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Diel Variation in Fig Volatiles Across Syconium Development: Making Sense of Scents

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Abstract Plants produce volatile organic compounds (VOCs) in a variety of contexts that include response to abiotic and biotic stresses, attraction of pollinators and parasitoids, and repulsion of herbivores. Some of these VOCs may also exhibit diel variation in emission. In *Ficus racemosa*, we examined variation in VOCs released by fig syconia throughout syconium development and between day and night. Syconia are globular enclosed inflorescences that serve as developing nurseries for pollinating and parasitic fig wasps. Syconia are attacked by gallers early in their development, serviced by pollinators in mid phase, and are attractive to parasitoids in response to the development of gallers at later stages. VOC bouquets of the different development phases of the syconium were distinctive, as were their day and night VOC profiles. VOCs such as α-muurolene were characteristic of the pollen-receptive diurnal phase, and may serve to attract the diurnally-active pollinating wasps. Diel patterns of release of volatiles could not be correlated with their predicted volatility as determined by Henry's law constants at ambient temperatures. Therefore, factors other than Henry's law constant such as stomatal conductance or VOC synthesis must explain diel variation in VOC emission. A novel use of weighted gene co-expression network analysis (WGCNA) on the volatilome resulted in seven distinct modules of co-emitted VOCs that could be interpreted on the basis of syconium ecology. Some modules were characterized by the response of fig syconia to early galling by parasitic wasps and consisted largely of green leaf volatiles (GLVs). Other modules, that could be characterized by a combination of syconia response to oviposition and tissue feeding by larvae of herbivorous galler pollinators as well as of parasitized wasps, consisted largely of putative herbivore-induced plant volatiles (HIPVs). We demonstrated the usefulness of WGCNA analysis of the volatilome in making sense of the scents produced by the syconia at different stages and diel phases of their development.

Keywords Diel variation · Diurnal volatiles · Fig volatiles · Henry's law constant · Herbivore-induced plant volatiles (HIPVs) · Nocturnal volatiles · Volatilome analysis · Weighted gene co-expression network analysis (WGCNA)

Introduction

Plants emit volatile organic compounds (VOCs) from various vegetative and reproductive tissues (Steeghs et al., 2004; Knudsen et al., 2006; Borges et al., 2008, 2011). These VOCs include terpenes, oxylipins, and benzenoids besides low molecular weight compounds such as methanol and acetaldehyde (Dudareva and Pichersky, 2000; Dudareva et al., 2004, 2006; Loreto and Schnitzler, 2010). While VOC emission can be affected by light and/or temperature (Kesselmeier and Staudt, 1999; Ibrahim et al., 2010), their production may also be under circadian control (Wilkinson et al., 2006; Nagegowda et al., 2010).

Many flowers and fruits produce VOCs at times of the day and night when pollinators or seed dispersers are most active (Matile and Altenburger, 1988; Loughrin et al., 1990; Helsper et al., 1998; Raguso et al., 2003; Raguso, 2008; Borges et al., 2011). Herbivore-induced plant volatiles

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(HIPVs) including egg-induced VOCs may be released during the day or at night and could attract plant bodyguards in the form of predators and parasitoids, repel herbivores, as well as prime neighboring plants or plant tissues for defense against herbivore attack (de Moraes et al., 2001; Frost et al., 2007; Heil and Ton, 2008; Rodriguez-Saona et al., 2009; Dicke and Baldwin, 2010; Hilker and Meiners, 2011).

While plant VOCs clearly mediate interactions with pollinators, seed dispersers, herbivores, parasites, and other plants as well, it is still debated whether all plant VOCs at specific developmental or diel phases are actively produced and emitted for such purposes (Pichersky et al., 2006). The physicochemical and physiological properties of VOCs, such as their gas–water partition coefficients or their role as precursors of essential compounds such as sterols and hormones, as well as their role in protection against abiotic stresses may influence their individual emissions (Niinemets et al., 2004; Owen and Peñuelas, 2005; Noe et al., 2006; Loreto and Schnitzler, 2010).

It is, therefore, necessary to examine additional factors that may influence individual VOC emission as well as co-emission patterns. For example, many VOCs such as terpenes are hydrophobic, and therefore, their partitioning between the gas and liquid phase in plant extracellular spaces could affect their release from pre-existing pools (Loreto and Schnitzler, 2010). This equilibrium between the gaseous and aqueous phase of a VOC is captured by its Henry's law constant (k_H), which describes the solubility of a gas in water and thereby its volatility. Since Henry's law constant is also affected by temperature (Copolovici and Niinemets, 2005), and if one assumes *ceteris paribus* (i.e., that other factors such as VOC diffusivity through cell membranes, or *in situ* VOC synthesis are kept constant), it is possible to examine whether aqueous/gas phase partition coefficients at ecologically relevant temperatures are correlated with VOC emission. Such investigations could constitute basic null hypotheses in the understanding of VOC emissions. Since the volatilome of a plant is usually complex and composed of many compounds, finding sets of co-emitted VOCs across development phases is another important aspect of understanding VOC emission complexity.

We adopted a novel use of weighted gene co-expression analysis (WGCNA) from the microarray analysis tool-kit to find patterns of co-emission of VOCs. We elected to examine diel variation in VOCs produced by an enclosed inflorescence called a syconium within the fig nursery pollination system. The *Ficus* syconium (globular enclosed inflorescence) is the structure within which seeds are produced and wasps (Hymenoptera: Agaonidae) develop. In typical monoecious figs, pollinating female wasps enter the syconium, pollinate some female flowers resulting in seeds, and gall other flowers into each of which an egg is laid. The pollinator offspring develop within the galled flowers by feeding on plant tissue, and thus constitute a nursery pollination system (Cook and Rasplus, 2003). Non-pollinating fig wasps that include gallers and parasitoids arrive for oviposition into the syconium either before or after the pollinator in a specific sequence based on their trophic position, size, longevity, and other life history parameters (Kerdelhué and Rasplus, 1996; Proffit et al., 2007; Ghara and Borges, 2010; Ranganathan et al., 2010). The fig syconium has distinct development phases: A phase or pre-pollen receptive phase; B phase or pollen receptive phase when pollinators oviposit and perform pollination services; C phase or interfloral phase when pollinating and parasitic wasps complete their development; D phase when developed wasps eclose and leave the syconium; and E phase when seeds are dispersed (Galil and Eisikowitch, 1968). The fig–fig wasp system is highly species-specific with usually only one pollinator species and a set of parasitic wasp species associated with each species of fig (Herre et al., 2008).

We examined the VOCs emitted by the developing fig inflorescences (syconia) that are host to herbivorous pollinating wasps, as well as a community of parasitic galler and parasitoid wasps that develop in the syconium. In such a community, attraction of pollinators and parasitism by wasps is mediated by VOCs (Grison-Pigé et al., 2002a,b; Proffit et al., 2007, 2008; Hossaert-McKey et al., 2010). VOC signatures were compared across the development of fig syconia as well as between day and night in distinct development phases to determine whether there are phase-specific as well as diel-specific VOCs, as might be expected since pollinators, for example, only visit pollen-receptive figs during the day (Ghara and Borges, 2010; Ranganathan et al., 2010). Since galler (herbivorous) wasps oviposit early in syconium development while parasitoid wasps do so later, it is possible that the VOC profiles of the syconium in later development stages contain HIPVs. Therefore VOC profiles were examined at four stages of syconium development to detect early galler-, pollinator-, and parasitoid-relevant VOC signatures. To make further sense of the VOC emissions, we also asked whether the release of a VOC is related to its physicochemical properties, such as its gas–water partition coefficients (i.e., Henry's law constants) at day and night temperatures. We also examined whether there are discernible co-emission patterns of VOCs during syconium development.

Methods and Materials

The Study System The fig wasp community of *Ficus racemosa* L. (Section: Sycomorus) from South India was chosen for the study, as this species has emerged as a useful model system in chemical ecology (e.g., Proffit et al., 2007; Ranganathan and Borges, 2009; Krishnan et al., 2010;

Borges et al., 2011; Ghara et al., 2011). The community comprises one pollinating wasp species (*Ceratosolen fusciceps*) and six species of non-pollinating fig wasps (gallers: *Apocryptophagus stratheni*, *Apocryptophagus testacea*, *Apocryptophagus fusca*; parasitoids: *Apocryptophagus agraensis*, *Apocrypta* sp. 2, *Apocrypta westwoodi*) (Ghara and Borges, 2010). Although gallers and parasitoids oviposit in the syconium from A to C phases, we sub-divided the C phase into the longer C1 phase (when parasitoids continue to oviposit) and a short C2 phase (when oviposition by all wasps has ceased). The syconium takes about 50 days to develop from A through C phases (Ranganathan et al., 2010). The wasps arrive in the following sequence and are present at the following phases for oviposition: *A. stratheni* (pre-A phase), *A. testacea* (A phase), *A. fusca*+*C. fusciceps* (B phase), *Apocrypta* sp 2 (late A, B, and C1 phases), *A. agraensis*+*Apocrypta westwoodi* (C1 phase) (Ghara and Borges, 2010; Ranganathan et al., 2010).

Volatile Collection Volatiles of *F. racemosa* were collected from A–C2 phases from 10 trees, one fig bunch per tree, within the campus of the Indian Institute of Science, Bangalore (12º 58′ N and 77º 35′ E). Two samples per phase from the same syconia were collected (1100 h and 2300 h on the same day) by dynamic headspace adsorption *in situ*. Figs were enclosed in a polyethylene terephthalate (Nalophan™) bag (Kalle Nalo GmbH, Wursthullen, Germany) through which a constant airflow over an Alltech Super Q® volatile collection trap (ARS Inc., Gainesville, FL, USA) was maintained for 1 h per sample at incoming and outgoing flow rates of 111 ml min^{-1} and 94 ml min^{-1}, respectively, using glass flowmeters (Aalborg Instruments and Controls, Orangeburg, NY, USA) controlled by a micropump (KNF Neuberger GmbH, Germany; model number NMP50KNDC 12VDC). Incoming air was cleaned using activated charcoal filters (Sigma-Aldrich). VOC traps were eluted with 150 μl of dichloromethane, and the eluate concentrated by solvent evaporation at room temperature to 10 μl, to which 0.5 μl of the internal standard cumene was added at a concentration of 200 ng ml^{-1}.

A 4.0 μl quantity of this mixture was injected into a gas chromatography–mass spectrometry instrument (Agilent-HP GC model 6890N, MS model 5973N) operating throughout in a splitless mode fitted with an HP-5MS column of 60 m length and 250 μm internal diam with a 0.25 μm film thickness. Helium was used as carrier gas at a flow rate of 1.5 ml min^{-1}. Optimal VOC separation was achieved using the following temperature program: 40 °C held for 3 min, followed by a rise to 100 °C at 3 °C per min, 140 °C at 2.7 °C per min, 180 °C at 2.4 °C per min, and 210 °C at 6 °C per min. The inlet temperature was set at 250 °C, and the inlet transfer line from GC to MS was set at 280 °C. Compound identification was based on retention times that were converted into Retention Indices (see Supplementary Information) as well as the National Institute of Standards and Technology (NIST) library (Version 2.0a) of mass fragmentation spectra. Quantification of all compounds (pg g^{-1} h^{-1}) was based on their peak areas relative to the peak area of known amounts of internal standard added to the sample. Since VOCs were collected *in situ* from the same syconia throughout their development and could not be detached for weighing, representative syconia from neighboring syconial bunches were weighed to obtain syconial mass at each developmental phase. For each group (developmental phase or diel time of collection), only those VOCs that were present in at least 90 % of each set of samples were considered. This was to obtain a conservative representation of phase- and diel-specific VOCs and to reduce inter-tree variation.

Syconial Phase-Specific and Diel Volatile Signatures The MDSplot (multi-dimensional scaling) function of the randomForest software package for R was used to perform an unsupervised classification of the samples based on proportional abundance (%) and quantity (pg g^{-1} h^{-1}) data. Random Forest is a machine-learning algorithm used in data-rich fields such as bioinformatics or chemoinformatics to select the most appropriate classification variables from surrounding noise in a highly variable dataset (Ranganathan and Borges, 2010, 2011). Two classifications were performed using the varSelRF software package for R to differentiate pre-receptive (A), receptive (B), inter-floral1 (C1), and inter-floral2 (C2) phases. The varSelRF algorithm finds the smallest set of predictor variables (VOCs in this case) for each classification. In an *all vs. all* classification where the sample identities were not masked, predictor VOC variables were found that distinguished all phases from each other. In the *one vs. the rest* classification, the sample identity of *the rest* was masked compared to samples from the *one* phase under consideration. The characteristic set of VOCs that defined the *one* phase from *the rest* was determined (see Ranganathan and Borges, 2010, 2011 for more details). The same approach was used to analyze diel differences in VOC emission. All statistical analyses were performed using the freely available statistical program R version 2.13.1 (R Development Core Team, 2010). The packages for R mentioned here are also freely available from CRAN, the Comprehensive R Archive Network (http://cran.r-project.org/).

Henry's Law Constants and Diel VOC Production Henry's law constants of the VOCs were calculated using the HENRYWIN™ module (version 3.20) of the United States Environmental Protection Agency's Estimation Program Interface (EPI) Suite (Meylan and Howard, 2005). The Chemical Abstracts Service (CAS) Registry number of each

VOC was obtained from http://www.pherobase.com. Using this CAS number, the Simplified Molecular Input Line Entry Specification (SMILES) format was acquired from http://www.thegoodscentscompany.com. This SMILES format was used as the input for the HENRYWIN software. The bond contribution method was employed since the group contribution method was not applicable for all VOCs in our samples. The bond contribution method is the overall best-performing method as implemented in this software (Dearden and Schüürmann, 2003). The inverse of Henry's Law constant, $k_{H,inv}$=partial pressure of the gas/aqueous concentration, describes the volatility of a compound. Analysis and results of Henry's coefficient in this paper are presented as $H=k_{H,inv}$=partial pressure of the gas/aqueous concentration (Pa $m^3 mol^{-1}$).

A log relationship was established for the temperature dependence of Henry's law constants (0–50 °C at 5° intervals) of the 54 VOCs present in our samples. Using this equation, Henry's law constants were calculated at the median diurnal (H_d) and nocturnal (H_n) temperatures during the sampling period for each syconium phase (A–C2). Temperature data were obtained from the Centre for Atmospheric Sciences (CAOS) in the Indian Institute of Science campus where the fig VOCs were collected. Since the 54 VOCs had widely varying Henry's law constants, the relative decrease in the constant was calculated for each VOC, i.e., by what proportion did the Henry's law constant for a VOC drop with a known temperature difference between diurnal and nocturnal hours (temperatures are lower at night compared to the day). We reasoned that if VOC emissions (expressed as pg g^{-1} h^{-1}) are solely directed by their Henry's law constants, then their temperature-dependent decrease at night should fall within the confidence limits of the predicted decrease according to Henry's law.

Determination of Co-emitted Sets of VOCs To determine whether certain VOCs are co-emitted during the different phases of syconium development, perhaps due to membership in common biosynthetic pathways, we adopted a method being employed in microarray analysis to analyze co-expression of genes. The weighted gene co-expression network analysis (WGCNA) (Zhang and Horvath, 2005; Langfelder and Horvath, 2008; Weston et al., 2008) was used to perform a volatilome analysis where VOC emission (pg g^{-1} h^{-1}) was employed as an analog of gene expression in a microarray proteome or transcriptome analysis. Co-emission VOC networks were built using the WGCNA package version 1.13 of R (Langfelder and Horvath, 2008). A pair-wise correlation matrix was created between VOCs by using the entire data set of day and night samples across all developmental phases (A–C2). This correlation matrix was used to construct an adjacency matrix (Zhang and Horvath, 2005) with a soft-thresholding power of 7 as determined by the scale-free topology criterion. In network analysis, an adjacency matrix encodes the connection strength between nodes, i.e., VOCs in our case. The soft-thresholding strategy of WGCNA raises co-expression values (i.e., correlations) to a power 'β' such that high correlations are favored over low correlations. The appropriate soft-threshold value was chosen by plotting the change in scale-free topology as a function of soft-thresholding values ranging from 1 to 20. The value of 7 was selected, as it was the lowest power for which the scale-free topology fit index reached its highest value. The blockwiseModules function in WGCNA within the software package R was then employed to detect VOC modules that are defined as blocks or groups of nodes (i.e., VOCs) with high topological overlap. This function uses a hierarchical clustering algorithm via the *hclust* procedure. The branches of the dendrogram thus generated represent VOC modules, i.e., sets of highly co-emitted VOCs. To detect optimum module numbers, i.e., tree branches, the cuttreeDynamic function of the Dynamic Tree Cut algorithm was employed. To establish a relationship between the detected modules and the trait in question, i.e., emission of particular modules of VOCs at certain developmental and diel phases, each VOC from every sample was coded as belonging to a particular developmental or diel phase. The trait here considered was the developmental time of the fig syconium. Since the development of these phases was monitored on a daily basis for our sampled fig syconia, the day of VOC collection from each fig syconium initiation date was used as the trait value. Therefore, for example, we coded A-phase diurnal samples as 13.5 and A-phase nocturnal samples as 14.0 since 13 d had elapsed from syconium initiation until sample collection. The 0.5 d difference corresponds to the 12 h gap between day and night sample collections. The C2 samples were similarly coded, for example, as 41.5 and 42.0. By using the actual developmental time as a variable, we expected to capture differences in VOC signatures between syconium developmental phases. Once VOC modules were detected, the moduleEigengenes function was used to calculate module eigen values for each sample that was correlated with the trait (i.e., the developmental time) by using the in-built Pearson product moment correlation coefficient. The module eigen value is defined as the first prinicipal component of a module, i.e., the composite expression of the members belonging to the module. Since our trait values increased with time, a positive correlation between module eigen values and the trait would indicate sets of VOCs that were co-emitted at later developmental phases, while a negative correlation would indicate co-emission at earlier developmental stages. Non-significant correlations would indicate lack of phase specificity in co-emission of VOCs constituting a module.

Results

Syconial Phase-Specific Volatile Signatures A total of 54 VOCs was identified from A–C2 phases. These included aliphatic compounds, benzenoids, monoterpenes, and sesquiterpenes (Supplemental Tables S1 and S2). The scent bouquet of all samples was clearly distinguishable based on phases (A, B, C1, and C2) and diel differences (Fig. 1). The distances between clusters based on phases was greater compared to those based on diel time of collection, although this was true only with VOC quantity data (Fig. 1; Manhattan distances between cluster medoids using GLM analysis: for VOC proportions, phase distance=0.60±0.14 (mean± SE), diel distance=0.49±0.13, t=0.768, P=0.455; for VOC quantities, phase distance=0.70±0.14, diel distance=0.16± 0.12, t=3.740, P=0.002). In this section, therefore, only results of diurnal VOCs will be reported.

In an *all vs. all* classification of diurnal A–C2 samples using VOC proportions (N=40, 10 samples per phase), only 10 VOCs (out of 54) with model frequency of 100 % and prediction error of 0.175 (1.7 out of 10 samples were misclassified) were enough to correctly assign their membership to their particular phases. These VOCs were α-muurolene (tentatively identified), hexyl acetate, linalool, methyl benzoate, methyl salicylate, nonanoic acid, perillene, *trans*–linalool oxide, 4-ethyl acetophenone, and 6-methyl-5-hepten-2-ol. The same 10 compounds served as predictor VOCs using quantity data with a model frequency of 92 % and a prediction error of 0.126.

The most abundant VOC in A-phase diurnal samples was the monoterpene 3-carene (Table 1). This VOC along with dodecane, δ-cadinene, and α-terpineol formed the unique set of VOCs with a model frequency of 100 % and .632+ prediction error of 0.081, which distinguished A-phase diurnal samples from the other diurnal samples in a *one vs. the rest* classification (Table 2). B-phase diurnal samples were dominated by (*Z*)-3-hexenyl acetate (Table 1). However, α-muurolene (tentatively identified) present at a minor concentration of 2.83 % formed a distinguishing feature of the bouquet, with a model frequency of 100 % and prediction error of 0.063 (Table 2). Inter-floral phase samples (C1 and C2) were dominated by (*E*)-β-ocimene whose levels increased consistently across the developmental phases reaching 42.65 % of the VOC emissions in the C2 phase (Table 1). C1-specific VOCs were *trans*-linalool oxide, perillene, and hexyl acetate with a model frequency of 100 % and prediction error of 0.052 (Table 2). C2-specific VOCs were methyl benzoate, 6-methyl-5-hepten-2-ol, with a model frequency of 100 % and prediction error of 0.044 (Table 2). The *one vs. the rest* results obtained with VOC quantity data were similar but with lower model frequencies and with four more compounds needed to distinguish the A phase from the rest, and also linalool being included as a predictor variable in the C2 phase (Table 3). In these analyses, the absence of a VOC also contributed to the unique phase specificity (Tables 2 and 3).

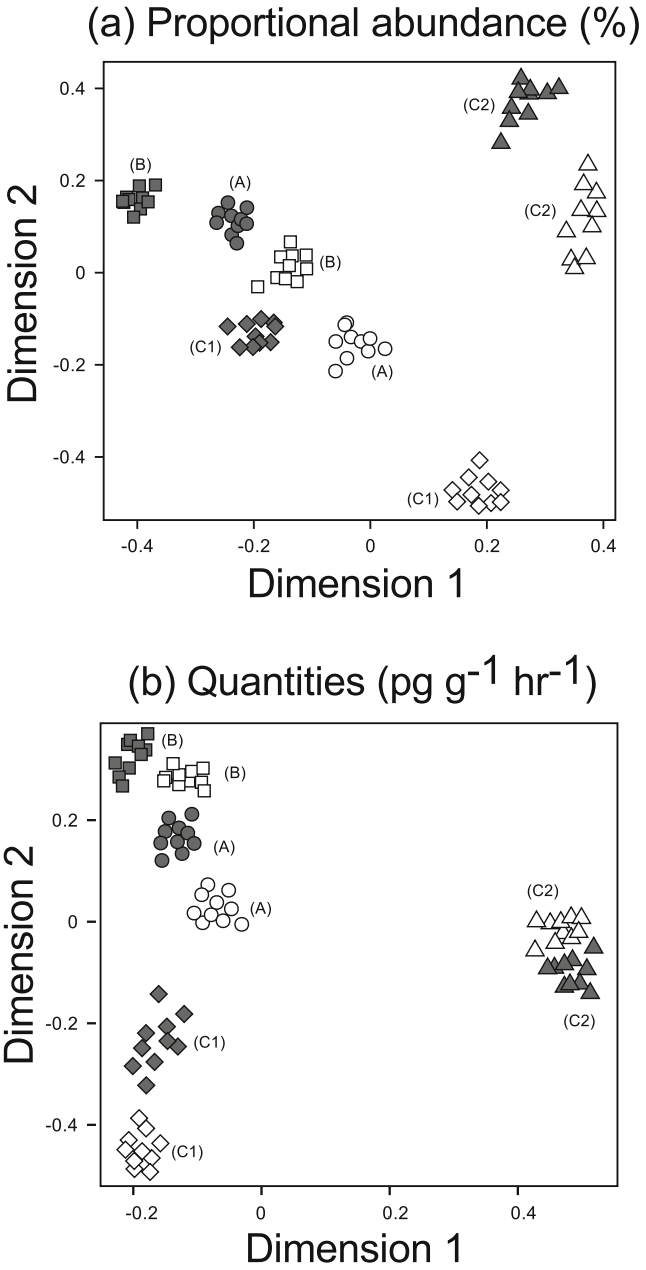

Fig. 1 Multidimensional scaling (MDS) plots of *Ficus racemosa* samples using **a** proportional abundance [expressed as %] and **b** quantities [expressed in pg g^{-1} h^{-1}]. The different developmental phases of the samples were pre-receptive (A; circles), receptive (B; squares), inter-floral1 (C1; diamonds) and inter-floral2 (C2; triangles). Samples collected during the diurnal phase (white) and the nocturnal phases (grey) are also indicated

Diel Differences in VOC Signatures Nocturnal samples always had fewer volatiles (61 %) compared to matched diurnal samples (33 nocturnal VOCs compared to 54 diurnal ones;

Table 1 Most abundant volatile organic compounds (VOCs) from *Ficus racemosa* syconia at different developmental phases (A, B, C1 and C2) and at different diel [D=Diurnal and N=Nocturnal] phases

Developmental phase [D | N]	VOC	Proportional abundance (%)[a]
pre-receptive (A) [D]	3-carene	13.01±4.38
[N]	α-pinene	23.80±5.42
receptive (B) [D]	(*Z*)-3-hexenyl acetate	15.49±7.82
[N]	(*E*)-β-ocimene	19.89±8.41
inter-floral1 (C1) [D]	(*E*)-β-ocimene	12.40±3.65
[N]	(*E*)-β-ocimene	16.22±5.26
inter-floral2 (C2) [D]	(*E*)-β-ocimene	42.62±8.52
[N]	(*E*)-β-ocimene	59.12±9.85

[a]mean ± SE

Supplemental Tables S1 and S2). There was no VOC that was exclusively emitted at night. The number of nocturnal VOCs was significantly different from the diurnal VOCs in A- and B-phase samples (Table 4). Although this pattern was consistent in inter-floral phases (C1 and C2), it was not statistically significant (Table 4). The quantity of emitted VOCs was significantly higher in the day compared to the night only in the A phase (Table 4).

Since 39 % of VOCs were absent in the night, diel variation in samples of all phases were characterized mostly by nocturnal absences of compounds (Tables 5 and 6). There was considerable improvement in model frequency prediction of day–night VOC signatures in C1 and C2 phases when VOC quantities instead of proportions were used (Tables 5 and 6).

Henry's Law Constants and Diel VOC Production We correlated day time emission of each VOC in each phase (pg g^{-1} h^{-1}) against its day time H value, and we found no significant relationship in any phase (results not shown), although there was a positive trend in the B phase. For every VOC in each phase (A–C2), we calculated H_d/H_n as well as V_d/V_n (VOC emission in the night/VOC emission in the day) only for VOCs that were emitted in the day as well as in the night (Supplemental Table S3). If the relationship $H_d/H_n=V_d/V_n$ held true for any compound, then its V_d/V_n values would fall within the confidence intervals of H_d/H_n. Compounds with V_d/V_n values above the confidence intervals of H_d/H_n would be emitted more in the day and *vice versa* (Supplemental Table S3). In A phase, V_d/V_n values were above the H_d/H_n confidence intervals for all compounds. In B phase, these values were below the confidence intervals only for (*E*)-β-ocimene and decanal while the emissions of all other compounds were above these intervals. In the C1 phase, the emissions of compounds such as hexadecane, α-pinene, dodecane, pentadecane, camphene, and limonene were

Table 2 Model frequency, .632+ prediction error and predictor compounds of diurnal emissions of volatile organic compounds (VOCs) of *Ficus racemosa* according to the Random Forests algorithm based on proportional abundance (%) in a *one versus the rest* classification

Developmental phase	Model frequency	.632+ prediction error	Predictor VOC	Abundance[a, b] (%)
pre-receptive (A)	100 %	0.081	octanal	–
			pentadecane	–
			(*Z*)-β-ocimene	–
			3-carene	13.01±4.38
			dodecane	1.78±0.26
			α-terpineol	0.94±0.41
			δ-cadinene	0.92±0.37
receptive (B)	100 %	0.063	4-ethyl acetophenone	–
			methyl salicylate	–
			α-muurolene	2.83±1.62
inter-floral1 (C1)	100 %	0.052	*trans*-linalool oxide	2.51±0.48
			perillene	0.87±0.22
			hexyl acetate	0.73±0.18
inter-floral2 (C2)	100 %	0.044	methyl benzoate	3.58±0.67
			6-methyl-5-hepten-2-ol	1.57±0.53

[a] (mean ± SE)

[b] the absence of a VOC is also considered a feature unique to the group of interest

Table 3 Model frequency, .632+ prediction error and predictor compounds of diurnal emissions of volatile organic compounds (VOCs) of *Ficus racemosa* according to the Random Forests algorithm based on quantities in a *one versus the rest* classification

Developmental phase	Model frequency	.632+ prediction error	Predictor VOC	Quantity[a, b] (pg g^{-1} h^{-1})
pre-receptive (A)	95 %	0.08	acetophenone	20.32±5.19
			α-pinene	27.1±6.26
			α-terpineol	3.98±1.56
			δ-cadinene	10.48±4.72
			γ-terpinene	11.50±6.12
			hexadecane	–
			octanal	–
			pentadecane	–
			(*Z*)-β-ocimene	–
			4-ethyl acetophenone	7.01±2.70
			4-ethyl benzaldehyde	5.26±2.78
receptive (B)	86 %	0.074	4-ethyl acetophenone	–
			methyl salicylate	–
			α-muurolene	6.62±2.49
inter-floral1 (C1)	93 %	0.045	*trans*-linalool oxide	6.64±1.65
			perillene	4.79±2.81
			hexyl acetate	4.38±2.79
inter-floral2 (C2)	96 %	0.052	methyl benzoate	31.81±12.78
			6-methyl-5-hepten-2-ol	12.42±5.87
			linalool	3.90±1.26

[a] (mean±SE)

[b] the absence of a VOC is also considered a feature unique to the group of interest

below confidence intervals, while all others were above the intervals. Similarly in the C2 phase, emissions of compounds such as nonane, heptanal, 1,4-cineole, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenol, γ-terpinene, (*Z*)-β-ocimene, 3-carene, *cis*-linalool oxide, (*E*)-β-ocimene, and terpinolene were below the confidence intervals, while the rest were above these intervals. This indicates that only a few compounds were emitted to a greater extent during the night, above their expected proportional differences between day and night as dictated by Henry's Law constants; the rest of the compounds were emitted to a greater extent in the day.

Determination of Co-emitted Sets of VOCs The WGCNA analysis detected 7 modules of co-emitted VOCs (Figs. 2 and 3). Of these, two modules (red and yellow) were positively correlated with the trait in question, i.e., were more representative of co-emitted VOCs in later developmental phases, while one module (green) was negatively correlated with developmental time (Fig. 3). The green module which

Table 4 Diel patterns of emissions of volatile organic compounds (VOCs) in *Ficus racemosa*

Developmental phase	Number of VOCs[a]		Total VOC emission (ng g^{-1} h^{-1}) [b, c]	
	diurnal	nocturnal	diurnal	nocturnal
pre-receptive (A)	31	9***	0.86±0.23	0.07±0.02***
receptive (B)	28	13*	0.48±0.13	$0.14±0.02^{ns}$
inter-floral1 (C1)	42	38^{ns}	0.42±0.22	$0.19±0.06^{ns}$
inter-floral2 (C2)	45	42^{ns}	0.65±0.22	$0.43±0.16^{ns}$

[a] comparison of number of VOCs in diurnal and nocturnal samples using a χ^2 test

[b] values are means ± SE

[c] comparison of total VOC emission between diurnal and nocturnal samples using a Wilcoxon matched pairs signed rank test

*** $P<0.001$, * $P<0.05$, ns=$P>0.05$

Table 5 Model frequency, .632+ prediction error and predictor compounds of diurnal *vs*. nocturnal emissions of volatile organic compounds (VOCs) of *Ficus racemosa* according to the Random Forests algorithm based on percent abundance in a *one vs*. *the rest* classification

Developmental phase	Model frequency	.632+ prediction error	Predictor VOC	Abundance (%) [a, b]	
				diurnal	nocturnal
pre-receptive (A)	100 %	0.273	decanal	7.43±2.71	–
			nonanal	6.87±2.18	–
			acetophenone	2.73±0.54	–
			dodecane	1.78±0.26	–
receptive (B)	100 %	0.165	(Z)-3-hexenyl acetate	15.49±7.82	–
			α-muurolene	2.83±1.62	–
			camphene	1.38±0.60	–
inter-floral1 (C1)	59 %	0.069	4-ethyl acetophenone	0.90±0.15	–
			undecanal	0.73±0.11	–
			hexyl acetate	0.73±0.18	–
			heptanal	0.52±0.09	–
			nonane	0.27±0.05	–
inter-floral2 (C2)	62 %	0.077	methyl benzoate	3.58±0.67	0.77±0.16
			nonane	–	0.12±0.04
			trans-α-bergamotene	1.34±0.30	–
			4-ethyl acetophenone	0.29±0.09	–

[a] (mean ± SE)

[b] the absence of a VOC is also considered a feature unique to the group of interest

represented VOCs being emitted early in syconium development time (i.e., negative correlation with the trait) was dominated by GLVs such as (*Z*)-3-hexenol. The red and yellow modules, which represented VOCs emitted later in syconium development, were dominated by compounds well known to be HIPVs in the literature such as (*E*)-β-ocimene (Fig. 3). The brown and blue modules displayed a negative but non-significant correlation with the trait, and they represented VOCs that were co-emitted throughout the phases, i.e., were not phase specific (Fig. 3). The black module exhibited a positive but non-significant correlation with the trait (Fig. 3). The grey module contained VOCs (α-muurolene, *trans*-linalool oxide, and hexadecane) that did not form a large group but only a small branch of the clustering tree (Figs 2 and 3). VOCs contained in the grey module were absent in

Table 6 Model frequency, .632+ prediction error and predictor compounds of diurnal *vs*. nocturnal emissions of volatile organic compounds (VOCs) of *Ficus racemosa* according to the Random Forests algorithm based on quantities in a *one vs*. *the rest* classification

Developmental phase	Model frequency	.632+ prediction error	Predictor VOC	VOC quantity (pg g^{-1} h^{-1}) [a, b]	
				diurnal	nocturnal
pre-receptive (A)	87 %	0.343	dodecane	16.23±4.58	–
			methyl salicylate	26.06±8.02	–
receptive (B)	99 %	0.327	α-isomethyl ionone	13.02±6.42	–
			α-muurolene	6.62±2.49	–
			methyl dihydrojasmonate	7.54±3.55	–
inter-floral1 (C1)	100 %	0.377	4-ethyl acetophenone	3.59±1.82	0.15±0.10
			undecanal	2.97±1.33	0.53±0.35
inter-floral2 (C2)	99 %	0.184	methyl dihydrojasmonate	8.81±4.85	–
			allo-aromadendrene	2.32±0.53	0.35±0.30
			4-ethyl acetophenone	1.53±0.46	–

[a] (mean ± SE)

[b] the absence of a VOC is also considered a feature unique to the group of interest

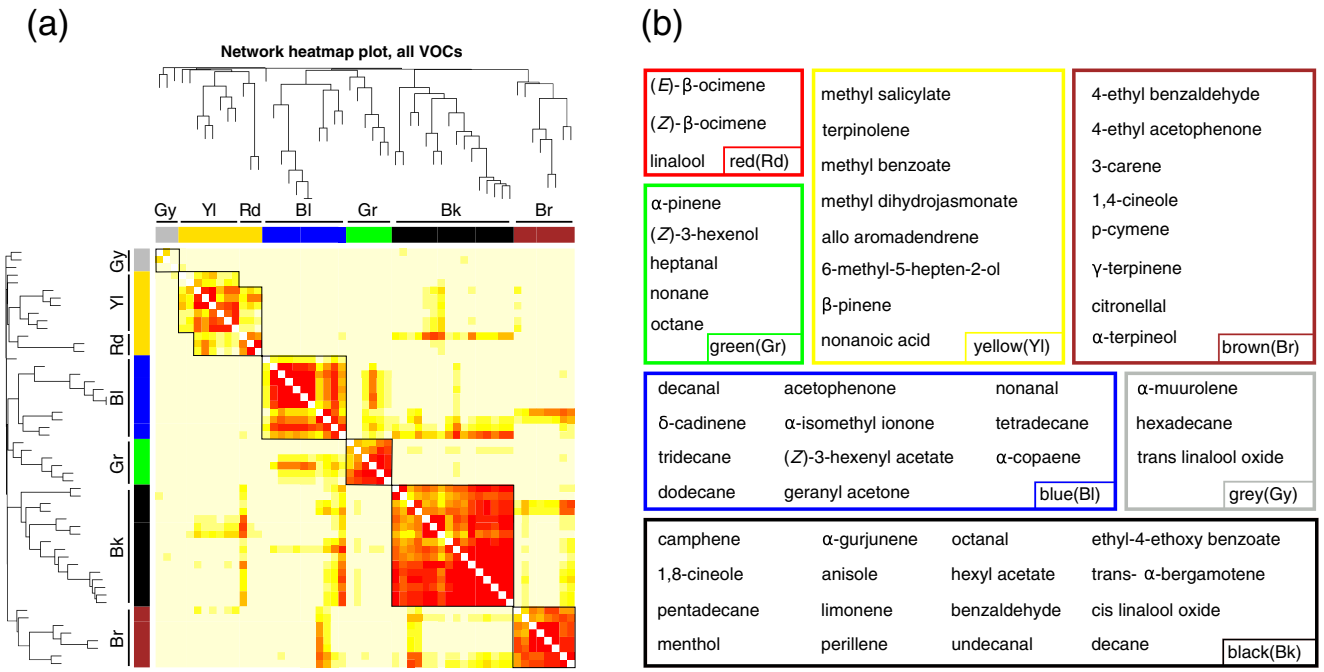

Fig. 2 **a** Network heatmap of all *Ficus racemosa* volatile organic compounds (VOCs) generated using the WGCNA method. Seven modules of co-emitted VOCs were detected: green, blue, brown, yellow, red, black, and grey. **b** Membership of VOCs in the seven detected modules

the A phase and were emitted only from the B phase onwards (Fig. 3).

Discussion

All headspace samples of *F. racemosa* showed clear clustering based on phase-specific and diel-specific VOCs. This pattern was consistent when using either VOC proportions or quantities. Qualitative and quantitative information on the identity of the developmental phase of the syconia could be vital for ovipositing fig wasps and in particular for the short-lived pollinator *C. fusciceps*. This is consistent with the idea that a set of few compounds in different blends (ratios or quantities) could confer phase or diel specificity, which could be particularly important in species-specific interactions (Raguso, 2008; Svensson et al., 2010) as is necessary in the highly species-specific interactions between figs and fig wasps.

Phase-Specific VOCs The *all vs. all* Random Forest classification looks for the minimum set of variables (VOCs in this case) that could successfully differentiate the groups (A–C2 phases). A similar approach could also be employed by fig wasps as they encounter trees of the right fig species but at varied developmental phases. With a minimal set of chemoreceptors sensitive to the minimal set of predictor VOCs, these different blends could confer vital information to the fig wasps about the suitability of the trees for oviposition. On the other hand, a fig wasp that arrives at a particular developmental phase, e.g., a pollinator in search of trees in pollen-receptive phase, could have receptors only for the phase-specific compounds and not for any other compounds present before and after pollination. This is the approach used in a *one vs. the rest* classification. Consequently, the predictor variables generated by the Random Forests algorithm (Tables 2 and 4) can provide candidate VOCs for further behavioral testing on fig wasps to determine the specific signals or cues employed by them to reach fig trees or syconia of the appropriate phase at the appropriate time of the day or night. For example, α-muurolene also was found to be a B phase-specific sesquiterpene present only in receptive phase samples and also only in the diurnal samples. This coincides with the fact that the pollinator, *C. fusciceps*, is active only during the diurnal part of the B-phase. It is possible that α-muurolene may constitute the signal or a component of the signal that figs produce to attract pollinators. However, α-muurolene was not found in the blend of VOCs produced by the receptive phase of *F. racemosa* in Yunnan, China (Proffit et al., 2009). This could indicate geographical variation in VOC profiles and signals.

Many GLVs and putative HIPVs were detected during the phases. While methyl salicylate is commonly considered to be an HIPV, it was completely absent in the B-phase bouquet but present in all other phases before and after pollination. (*E*)-β-ocimene, which is a well-known HIPV in many plant systems (Lucas-Barbosa et al., 2011), dominated the VOC profile right after pollination (Table 1). This pattern was evident even in nocturnal samples of B-phase extractions. This VOC could play a role in post-pollination scent changes that could discourage visits by other

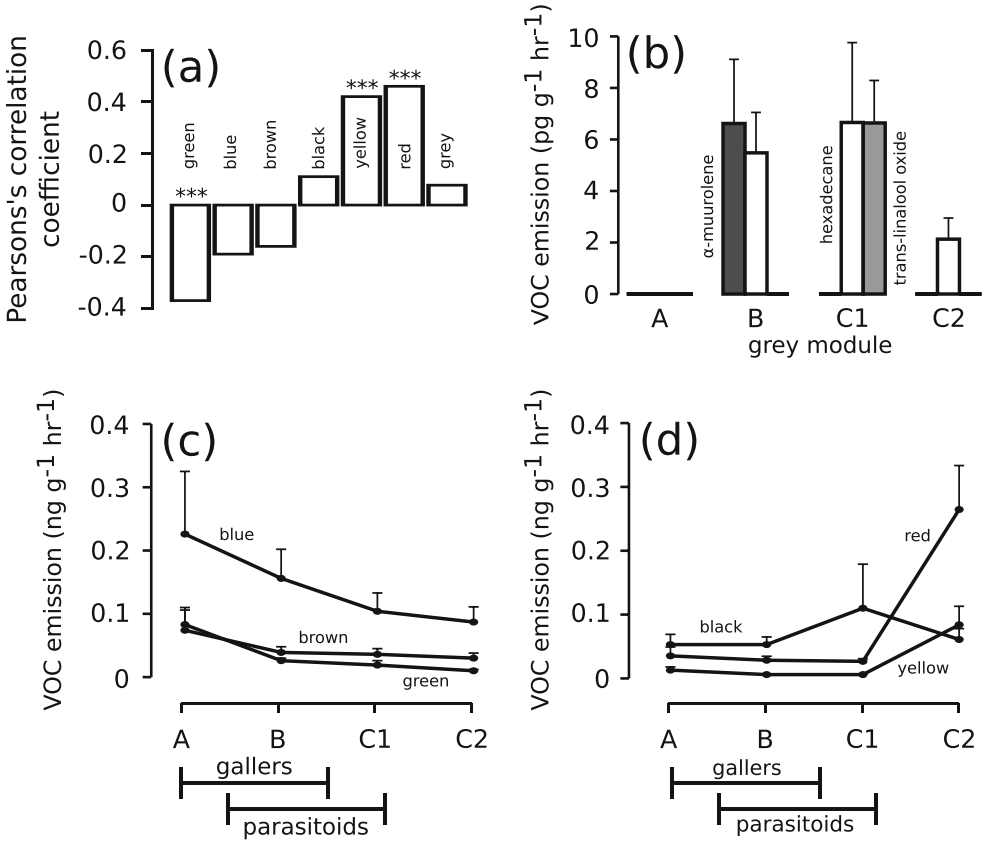

Fig. 3 Relationship between modules of co-emitted VOCs and the development age of the syconium **a** Pearson's correlation coefficient between module VOC emission and the development age of the syconium, *** $P < 0.001$, otherwise $P > 0.05$; **b** Emission of VOCs belonging to the grey module across the development phases of the syconium: A (pre-receptive), B (receptive), C1 (inter-floral1 or early to mid inter-floral), C2 (inter-floral2 or late inter-floral); **c** Emission of VOCs belonging to the green, blue and brown modules (mean + SE of values summed for all VOCs) across the phases: A, B, C1, and C2; **d** Emission of VOCs belonging to the black, yellow, and red modules (mean + SE of values summed for all VOCs) across the phases: A, B, C1, and C2. These development phases (A, B, C1, and C2) when gallers and parasitoids are ovipositing into the syconium are schematically indicated at the bottom of the figure; this trait was measured in days. All color modules in this figure are as defined in Fig. 2

pollinators and also act as a cue for non-pollinating parasitic fig wasps. The total VOC emission from syconia at the post-pollination phase increased to about 8–9 times their levels in receptive phase (Supplemental Table S2). This is in contrast to other systems such as thistles (*Cirsium* spp.) (Theis and Raguso, 2005) where scent emission declined after pollination. Post-pollination declines in floral scent in most flowering species is understandable since pollinator services are no longer needed after pollination. In nursery pollination systems such as figs, post-pollination increases in VOCs are probably a response to the developing herbivorous and carnivorous fig wasp larvae within the syconium nursery.

Diel-Specific VOCs The nocturnal samples consistently had fewer numbers of VOCS as compared to the diurnal samples (Table 3). This could mean that VOC emission (and/or synthesis) has a circadian rhythm that is light entrained as seen in other studies (Hendel-Rahmanim et al., 2007). Our sampling regime of two sample periods in the diel cycle is certainly not sufficient to reveal the entire rhythm, but our data do suggest that there are diel differences. These need to be examined in greater detail. Circadian rhythms usually are also coupled with temperature differences between the diurnal and nocturnal hours. Furthermore, VOC emission is also affected by ambient temperatures (Sagae et al., 2008; Ibrahim et al., 2010). Ambient temperatures in our study were higher (by approximately 7 °C) during the day compared to the night. This may have affected VOC emission; however, total quantities of VOCs produced were not significantly different between day and night except in the A phase. The lower emission of some VOCs during the nocturnal hours also could imply that the fig tree is sending out long-range signals directed to diurnal interactants such as the strictly diurnal pollinator. Although some gallers and parasitoids in *F. racemosa* exhibit nocturnal activity, nocturnal wasps constitute only 4.5 % of the population (Ranganathan et al., 2010); therefore, the relevant signals or cues likely reside in the diurnal VOC bouquet. This is consistent with findings from other studies that have shown that peak VOC emission, especially of compounds that

pollinators can perceive, coincides with pollinator diel activity (Morinaga et al., 2009; Balao et al., 2011), as well as with results that indicate functional synchronization of VOC diel rhythms in tritrophic systems consisting of plants, herbivores, and parasitoids (Zhang et al., 2010).

Henry's Law Constants and Diel VOC Production Our investigations showed that VOC emission from available VOC tissue pools could not be explained solely by parameters such as Henry's law. Therefore, other factors such as VOC synthesis, conductance through cell compartments, light, stomatal conductance or openness must dictate diel differences in VOC emission (Niinemets and Reichstein, 2003; Niinemets et al., 2004, 2010; Noe et al., 2006). More work needs to be done in this area, especially modifying simplistic models to encompass more realistic measurements and predictions.

Patterns of VOC Co-emission The WGCNA analysis identified modules that were interpretable based on the ecology of the fig syconium (Figs 2 and 3).

Early galling wasps deposit eggs into syconial primordia in the A phase and probably trigger the release of GLVs such as (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate (present in the blue and green modules) based on tissue damage (Figs 2 and 3).

As pollinators enter the syconium and deposit eggs in the B phase, larvae of the early gallers that had oviposited in the A phase as well as pollinator larvae begin to develop and feed on galled plant tissue. This probably results in the release of compounds, i.e., (*E*)-β-ocimene, (*Z*)-β-ocimene, methyl salicylate, methyl dihydrojasmonate (present in the red and yellow modules, Fig. 2), that are well known as HIPVs in other studies, (Gouinguené and Turlings, 2002; Dicke et al., 2009; Dicke and Baldwin, 2010; Holopainen and Gershenzon, 2010; Snoeren et al., 2010; Lucas-Barbosa et al., 2011; Tholl et al., 2011). It is extremely interesting, therefore, that the quantities of (*E*)-β-ocimene continue to rise throughout the development of the syconium indicating a possible response to herbivory by the plant caused by wasp larvae feeding within (Fig. 3). Furthermore, parasitoids are attracted to the fig syconium largely from the B phase onwards (Ranganathan et al., 2010), which is precisely when the putative HIPVs increase in quantity; such compounds may thus also be responsible for attraction of parasitoids to the fig syconia. The WGCNA results indicated co-emitting VOCs in the black and brown modules (Fig. 2) that were emitted in all phases; such co-emission cannot yet be explained based on our knowledge of this system. The identification of a small cluster, i.e., the grey module (Fig. 2) which contained a compound such as α-muurolene that is only emitted in the day during B-phase and, although being a sesquiterpene, is not co-emitted with any other sesquiterpene during syconium development, suggests that it may serve as a pollinator attractant even though it is emitted in minor amounts. The role of minor compounds as attractants is becoming increasingly acknowledged (D'Alessandro et al., 2009) and warrants further investigation. Furthermore, the grey module also contained other well known HIPVs such as hexadecane and *trans*-linalool oxide (Fig. 3), and comprised compounds that were co-emitted to a greater extent from the middle phases of syconium development onwards (Fig. 3). Since a fig syconium is subject to parasitism and herbivory even before the pollination stage, the "real" signal for the pollinator is likely mixed with an HIPV signature, which may attract other gallers and parasitoids. Despite its complexity, the high specificity between interactants in this system holds out the possibility for future discovery of the relevant signals.

Experiments are needed on potential pollinator attractants as well as on fig syconia in which early gallers and parasitoids are denied oviposition to determine the feedback loops between oviposition, herbivory, and VOC emisssion. Analyses such as we have performed in our present study including testing some physicochemical properties of VOCs as well as co-emission patterns in VOC emissions, have, however, provided many testable hypotheses for future understanding of this complex system.

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