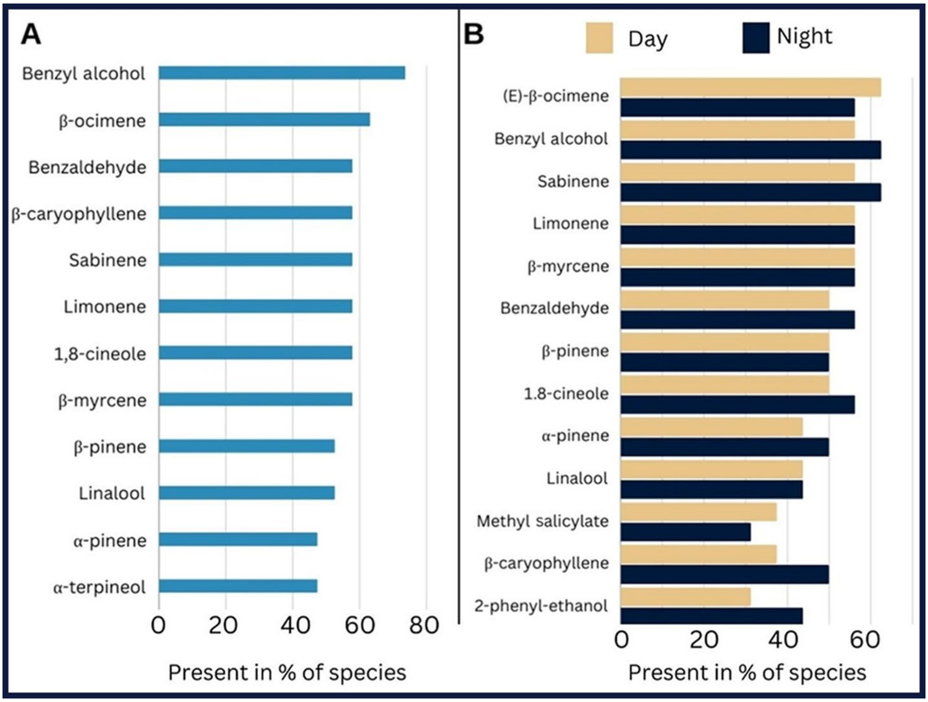

Fig. 3 Most ubiquitous compounds. **A**, percentage of species with the most ubiquitous compounds with total VOCs according to the Main Database – Table S2. **B**, percentage of species with the most ubiquitous compounds in emissions during day and night according to Table S3

acid derivatives class, which only appears in day emissions (Figs. 2B and 4A). The ubiquitous compounds for the hawkmoth group species in night emissions were β-caryophyllene, α-humulene (present in 75% of species), and benzaldehyde, benzyl alcohol, sabinene, β-myrcene (present in 62.5% of species). The nitrogenous compound 2-methylbutyl oxime is present in 50% of those species (Table S3). On the other hand, the hummingbird group showed lower night emissions for most classes, except for sesquiterpenes and oxygenated sesquiterpenes subclasses (Fig. 4B). A higher number of VOCs derived from fatty acid derivatives and alkanes were present in the daytime emissions. The most ubiquitous VOCs, present in more than 60% of hummingbird group species in daytime emissions, were benzyl alcohol, methyl salicylate, sabinene, β-myrcene, α-pinene, (E)-β-ocimene, linalool, α-terpineol. Limonene, β-pinene, and 1,8-cineole were present in 100% of the species. The irregular terpenoids were present only at night or day for hawkmoth group and hummingbird group species, respectively (Fig. 4A and B).

Species of the small moth group show high night emissions of benzenoids and terpenoids, especially monoterpenes, and higher daytime emissions of total terpenoids, fatty acid derivatives, and alkanes (Fig. 4C). Oxygenated sesquiterpenes were absent. Since only three

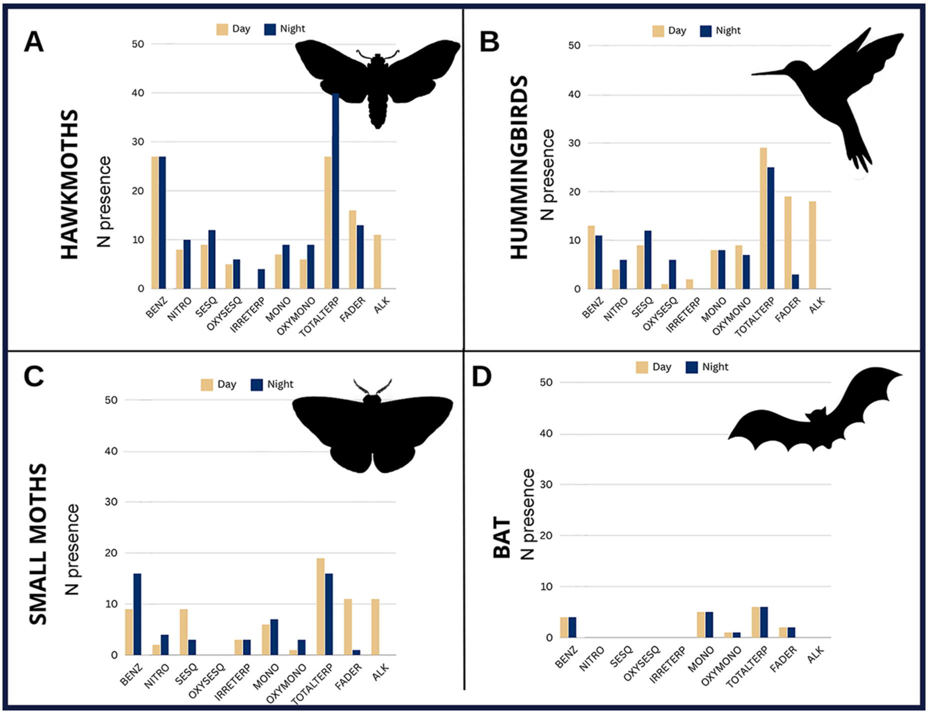

Fig. 4 Composition of VOCs in floral scent of *Nicotiana* species pollinated by (**A**) hawkmoth, (**B**) hummingbirds, (**C**) small moth, and (**D**) bat. The total VOCs for each pollinator group were presented according to circadian rhythm emissions, AM yellow and PM dark blue. Hawkmoths Group, Day, 5 species, *N*=89 compounds, and Night, 8 species, *N*=90 compounds. Hummingbirds Group, Day, 5 species, *N*=81 compounds, and Night, 4 species, *N*=45 compounds. Small Moths Group: Day, 3 species, *N*=52 compounds, and Night, 2 species, *N*=37 compounds. Bats Pollinated Group: Day and Night, 1 species, *N*=12 compounds. It did not include species with uncertain pollinators (*N. tomentosiformis*), and Sunbirds – *N. africana* because only one VOC was reported (Methyl heptanoate, Raguso et al. 2006)

species of small moth groups were recovered in this study, we took those compounds as most ubiquitous in at least two species. Those compounds are benzyl alcohol, phenylacetaldehyde benzyl benzoate, nicotine, and 4-oxo-isophorone, among others (Table S3). For only one bat-pollinated species, *N. otophora*, nitrogenous compounds, sesquiterpenes, oxygenated sesquiterpenes, and irregular terpenoids were absent (Fig. 4C).

When comparing the compounds produced by the functional pollinator groups, the one-way ANOVA (Table S4) test found no significant difference in the number of compounds in each chemical class or the total number of compounds. The post hoc test performed per chemical class also did not show significant differences for any pollinators' functional groups, with high p-values for all pairwise comparisons (Table S5).

Ordination Analysis. The results of CAP analysis of floral scent bouquets among pollinator groups are shown in Fig. 5 for total emissions (Fig. 5A) and separate temporal emissions (Fig. 5B and C). In the daytime emissions, the first constrained principal coordinate (CAP1) explained 72% of the total variation, mostly separated by 1.8 cineole; however, there was no significant statistical explanation of the variation among the groups of pollinators in the CAP intrinsic ANOVA test ($p=0.145$). In contrast, nighttime and total temporal emission CAP have significant statistical explanations in the permutation test ANOVA ($p=0.024$ and $p=0.05$, respectively). The nighttime emission CAP2 sums 67% of the variation, and the monoterpenoids and benzenoids mostly separate this axis. The CAP2 axis divides the species into all the day-pollinated species (hummingbird and sunbird pollinated group), *N. otophora*, *N. plumbaginifolia*, and *N. longiflora* in the upper quadrant, and all the night-pollinated species (hawkmoth and small moth groups).

The same constrained analysis was performed using the known phylogenetic relationship of *Nicotiana* species as a priori groups (*Nicotiana* sections), also using the total emissions (Fig. 6A) and daytime and nighttime emissions (Fig. 6B and C). In the total emissions (Fig. 6A, dataset DS1), the CAP1 sums 79% of the variation and is separated by benzyl acetone and the oxygenated monoterpene 1.8-cineole. However, there was no significant statistical explanation of the variation among a priori groups in the ANOVA test ($p=0.051$). There was, however, significant statistical variation in both daytime and nighttime emissions separately (Fig. 6B, $p=0.02$; Fig. 6C $p=0.016$, respectively). The nighttime emissions (Fig. 6B) CAP1 explains 91% of the variation, and 1.8-cineole separates all *Alatae* species in the same quadrant. The daytime emissions (Fig. 6A) in the constrained first axis (CAP1) sum more than 100% of the variation and also separate most of the *Alatae* species from the others by 1.8-cineole and β-myrcene to the negative quadrant.

The hierarchical cluster analysis was performed for the three separate datasets of temporal emission, and the dendrograms are shown in Fig. S1. In the total temporal emissions (Fig. S1A), section *Alatae* has many species clustered together, with the only exception being *N. bonariensis*, which is clustered closest with *N. suaveolens*. In the dendrogram for the daytime and the nighttime emissions, separately (Figs. S1B and C), species from *Alatae sections* hummingbird-pollinated clustered closest. *Nicotiana alata* and *N. forgetiana* are clustered together in all the dendrograms. The results of the daytime emissions cluster (Fig. S1B) recovered species from different sections together, like *Nicotiana suaveolens* and *N. sylvestris*, and *N. bonariensis* and *N. otophora.* The same occurs in the emissions (Fig. S1C) with the species *N. otophora* and *N. sylvestris*.

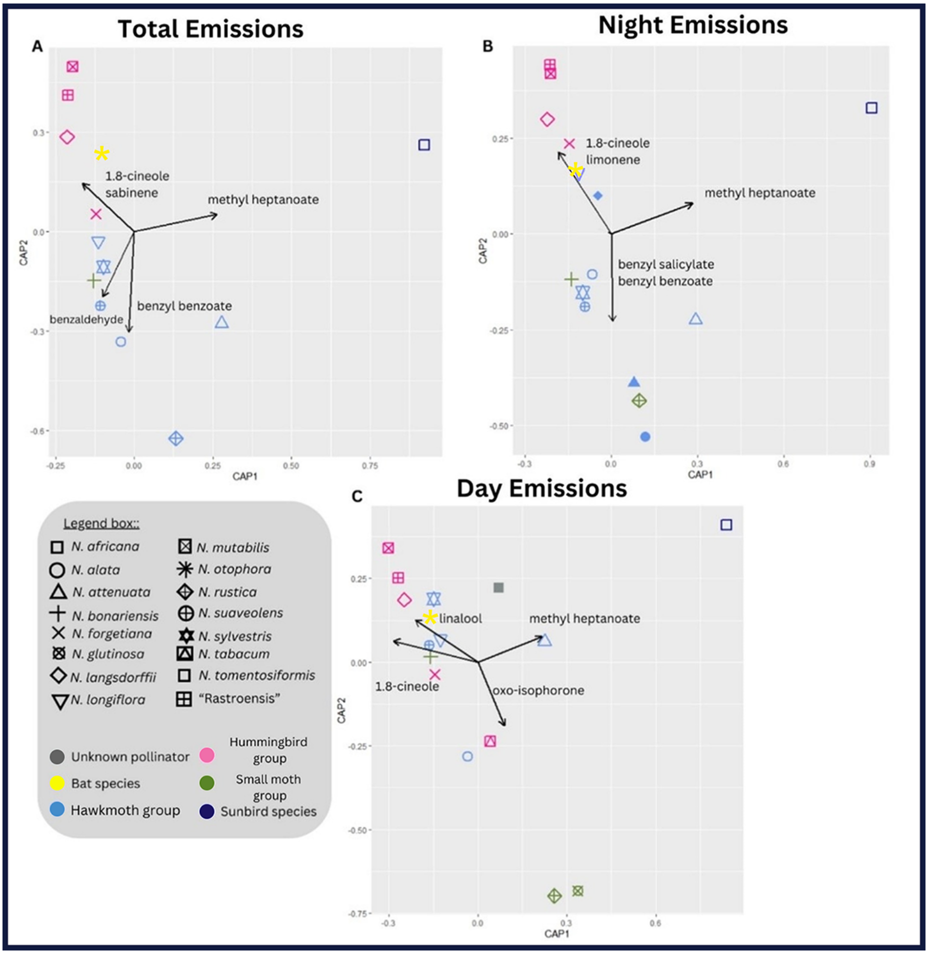

Fig. 5 Canonical analysis of principal coordinates (CAP) separating *Nicotiana* species floral scent by pollinator groups according to the temporal emission: total temporal VOCs emission (**A**) and time of day (daytime and nighttime, **B** and **C**). The analyses used Bray-Curtis distances. Pollinator groups are indicated by color and species are indicated by shape

Discussion

In this review, we intended to explore the chemical diversity of wild tobacco's floral blends, species for which interactions with pollinators are key for species diversification. Pollinators probably play a dual role in genus evolution, acting both as a driver of floral specialization and also as a vector of hybridization, where both processes contribute to species diversity (Kaczorowski et al. 2005; Kelly et al. 2009; Clarkson et al. 2017; Turchetto et al. 2022).

We found 179 floral VOCs from 19 species of *Nicotiana*, covering 61% of the sections (Table 1). Despite fair relative coverage across the studied group, some gaps were detected. At the present moment, there are ~43% allopolyploid species in *Nicotiana* (Knapp 2020), and only six of them have their floral VOCs emissions studied, including a lack of studies

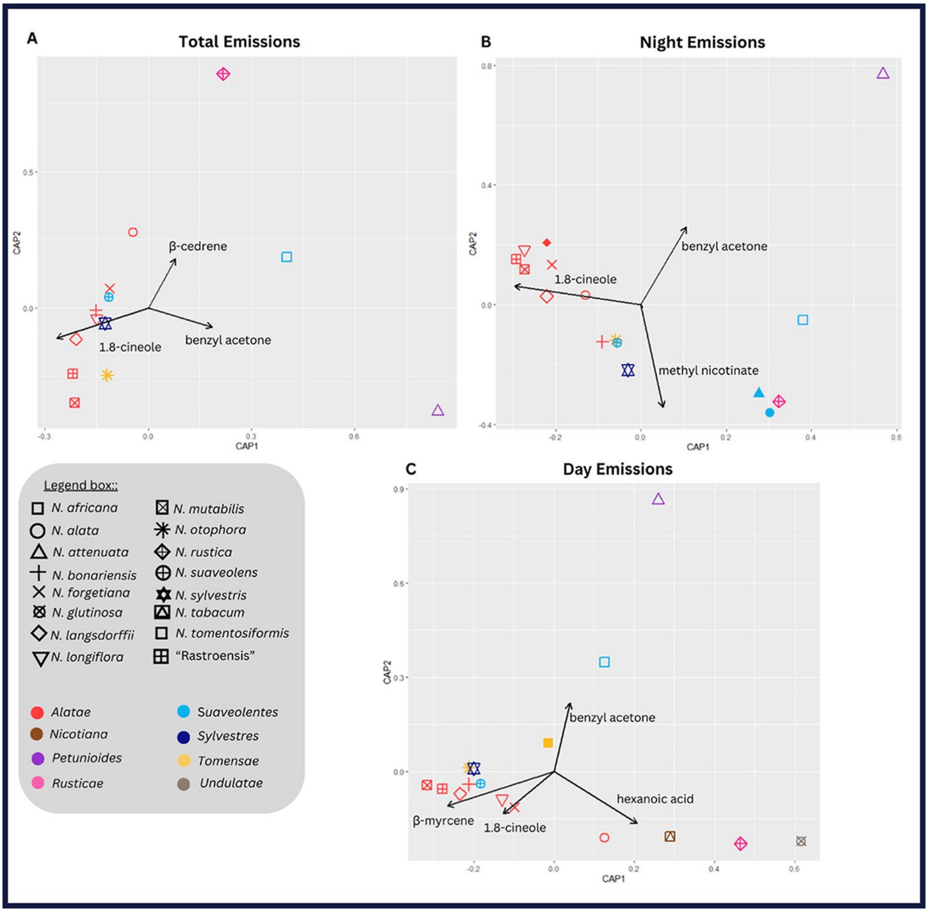

Fig. 6 Canonical analysis of principal coordinates (CAP) separating *Nicotiana* species floral scent by phylogenetic groups according to the temporal emission: total temporal VOCs emission (**A**) and time of day (daytime and nighttime, **B** and **C**). The phylogenetic groups are according to subdivision of the *Nicotiana* genus in different sections according to Knapp et al. (2004). The analyses used Bray-Curtis distances. Phylogenetic groups are indicated by color and species are indicated by shape

on the section with the highest number of allopolyploid species (*Suaveolentes*). Transgenerational genomic events such as allopolyploidization tend to generate changes in the gene methylation due to "genomic shock", and the epigenetic modifications may cause different gene expressions and regulations of VOCs biosynthetic pathways (Picazo-Aragonés et al. 2020). The impact of allopolyploidization on different Nicotiana's floral traits has been the aim of studies focusing on flower morphology and color. Transgressive phenotypes were observed in allopolyploid species of *Nicotiana* (McCarthy et al. 2016, 2017, 2019), suggesting a shift in the pollination system. These major changes in the floral phenotypes following genomic events (color change related to VOC's blend modification) can be linked to the competition of enzymes for substrate in the pigment biosynthetic pathways (Ben Zvi et al. 2008; Berardi et al. 2021). The impact of hybridization and polyploidization in the VOCs expression has been studied in other species, and the same variations were found, such as the presence of transgressive phenotypes in natural hybrids of *Ipomopsis* (Bischoff et al. 2014) and different emissions in hybrids of *Hedychium* (Wei et al. 2023). This begs the question of the gap in the knowledge of *Nicotiana*'s allopolyploids that still needs to be addressed.

Also, very few *Nicotiana* species had their total temporal emissions described. Furthermore, all studies were performed with greenhouse-germinated plants, addressing the innate patterns of floral VOCs by excluding biotic and abiotic environmental factors. If, on the one hand, this allows us to compare the floral VOCs among species; on the other, by excluding those natural components, edaphic and temperature variations, secondary pollinators, and antagonists' interactions, we are deprived of the complete understanding of *Nicotiana*'s floral VOCs. Here, we showed the different signals of phylogeny and pollinators influences on floral VOCs emissions. The more considerable bias in understanding pollinators' role in floral VOCs emissions is the lack of sampling of many taxa within the genus. Moreover, there are no pollination biology experiments in the field for many groups, such as the allopolyploid Suaveolentes section, despite the expectation that their phenotype indicates that moths will pollinate them.

Emissions of floral scent by one specific flower are not an isolated trait; instead, they form a channel of communication with the rest of the plant and the environment. Thus, the floral blend and its relationship with floral visitors in one species or local population can be impacted by many factors. Environmental factors, such as drought and overly wet seasons, can influence the flowering time for species and the regulations of biosynthetic pathways of VOCs. Under different climatic conditions, plants can adapt VOC floral emissions, changing the floral VOC's relative richness and emission time to fit the conditions better. Not only that, but studies in situ performed with the same species in different localities show significant variations that could be connected to phenotypic plasticity due to climatic conditions (Brody 1997; Delle-Vedove et al. 2017; Campbell et al. 2018; Farré-Armengol et al. 2020; Bing et al. 2021), interactions with herbivores, local plant community (Kigathi et al. 2019; Burkle et al. 2020), and microbiome associated with plants (Helletsgruber et al. 2017). These complex relationships between floral scent and floral visitors, the main vectors of gene interchange between populations and species, can potentially impact the diversification processes in angiosperms, such as pollination-mediated speciation and hybridization. This pool of information is critical in a changing world that impacts species' geographic distribution and relationship with the local community since environmental change can influence plant-pollinator interactions across spatial and temporal scales.

All *N. attenuata* plants analyzed in different studies originated from the same genotype, and this also was true for most species from section *Alatae*, in which the seeds cultivated come from the same accession or locality. Polymorphisms in floral emissions were previously found for different populations and species; these variations could be driven not just by external pressures, such as herbivory and florivory, but also by natural intraspecific variations (Delle-Vedove et al. 2017), or other factors not yet investigated as local plant community and introgression events. Phenotype variations as a response to local pollinators' pressure have already been studied for the corolla shape of different populations of *N. glauca* (García et al. 2020), remarking the genus plasticity and adaptation capacity for pollinator's best fit. To fully comprehend VOCs emissions in *Nicotiana*, we need further integration of population genetics in floral blend emissions studies, as proposed by Whitehead and Peakall (2009). Since the innate expressions of the species are already reported, as shown in our review, the expansion for in situ and studies from different populations should fill the gaps in our knowledge of *Nicotiana*'s floral emissions.

In addition, many key *Nicotiana* species still lack information about their floral emissions. Especially regarding polyploid species, which are even less represented in this review. Thus, jointly evaluating the information on volatiles can be crucial to understanding the evolution of a particular group of plants, especially those with an intimate relationship with pollinators as a drive of diversification, such as *Nicotiana*.

The role of pollinator and phylogenetic relationship in floral VOC emissions

In order to debate the relation of phylogeny and pollination with floral VOCs, we should first address the greater panorama of floral VOCs in *Nicotiana*. Terpenoids and benzenoids were the most common classes in *Nicotiana* floral blends, which is congruent with the knowledge of total floral blends in Angiosperms (Knudsen et al. 1993). Some of the most ubiquitous terpenoids are commonly found in hawkmoth group species, 1,8-cineole and E-β-ocimene (Knudsen and Tollsten 1993; Schlumpberger and Raguso 2008). However, other ubiquitous terpenoids (limonene, sabinene, α- and β-pinene, β-myrcene, α- terpineol) belong to the assemblage of compounds of 1,8-cineole synthase metabolism of geranyl diphosphate (Wise et al. 1998). That relation of compounds raises the question of the current understanding of floral blends concerning the ecological function of a single compound based on its presence and concentrations, as already raised by Raguso et al. (2003). The interconnected expression of compounds that belong to the same biosynthetic pathway can offer more insights into plant relations. We might consider higher expressions of minor products of pathways, linking potential ecological functions to compounds that are subproducts for others. Considering biosynthetic pathways branches and not individual compounds could further our understanding of the potential ecological functions of ubiquitous compounds (Barkman 2001). Moreover, it is also important to consider these VOCs as correlated traits, since a single gene and enzyme (1,8-cineole synthase) were responsible for this trait, to investigate whether they are expressed outside of *Alatae* section since is present in *N. suaveolens* but not universally present in section Suaveolentes (Roeder et al. 2007).

Emissions of *Nicotiana's* moth-pollinated species, both hawkmoth and small moth groups, had similar VOC profiles and were grouped in the CAP analysis. Those two pollination groups involve flowers with white or "dull colors" for human eyes with flavonols as UV-pigments (McCarthy et al. 2015; van der Kooi et al. 2016). Those hues contrast with the nocturnal environment, and similar scents often referred to as "white floral scents" (Knudsen and Tollsten 1993; Knudsen et al. 1993; Raguso and Willis 2002). That specific blend is rich with linalool, benzyl alcohol, 2-phenyl alcohol, acyclic terpene alcohols, and hydrocarbons, all present in the emission bouquets recovered here. Other expected consistency with previous findings, hawkmoth group flowers had oxygenated sesquiterpenes in their emissions, with a slight enhancement in nighttime's emissions, while small moth group flowers had none of those compounds reported in their emissions (Knudsen and Tollsten 1993; Kaiser 1993). However, the compounds commonly related to small moths' attraction, lilac aldehydes, and lilac alcohols, were not reported in small moth group flower emissions (Dötterl et al. 2006), although there is little to no information on the pollination biology of the small moth group species reported in this work. These species *(N. bonariensis, N. rustica* and *N. glutinosa*) have their pollinators inferred by morphology. Therefore, even though there is no indication of small moths attracted to those flowers' blends, they are consistently assumed to be night-pollinated species in the specialized literature.

Interestingly, the differentiation in the floral blend of some hawkmoth group species, *N. alata* and *N. attenuata* shows that even in a specialized system, there is space for variation in the ecological relationships. The relationship between floral blend emissions of *N. attenuata* and herbivory and pollinators are fascinating. Previous studies have found low emissions of benzyl acetone in flowers at night when leaves of plants are attacked by larvae of *Manduca sexta*, impacting the visits of nocturnal adults of the same species that act as pollinators. These relationships change the antheses of the flowers, which remain open the next day morning and thus are pollinated by hummingbirds (Kessler et al. 2010). These examples open the question of another sporadic visitor potentially serving as a vector to hybridization if pairs of species grow close to each other. These nuanced details of the environmental impact of floral VOCs emissions could be addressed in future studies of other *Nicotiana* species with comparable technological tools as have been addressed to *N. attenuata*.

Compounds commonly related to bat pollinations, sulfur-bearing compounds, are not reported for *N. otophora* or any other *Nicotiana* species (von Helverson et al. 2000; Jassbi et al. 2017). Besides bats, hawkmoths were also reported as night visitors for *N. otophora* (Nattero et al. 2003). However, despite visual records of the hawkmoth's visitations, this species does not present most compounds usually associated with the "white flower scent" and hawkmoth pollination, like 2-phenyl ethanol and most acyclic terpene alcohols. Oxygenated sesquiterpenes, often in hawkmoth group flowers, are also absent (Knudsen and Tollsten 1993). Notably, few chemical studies were conducted about *N. otophora*'s floral blend; only one study was recovered, so those numbers could be deflated and not represent the totality of emissions.

Overall, species from *Alatae* section have similar daytime emissions patterns, tending to emit more species-specific emissions at nighttime (Raguso et al. 2003). Therefore, it is unsurprising that all *Alatae* species are grouped in the analysis with day emissions dataset. However, the night emissions also demonstrate high clustering, separated by the 1.8-cineole. Interestingly, *N. alata* and *N. forgetiana* appear together in a hierarchical cluster, showing how chemically similar those two species are, even though they belong to different pollination groups with opposed attracting cues and circadian rhythm and also are not phylogenetic sister groups (Clarkson et al. 2004, 2017; McCarthy et al. 2019). Indeed, it is known that these two species produce viable hybrids in controlled conditions (Ippolito et al. 2004), and a putative hybrid population has been found in a contact zone of *N. alata* and *N. forgetiana* in nature (Teixeira et al. 2022; Couto et al. 2023). Apart from *N. forgetiana*, other species also hummingbird pollinated (*N. langsdorffiii*, *N. mutabilis*, and the putative new species "Rastroensis") are positioned together, forming a hummingbird cluster in all dendrograms. Flowers with a bird as their pollinator functional groups are usually scentless (Knudsen et al. 2004; Cronk and Ojeda 2008). As previously mentioned, *Alatae* has similar daytime emissions, meaning that both night- and day-pollinated flowers emit during the daytime, including flowers that are not expected to do so. The similarity in VOCs emissions during the day could have other ecological roles, such as herbivory deterrence (Knudsen et al. 2004) or phylogenetically constrained emissions, a hypothesis already raised by Raguso et al. (2003, 2006).

Nicotiana bonariensis is unrelated to any other *Alatae* species in the dendrograms ordinations. Even in the constrained analysis where the sections were used as a priori groups, this species was positioned closer to *N. otophora* and *N. suaveolens* in nighttime emissions (when the VOC blends are more species-specific). In the hierarchical clusters, it holds its positions near other white-flowered and moth-pollinated species, *N. sylvestris* and *N. suaveolens*. *Nicotiana bonariensis* exhibit floral traits related to the small moth pollination system and most of the expected emissions patterns associated with that system: the "white floral scent", absence of oxygenated sesquiterpenes, and nitrogenous compounds. However, no lilac aldehydes or lilac alcohols were found in its blends, and those compounds are related to the specific attraction of small moths (Dötterl et al. 2006). *N. bonariensis* does not seem to have emissions patterns related to its phylogenetic relations but is more related to the pollinator functional groups.

Nicotiana suaveolens was not positioned close to their section-related species in ordinations analysis, *N. cavicola*, *N. africana*, and *N. ingulba*. That could be due to the difference in chemical information offered on those species, *N. suaveolens* is present in seven studies, and the other species had their floral blend present in only one study. However, since the studies performed on *N. cavicola*, *N. africana*, and *N. ingulba* have an exploratory nature and include *N. suaveolens* in the species pool (Raguso et al. 2006), those results are directly comparable. *N. africana* had only one VOC reported (methyl heptanoate), which is congruent with the expected for flowers bird-pollinated (Knudsen et al. 2004; Cronk and Ojeda 2008). *N. cavicola* and *N. ingulba* only have reported night emissions and are closely related, forming a hummingbird group strongly related to *N. rustica* (monospecific section *Rusticae*). The referred small moth group of three species is more similar within themselves than the others, separating early from the remaining species. It is important to highlight the hybrid origin of the polyploid section *Suaveolentes*, with contribution from sections *Alatae, Sylvestres, Noctiflorae*, and *Petunioides* (McCarthy et al. 2016; D'Andrea et al. 2023). In previous studies on volatile emissions in nature, hybrids show a great diversification from the parentals, some of which exhibit intermediate emission of volatiles concerning the parental species (Bischoff et al. 2013). The number of studies on volatile emissions in the parental sections is still too scarce to create a solid argument; however, this raises an exciting path to further research. On the other hand, there is a similarity of floral blends in flowers with the same pollinator functional groups from different sections.

These results demonstrate that phylogeny does have some influence on VOC emissions to some extent since species pollinated by different pollinators present a great similarity in the emission bouquet, such as *N. forgetiana* and *N. alata*. However, the same can be inferred about the reflection of pollinators' functional groups on the selection of floral scent traits, with many species that are phylogenetically distant but a shared pollinator functional group having similar scent blends, such as *N. cavicola* and *N. rustica*.

Nicotiana is a genus with a great number of species with a hybrid origin (Chase et al. 2003; Kelly et al. 2013; Clarkson et al. 2010); therefore, it is crucial to understand how these species interact not only with their primary pollinator but also the secondary floral visitors. Seven *Nicotiana* species with specialized pollination have bee visitation recorded (Turchetto et al. 2022). Most of them belong to *Alatae* section and are pollinated by hummingbirds, hawkmoths, and small moths. The species outside *Alatae* also visited by bees is *N. rustica*, from the *Rusticae* section. The compounds Β-ocimene, linalool, farnesol, methyl benzoate, and benzyl acetate are related to many bees' attraction, such as honeybees and Euglossini bees (Bisrat and Jung 2022); however, these compounds are also commonly found in flowers (Knudsen et al. 1993). These compounds are ubiquitous among angiosperms, with no basis for species-specific attraction. However, this does not discard the possibility of bees' interaction once bees use floral signals like color and scent to create association patterns between florals and resources like pollen and nectar (Arenas and Farina 2012; Raguso et al. 2008a, b). Even though linalool is present in many flower scents, its emissions were still enhanced due to bee selection in *Penstemon digitalis*, as shown by Parachnowitsch et al. (2012). In fact, we have observed bees foraging pollen in a contact zone between *N. alata* and *N. forgetiana* (Turchetto et al. 2022), which were close in ordination analyses. It is relevant to consider that pollen odors can significantly impact bees foraging behavior (Rodrigues et al. 2018; Muth et al. 2016). In general, the floral volatiles studied here represent the amount of floral blend of the whole flower, and we did not know the differences between petals, pollen, or nectar emissions. Expanding our knowledge of *Nicotiana*'s pollen scent is important to grasp better how these flowers showcase their resources to the environment around them and how scent emissions of other parts of flowers impact the floral visitors, as demonstrated by Kessler and Baldwin (2007).

This review showed a complex relationship between floral VOCs in *Nicotiana*, which can be related to pollination mode, the evolutionary history of species, and species specificity. An important study gap to address in the future is to investigate what we can expect from plant floral VOC emissions and their relationship to the pollinator communities in the face of global change. For example, climatic changes can affect the geographic distribution, the connections of isolated plant populations and species, and also the herbivores and pollinators, impacting their complex interaction and, consequently, plant diversification. We strongly advocate the importance of integrated studies in predicting climate change and its impact on plant diversity. We consider intraspecific variations in genetic diversity and interaction networks of floral signals, such as volatiles, and the plant community of potential pollinators. Future intraspecific studies investigating the phenotypic plasticity of floral blend and the floral blend emitted by different components of flowers, such as pollen, petals, and nectar, should provide valuable information to better understanding and predict the impact of environmental changes on plant-pollinator interaction and consequently the plant diversification.

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Declarations

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Conflict of interest The authors declare no conflict of interest.

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