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Emission of pollinator-attractive signals in fig trees : evolution, spatio-temporal variation and biosynthesis

Li Cao

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Ecologie et Biodiversité

École doctorale GAIA

Unité de recherche CEFE

Emission des signaux attractifs pour les pollinisateurs chez les figuiers: évolution, variation spatio-temporelle et biosynthèse

Présentée par Li CAO

Le 03 Juillet 2023

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UNIVERSITÉ
DE MONTPELLIER

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Li

Abstract

Floral scents, forming a complex mixture of volatile organic compounds (VOCs), play a major role in attracting pollinators, and thus in plant reproduction, but also in defense against biotic and abiotic stresses. As different factors can influence the production of these VOCs, their emission is very variable, at the inter- and intra-specific levels but also with time. In this interdisciplinary study, we have integrated metabolomics, bioinformatics, biochemistry and molecular biology methodologies to study the evolution, spatio-temporal variation and biosynthesis of the emission of attractive signals for pollinators using fig trees and in particular the Mediterranean fig tree *Ficus carica* as a model. Each species of the genus *Ficus* (Moraceae) is pollinated by a specific pollinator of the family Agaonidae (Hymenoptera). They are tiny wasps that develop exclusively in the figs, i.e. closed inflorescences with a single entrance (the ostiole), of their host species and use the VOCs emitted by the receptive figs (i.e. at the stage of development when they are ready to be pollinated) to locate their host. In the case of *Ficus carica*, it has been shown that its specific pollinator (*Blastophaga psenes*) is attracted to receptive figs by a combination of four VOCs in a particular proportion. At the level of the genus *Ficus*, we compared phylogenetic and chemical data of 32 fig species and found a strong phylogenetic signal in the VOCs emitted by receptive figs. This signal could be due to either constraints in VOC biosynthetic pathways or selection to attract pollinators that evolved along with the host fig. However, using the same analysis, we found no effect of pollinator phylogeny. By comparing chemical profiles of receptive figs of eight fig varieties of *F. carica* in Morocco, we found that human domestication resulted in inter-varietal differences, although the chemical signal responsible for pollinator attraction was highly conserved between varieties. Also, in *F. carica*, we studied the emission patterns of VOCs responsible for pollinator attraction, by performing real-time measurements using a PTR-TOF-MS. We found that VOCs emitted by receptive figs followed a diurnal emission pattern, with a peak at midday corresponding to the period when the pollinator is most active. The VOC emission pattern in *F. carica* may be optimized to attract its

exclusive pollinators. We also highlighted that temperature and humidity can also influence the emission of these VOCs but in a non-linear manner. Moreover, still with these real-time measurements, we have highlighted a very fast change in the emission of floral VOCs after pollination. Chemical profile analyses and comparative transcriptomics between receptive and pollinated figs allowed us to highlight some genes whose expression is down-regulated after pollination. The heterologous expression in *Escherichia coli* of two genes coding for terpene synthases (TPS) allowed us to confirm their function. Finally, three complementary experiments (VOC analysis, transcript analysis and histology of different tissues) showed that the ostiole is the source of production of volatile compounds that attract fig wasps. This study examined some key factors affecting the emission of pollinator attractive signals in fig trees and elucidated some molecular mechanisms of the biosynthesis of these VOCs. Our work thus provides a solid basis for future studies on the evolution of VOC biosynthesis in *Ficus*.

Résumé

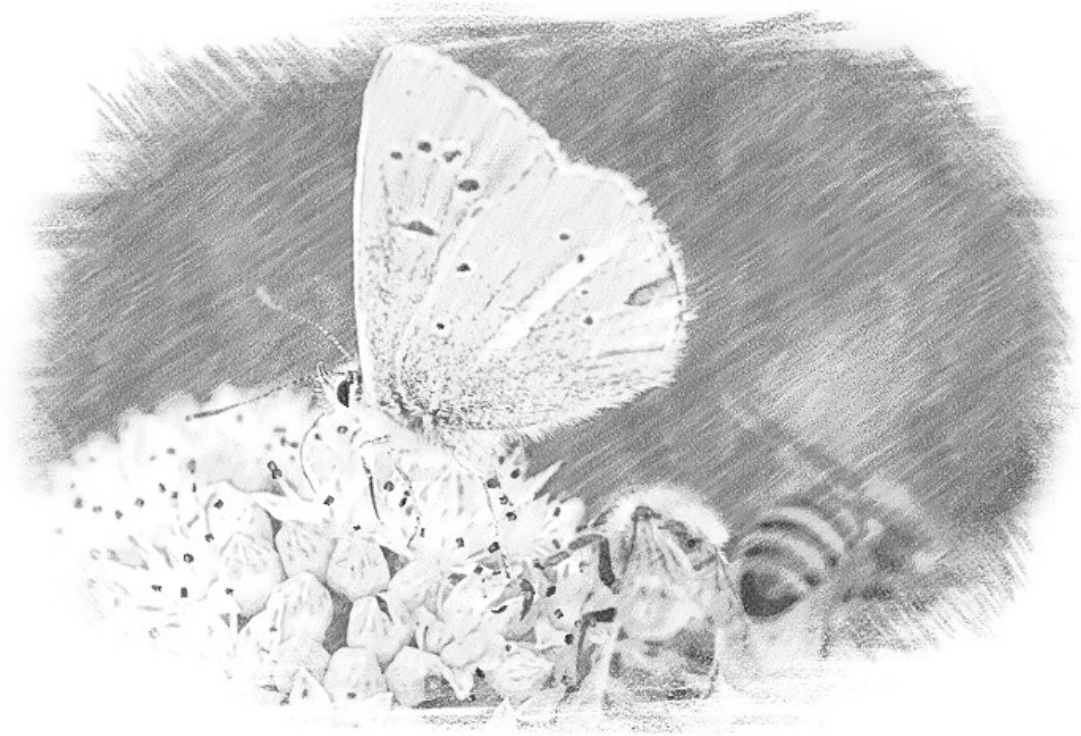
Les odeurs florales forment un mélange complexe de composés organiques volatils (COV) jouant un rôle majeur dans l'attraction des pollinisateurs, et donc dans la reproduction des plantes, mais aussi dans la défense contre les stress biotiques et abiotiques. Comme différents facteurs peuvent influencer la production de ces COVs, leur émission est très variable, aux niveaux inter- et intra-spécifiques, mais aussi au cours du temps. Dans cette étude interdisciplinaire, nous avons intégré des méthodologies de métabolomique, de bio-informatique, de biochimie et de biologie moléculaire pour étudier l'évolution, la variation spatio-temporelle et la biosynthèse de l'émission de signaux attractifs pour les pollinisateurs en utilisant les figuiers et en particulier le figuier méditerranéen, *Ficus carica*, comme modèle. Chaque espèce du genre *Ficus* (Moraceae) est pollinisée par un pollinisateur spécifique de la famille des Agaonidae (Hymenoptera), ce sont de minuscules guêpes qui se développent exclusivement dans les figes, qui sont des inflorescences fermées avec une seule entrée (l'ostiole) de leur espèce hôte et utilisent les COV émis par les figes réceptives (c'est-à-dire au stade de développement où elles sont prêtes à être pollinisées) pour localiser leur hôte. Dans le cas de *Ficus carica*, il a été démontré que son pollinisateur spécifique, *Blastophaga psenes*, est attiré vers les figes réceptives par une combinaison de quatre COVs dans une proportion particulière. Au niveau du genre *Ficus*, nous avons comparé les données phylogénétiques et chimiques de 32 espèces de figuiers et nous avons trouvé un signal phylogénétique fort dans les COVs émis par les figes réceptives. Ce signal pourrait être dû à des contraintes dans les voies de biosynthèse des COVs, ou à une sélection pour attirer les pollinisateurs qui ont évolué en même temps que la figue hôte. Cependant, en utilisant la même analyse, nous n'avons trouvé aucun effet de la phylogénie des pollinisateurs. En comparant les profils chimiques des figes réceptives de huit variétés de *F. carica* au Maroc, nous avons constaté que la domestication a entraîné des différences entre les variétés, bien que le signal chimique responsable de l'attraction des pollinisateurs ait été fortement conservé entre les variétés. En outre, chez *F. carica*, nous avons étudié les schémas d'émission des COVs responsables de

l'attraction des pollinisateurs, en effectuant des mesures en temps réel avec un PTR-TOF-MS. Nous avons constaté que les COVs émis par les figes réceptives suivaient un schéma d'émission diurne, avec un pic à midi correspondant à la période où le pollinisateur est le plus actif. Le schéma d'émission des COVs chez *F. carica* serait donc optimisé pour attirer ses pollinisateurs exclusifs. Nous avons également mis en évidence que la température et l'humidité peuvent également influencer l'émission de ces COVs, mais de manière non linéaire. De plus, toujours avec ces mesures en temps réel, nous avons mis en évidence un changement très rapide dans l'émission des COVs floraux après la pollinisation. Les analyses des profils chimiques et de transcriptomique comparative entre figes réceptives et pollinisées nous ont permis de mettre en évidence certains gènes dont l'expression est régulée à la baisse après la pollinisation. L'expression hétérologue dans *Escherichia coli* de deux de ces gènes codant pour des terpènes synthases (TPS) nous a permis de confirmer leur fonction. Enfin, trois expériences complémentaires (analyse des COVs, analyse des transcrits et histologie de différents tissus) ont montré que l'ostiole est la source de production des composés volatils qui attirent les guêpes du figuier. Cette étude a examiné certains facteurs clés affectant l'émission de signaux attractifs pour les pollinisateurs chez les figuiers et a élucidé certains mécanismes moléculaires de la biosynthèse de ces COV. Elle fournit ainsi une base solide pour de futures études sur l'évolution de la biosynthèse des COVs chez *Ficus*.

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General introduction

Plants, being immobile and sessile organisms, must adapt to the surrounding environment during their life cycle. To compensate for their immobility, plants have developed various mechanisms of interaction with their biotic and abiotic environment. Among these, the production and emission of complex mixtures of volatile organic compounds (VOC) by different organs, allows plants to convey chemical messages giving information on their status. Some of these VOCs, for example, constitute chemical signals to their pollinators and thus affect their reproduction. These VOCs will also allow plants to form symbiotic relationships with micro-organisms or to defend themselves against herbivores and to resist abiotic stresses.

1. Volatile Organic Compounds (VOCs) in plants

Plant VOCs are specialized metabolites that play a major role in the interactions between plants and their biotic environment (Figure 1). For example, they play a major role in attracting pollinators and seed dispersers, as well as other beneficial animals and microorganisms, and in defending plants from herbivores and pathogens (Agrawal, 2011, Beccera et al., 2009, Dudareva and Pichersky, 2008; Raguso, 2008; Junker and Parachnowitsch, 2015). Plant VOCs also play an important role in defense against abiotic factors. Under abiotic stress, in some species, the emission of isoprenoids is stimulated, either through direct neutralization of reactive oxygen species (ROS) (Loreto and Velikova, 2001) or indirectly by stabilizing cell membranes (Velikova et al., 2011) to improve plant resistance. Plant VOCs are mainly released from flowers, but also from fruits, leaves, stems, and even roots (Farré-Armengol et al., 2016). To date, more than 1 700 VOCs have been described in floral VOCs (Knudsen et al., 2006). These VOCs belong to four major chemical families based on their metabolic origin: terpenoids, phenylpropanoids/benzenoids, fatty acid and amino acid derivatives (Knudsen et al., 2006, Maffei, 2010). Different biosynthetic pathways, located in different parts of the plant cell (cytosol or plastid), are responsible for the production of these VOCs (Pichersky et al., 2006, Maffei, 2010, Picazo-Aragonés et al., 2020). Once produced, these VOCs can be released from different plant cells, or stored in a

particular structure such as glandular trichomes, secretory canals and cavities, secretory cells in flowers and roots (Maffei, 2010).

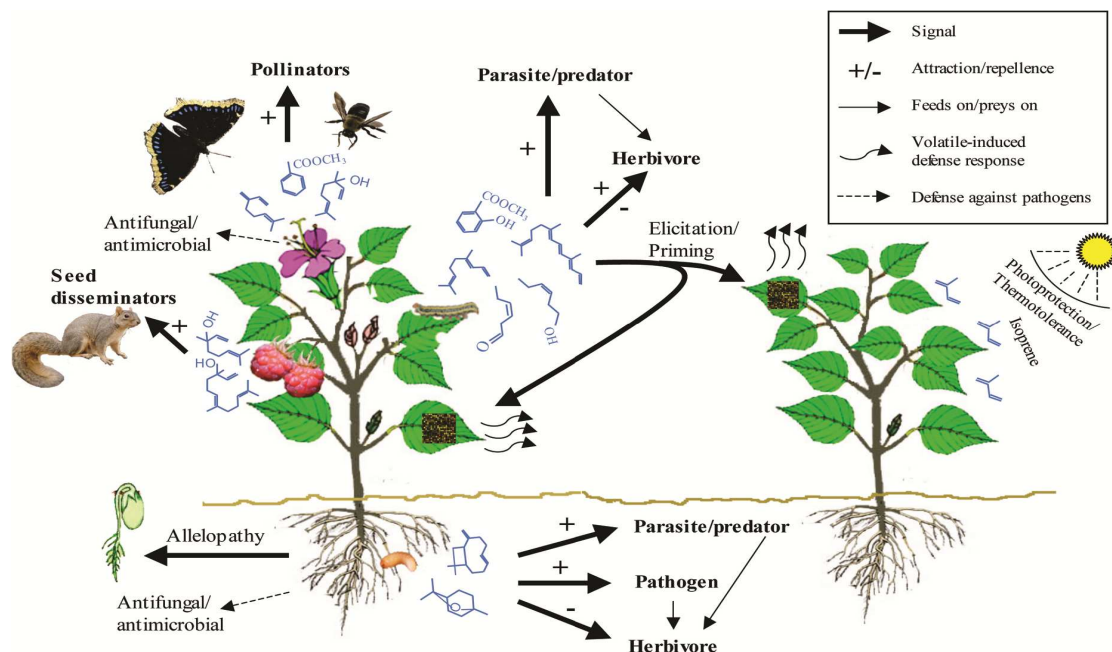


Figure 1. An overview of volatile-mediated plant interactions with the surrounding environment. Plant-animal interactions include the attraction of pollinators and seed disseminators by floral and fruit volatiles, attraction/repellence of herbivores, and attraction of natural enemies of attacking herbivores, both in atmosphere and rhizosphere. Aboveground plant-plant interactions comprise elicitation or priming of defense responses in healthy undamaged leaves of the same plant or in the neighboring unattacked plants. Belowground, these interactions include allelopathic activity on the germination and growth of competitive neighboring plants. Volatiles released from reproductive organs and roots also have antimicrobial activity thus protecting the plants from pathogen attack. In addition, isoprene, a leaf volatile confers photoprotection and thermotolerance. (from Dudareva et al., 2006)

1.1. Main biochemical pathways of plant VOCs

VOCs are produced by numerous synthetases and in different parts of the cell depending on the chemical classes (Figure 2) (Dudareva et al., 2006; Maffei, 2010; Dudareva et al., 2013; Picazo-Aragonés et al., 2020).

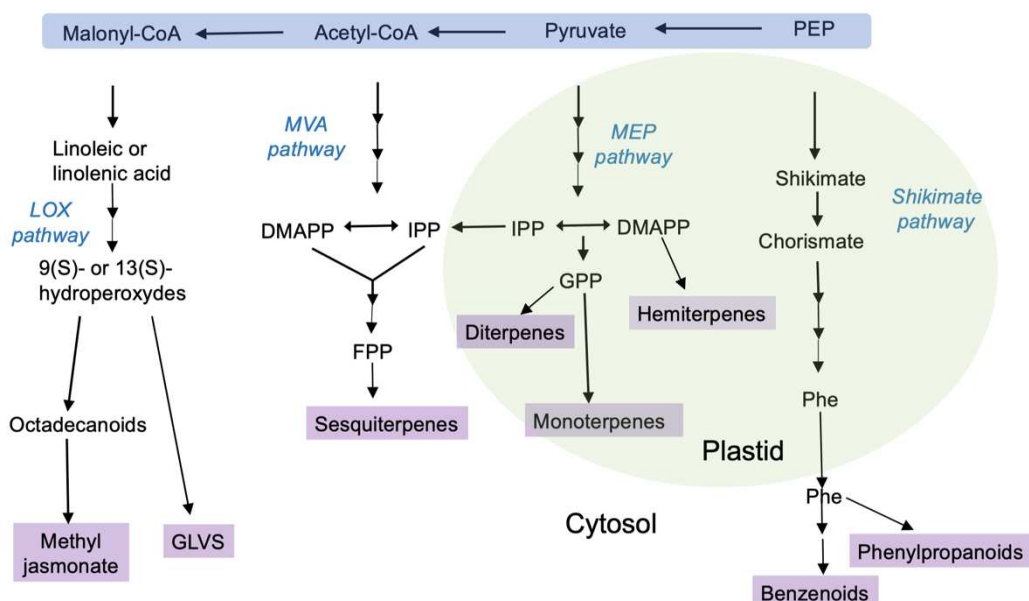


Figure 2. Overview of biosynthetic pathways leading to the emission of plant VOCs. Precursors for plant VOCs originate from central metabolism (represented in the blue box). The four major VOC biosynthetic pathways, namely the shikimate/phenylalanine, the methylerythritol phosphate (MEP), the mevalonic acid (MVA) and lipoxygenase (LOX) pathways lead to the emission of benzenoids/phenylpropanoids, monoterpenes, sesquiterpenes and methyl jasmonate/green leaf volatiles. Pathway names are italicized, VOCs are highlighted with a purple box as background. Stacked arrows illustrate the involvement of multiple enzymatic reactions. Abbreviations: DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; GLVs, green leaf volatiles; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate; PEP, phosphoenolpyruvate; Phe, phenylalanine. (Modified from Dudareva et al., 2006; Dudareva et al., 2013)

Terpenoids

All terpenoids originate from two common five-carbon precursors, isopentenyl diphosphate (IPP) and its isomers dimethylallyl diphosphate (DMAPP) (McGarvey and Croteau, 1995), which are synthesized from two independent and compartmentally separated pathways: the mevalonic acid (MVA) pathway and the methylerythritol phosphate (MEP) pathway. In plastids (Hsieh, et al., 2008), the MEP pathway providing precursors for hemiterpenes (C5), monoterpenes (C10) and diterpenes (C20), while in the endoplasmic reticulum and peroxisomes (Simkin et al., 2011; Pulido et al., 2012), the MVA pathway producing precursors for sesquiterpenes (C15).

Terpenoids are the largest class of floral volatiles (Knudsen et al., 2006). The plant genome contains many terpene synthase genes, encoding enzymes with different functions. The most exceptional property of these enzymes is the ability of many of

them to synthesize multiple products from a single prenyl diphosphate precursor (Degenhardt et al., 2009). For example, only two sesquiterpene synthases are responsible for nearly all of the 20 sesquiterpenes emitted by *Arabidopsis* flowers (Tholl et al., 2005). Some of TPSs can also use more than one substrate (Tholl, 2006; Bleeker et al. 2011). In these cases, their subcellular localization and the availability of a particular substrate determine the type of product formed (Nagegowda et al., 2008).

Shikimic acid derivatives

Shikimic acid derivatives consist of compounds containing an aromatic ring and constitute the second largest class of volatile compounds in plants. Lead from shikimate to phenylalanine and then to phenylpropanoid and benzenoid compounds (Maffei, 2010; Dudareva et al., 2013). These compounds can be generally divided into three classes according to the length of their side chains: phenylpropanoids (which have a three-carbon side chain and thus designated C₆-C₃ compounds), Phe-derived benzenoid compounds (C₆-C₁), as well as the C₆-C₂ phenylpropanoid-related compounds (Knudsen et al., 1993).

The complete pathways for formation of the phenylpropenes (C₆-C₃) have now been elucidated in *Clarkia breweri* (Koeduka et al. 2008), *Petunia hybrida* (Koeduka et al., 2006, 2008) and *Ocimum basilicum* (Koeduka et al., 2006; Vassão et al., 2006). The formation of benzenoids (C₆-C₁) have two different pathways. A CoA-dependent β -oxidative pathway was recently fully elucidated in *Petunia hybrida* and *Arabidopsis thaliana*. An alternative non- β -oxidative pathways, have been reported in *Capsella* genus, *Petunia axillaris*, *Arabidopsis* and snapdragon (Sas et al., 2016; Amrad et al., 2016; Ibdah et al., 2009; Long et al., 2009), although the biochemical steps leading to benzyl alcohol formation are still in question. The pathway of formation of phenylacetaldehyde (C₆-C₃) have been reported in petunia and rose petals (Kaminaga et al., 2006).

Fatty acid derivatives

Fatty acid derivatives are produced from C18 unsaturated fatty acids, which are subjected to deoxygenation by the enzyme lipoxygenase (LOX) (Feussner and

Wasternack, 2002). There are two different branches of LOX pathway: the allene oxide synthase (AOS) and the hydroperoxide lyase (HPL). The AOS pathway: Jasmonic acid (JA) product of AOS is converted to methyl jasmonate (Seo et al., 2001; Song et al., 1993). The HPL pathway: Short-chain C6- and C9-aldehydes and alcohols known as green leaf volatiles (GLVs), are important constituents of the floral volatile bouquet of plant (Schade et al., 2001; Suchet et al., 2011).

The different LOX iso-enzymes can be classified in two types according to their stereospecificity. Most of the time, the LOX isoenzyme orients the complete LOX pathway of the plant, such as the 9-LOX (oxygenating the ninth carbon of the fatty acid) in cucumber, the 13-LOX (oxygenating the thirteenth carbon of the fatty acid) in watermelon (Gigot et al., 2010).

1.2. Sites of production and storage of plant VOCs

After their production, in the various plant organs, VOCs are usually released by secretory cells outside the plant or into specialized intercellular spaces (Fahn, 1988; Maffei, 2010). VOCs at high concentrations can be toxic to the plants themselves. In some species, the strategy developed by plants to overcome this problem is to store VOCs in extracellular compartments, such as glandular trichomes, secretory ducts and cavities, secretory cells (e.g., tomato, citrus, pine, vetiver, and snapdragon). (Maffei, 2010; Tissier, 2012). Glandular trichomes (Figure 3A-1 and Figure 3B-6) can secrete a wide range of metabolites in defense against various biotic and abiotic attacks. Non-glandular trichomes, on the other hand, are unable to produce or store metabolites (Figure 3A-2 and Figure 3B-3). In some plants, VOCs are stored in resin canals (Figure 3C) and secretory cavities (Figure 3D) and represent a constitutive defense ready to be delivered in the event of tissue rupture. In other species, VOCs are present in the secretory cells of roots (Figure 3E) and flowers (Figure 3F). In some species, flowers produce their attractive VOCs in osmophores or cone cells located on the petals (Kolossova et al., 2001).

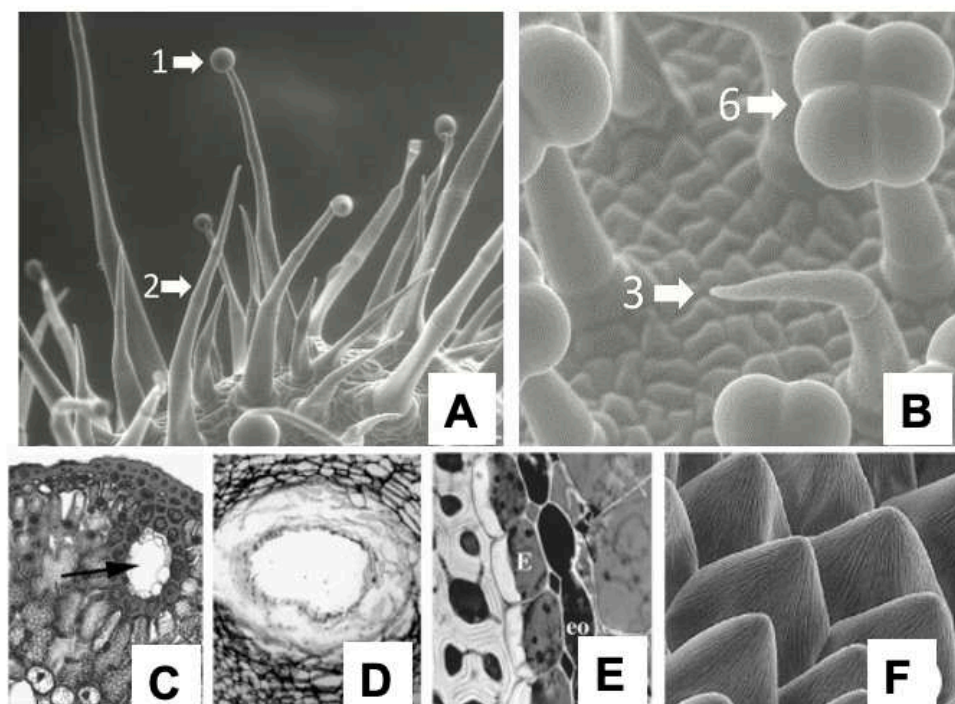


Figure 3. Secretory structures producing VOCs in plants. (A) Scanning electron microscopy (SEM) image of a young wild tomato (*Solanum habrochaites*, Solanaceae, accession LA2409) leaf. This picture illustrates the complexity of the leaf trichome landscape. At least five trichome types, numbered according to Luckwill (1943) can be observed: Type 1, tall glandular trichomes with a single secretory cell; Type 2, tall non-glandular trichomes. (B) Detail of a tomato leaf (*Solanum lycopersicum* LA 4024) showing: Type 3, short hooked non-glandular trichome; Type 6, glandular trichomes with four head cells. (C) Needles of gymnosperms show the presence of resin ducts; arrow indicates the cavity where resin is secreted. (D) Citrus fruit skin shows the presence of secreting cavities of lysigenous origin. (E) Cross-section of a *Vetiveria zizanioides* root stained with Sudan Black B, showing oil-producing cells within the last layer of the cortical parenchyma. (F) SEM of conical cells from the inner epidermis of *Antirrhinum* flower petals. E = endodermis; eo = essential oil-storing cells. (Modified from Maffei, 2010; Tissier, 2012)

2. Factors affecting the emission of plant VOCs

The emission rates of plant VOCs are determined on the one hand by their biosynthesis rates and on the other hand by their physicochemical characteristics (solubility, volatility and diffusivity). The biosynthesis of plant VOCs is modulated by internal and external factors (Peñuelas and Staudt, 2001). Internal factors are mainly genetic and biochemical factors. External factors can be biotic factors such as herbivore or pest attacks, selection pressure exerted by pollinator attraction, human domestication, or abiotic factors such as temperature, light, drought, environmental

pollution (Figure 4). While many studies have tested the effect of these different factors, often individually, on the emission of VOCs, studies on floral odors remain limited and a number of issues have yet to be explored.

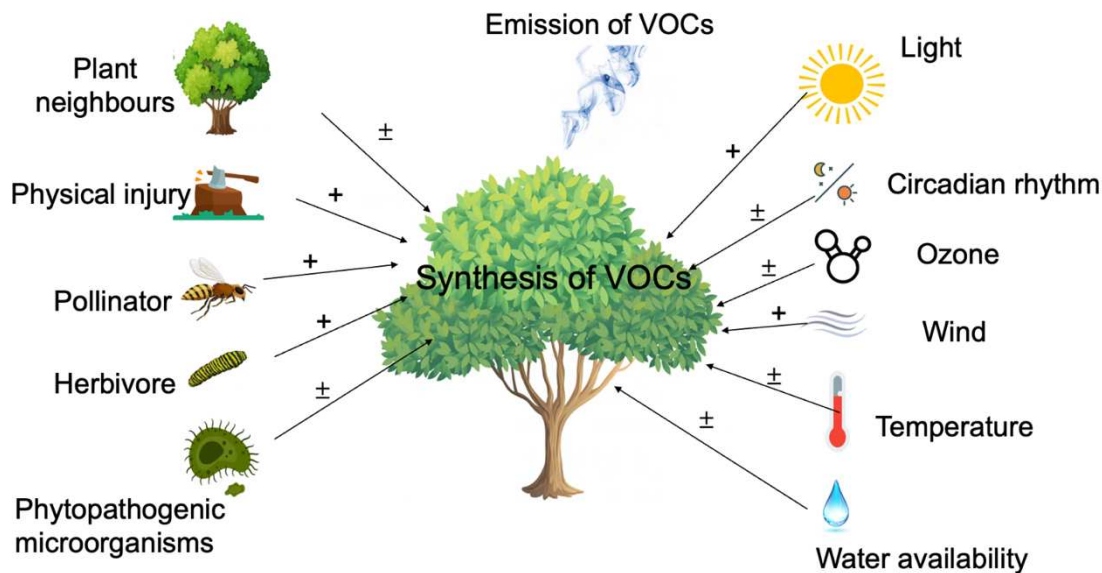


Figure 4. Factors affecting the emission of plant VOCs. The “+” symbols indicate clear overall enhancing of VOCs emission, the “±” symbols indicate both enhancing and decreasing effects. (Modified from Peñuelas and Staudt, 2001)

2.1. Internal factors: genetic and biochemical factors

VOC release from plants is generally regulated by the developmental stage (Picazo-Aragón et al., 2020) and follows a diurnal rhythm often linked to its endogenous biological clock, i.e. circadian rhythm (Zeng et al., 2017). Rhythmic VOC release under circadian control has been demonstrated in many plant species (Zeng et al., 2017). This circadian release of plant volatiles may be related to the rhythmicity of expression of genes encoding key or final enzymes in biosynthetic pathways or to the rhythmicity of enzyme activity. For example, in snapdragon flowers it has been shown that the rhythmicity of terpenoid emission is controlled by the endogenous clock via the methylerythritol phosphate (MEP) pathway (Figure 2) (key enzyme) (Dudareva et al., 2005). In another species, *Artemisia annua*, grown under constant light or dark conditions, transcript levels of QH6, a β -pinene synthase gene (final enzyme) also continue to fluctuate with circadian rhythms, resulting in diurnal patterns of β -pinene content and emission (Lu et al., 2002). Finally, for *Stephanotis* flowers, oscillations in

salicylic acid carboxyl methyltransferase activity (enzyme activity) coincided with nocturnal emission of methyl salicylate (Pott et al., 2003).

In addition, the efficiency of volatile emission may depend on the availability of the substrate but also on transcription factors (Fenske et al., 2015). For example, the benzoic acid substrate regulates the rhythmic production and emission of VPBs such as methyl benzoate in petunia flowers (Fenske et al., 2015). In addition, PhLHY transcription factors regulate the production of floral volatiles in petunia (Fenske et al., 2015).

2.2. External factors: biotic and abiotic factors

Many external factors such as biotic factors (pollinator, herbivore, microorganism and human) and abiotic factors (light, temperature, humidity and air pollution) influence the emission of plant volatiles.

Pollinator attraction

Selection pressures exerted by pollinator attraction have major effects on the composition of floral VOCs and their emission rates (Farré-Armengol et al., 2020). Some studies have shown convergent evolution of floral VOC composition in species that are distant but depend on the same group of pollinators for reproduction (Majetic et al., 2009). For example, dimethyl disulfide (DMDS) is an important odorant component in most bat-pollinated plant species (Knudsen and Tollsten, 1995; Helversen et al, 2000), and benzaldehyde, lilac aldehyde, and phenylacetaldehyde are typical constituents of flowers pollinated by moths (Dudareva et al., 2006; Dotterl et al., 2006; Plepys et al., 2002, Dobson, 2006). These cases of convergence have been demonstrated on a small number of species, pollinated by very different groups of animals. However, when a lineage of plants is pollinated by pollinators that are phylogenetically very close, the presence of a phylogenetic signal is expected for a given attractive trait. **However, the presence of phylogenetic signals in the evolution of floral odors has rarely been shown.**

In addition, the timing of pollinator activities affects VOC emissions. It has been reported that nocturnally pollinated plant species emit strong odors during the night and

daytime pollinated species during the day (Dötterl et al., 2006). Flowers of *Petunia axillaris* (Oyama-Okubo et al., 2005) and wild tobacco, *Nicotiana attenuata* (Kessler et al., 2008; Yon et al., 2016) emit volatile compounds at night to attract nighttime pollinators (Fenske et al., 2015; Yon et al., 2016). In addition, generally during development the emission of floral odors increases when pollinators are most active (Friberg et al., 2014; Jürgens et al., 2014). It is also expected that after successful pollination, floral VOC emission is reduced to limit the physiological costs associated with their biosynthesis and also to maximize their reproductive success by redirecting pollinators to unpollinated flowers of the same plant (Chen et al., 2009; Proffit et al., 2018). Thus, it has been reported in several species that a significant reduction in floral odors is observed after 24 hours (Dötterl et al., 2005) to several days (Schiestl and Ayasse, 2001) after pollination. **However, to our knowledge, no study has tested whether these changes can take place on a time step shorter than 24 hours.**

Defense against biotic stresses

Herbivores and pathogenic microorganisms can also affect VOC emission. It has been shown that VOCs can play a role in direct or indirect plant defense against insect herbivores (Ferry et al. 2007; Kim and Felton, 2013) and pathogens (Ameye et al., 2015). For example, plants can protect themselves directly from herbivores by secreting repellent VOCs (Turlings et al., 1990; Ferry et al., 2007). But they can also, play an indirect defense role by releasing VOCs that attract natural enemies of herbivores (Turlings et al., 1990; Ferry et al., 2007). In addition, GLVs (green leaf volatiles) have antimicrobial activity against bacteria. For example, C6-aroma compounds GLVs have potent antibacterial action that acts on both Gram-negative and Gram-positive bacteria (Nakamura et al., 2015).

Effects of Human Practices

Human-driven diversity can impact VOC emissions. Indeed, domestication could affect the evolution of VOC composition by direct selection of varieties with a particular chemical composition, by indirect effects of selection of varieties with other traits than VOCs or traits of other organs (e.g. impact on floral VOCs of traits related

to fruits), or simply by genetic drift. In fact, direct selection of varieties with a particular composition (i.e. production of different roses by horticulturists) has acted on VOCs in many aromatic plants grown for the perfume industry. For example, the essential oil of true lavender used for perfumes, *Lavandula angustifolia*, contains less camphor than the essential oil of wild lavender, *Lavandula latifolia*, and that of hybrid lavandin, *Lavandula x intermedia* (Guitton et al., 2018, Pokajewicz et al., 2022). On the other hand, in commercial breeding programs, because floral odor is rarely targeted, unlike traits such as cold and disease resistance, flower shape, or vase life, floral odor is often absent from modern varieties (Vainstein et al., 2001). In addition, during the domestication of fruit crops, selection for certain fruit traits related to a particular flavor, color, or odor likely resulted in the addition, elimination, and/or modification of various specialized metabolic pathways, resulting in the gain or loss of specialized metabolites (Dar et al., 2021). These specialized metabolites, such as glycosides or ethylene, but also specific enzymes, play a role in VOC production (Mostafa et al., 2022). **However, to date, no study has examined the effect of domestication on the signal emitted by plants to attract their pollinators.**

Effects of abiotic factors

The emission of VOCs can be influenced by abiotic factors that directly affect the biosynthesis of VOCs or indirectly as they affect the physiology of the plant, such as photosynthesis, or through their volatilization (Loreto et al., 1996; Niinemets et al., 2002; Rasulov et al., 2009).

In general, increasing temperature leads to an increase in leave VOC emissions. This increase is due to the temperature dependence of the physicochemical properties of VOCs and the enzymatic activities of terpene synthases (Holopainen and Gershenzon, 2010). This type of emission pattern has been demonstrated for the activities of key enzymes in the pathway and the expressions of several genes related to enzymes responsible for VOC biosynthesis under different temperatures in *Jasminum auriculatum* Vahl (Barman and Mitra, 2021). In several Mediterranean species, it has been shown that floral emissions increased with temperature to an optimum and

thereafter decreased (Farré-Armengol et al., 2014).

VOC emission increases with humidity due to increased stomatal conductance (Lange et al., 1971). Light intensity increases the emission of VOCs, especially monoterpenes. Because the monoterpene biosynthetic pathway, the methylerythritol phosphate (MEP) pathway, is partially located in chloroplasts and requires a carbon substrate and cofactors from photosynthesis (Loreto et al., 1996; Niinemets et al., 2002; Rasulov et al., 2009), sesquiterpene biosynthesis, which occurs primarily in the cytoplasm, is light independent (Lynch et al., 2020). The light responses of sesquiterpene emissions are not exactly known, although evidence of light dependence has been reported in some studies (Duhl et al., 2008; Geron and Arnts, 2010).

Air pollution is a factor that affects VOC emission. A number of pollutants, such as CO₂ or ozone (O₃), induce the production and/or emission of VOCs from leaves into plant tissue and the atmosphere. For example, increases and decreases in VOC emissions have been described in the presence of high CO₂ concentrations (Peñuelas and Llusà, 1997; others listed in Daussy, 2020).

Compared to VOCs from leaves, the effect of abiotic factors on the emission of floral VOCs has been much less investigated.

3. Objectives of the thesis and studied system

My thesis proposes to fill in some of the gaps we have on the effects of internal (genetic and biochemical) and external (biotic-pollinators, human practices and abiotic-temperature, light, humidity) factors on the emission of floral VOCs, in particular on VOCs responsible for pollinator attraction. For this, we chose to work on a plant whose reproduction depends on only one species of pollinator, for which the phylogeny of its genus is well known, of which certain species have been domesticated for a long time.

3.1 Study model

3.1.1. The genus *Ficus*

The genus *Ficus* (Moraceae) is quite diverse, with more than 800 specific fig/fig wasp interactions distributed in all continents. *Ficus* spp., bear figs that are enclosed

inflorescences termed 'syconia', which are characterized by an enclosed wall with a unique entrance (i.e. the ostiole) with the hundreds of tiny florets lining the inside of a central cavity. Each species of *Ficus* is generally pollinated by one species-specific pollinator of the family Agaonidae (Hymenoptera), which are tiny wasps that develop exclusively in the figs of their host species and use VOCs emitted by receptive figs (i.e. in the developmental stage where they are ready to be pollinated) to locate their host (Gibernau *et al.*, 1997; Grison-Pigé *et al.*, 2002; Proffit *et al.*, 2008). The genus *Ficus* appeared a long time ago (end of Superior Cretaceous, ca. 75 MY [Cruaud *et al.*, 2012]) and both partners share a long history of coevolution shaping floral traits and the insects' capacities of signal recognition. The genus is composed of 19 sections and the family Agaonidae includes about 20 genera, offering an adequate sample size for phylogeny-based analyses. Comparisons of the phylogenies of *Ficus* and their associated Agaonidae have revealed co-diversification between plants and pollinators: in general, species of *Ficus* belonging to different sections are associated with different pollinator genera (Figure 5) (Cruaud *et al.*, 2012). Numerous studies have shown that partner encounter is mediated solely by the emission of floral VOCs by receptive figs and that species-specificity is maintained by the emission by figs, and the recognition by the associated fig wasps, of species-specific volatile signals (Ware *et al.*, 1993; Proffit *et al.*, 2008; Chen *et al.*, 2009; Wang *et al.*, 2013).

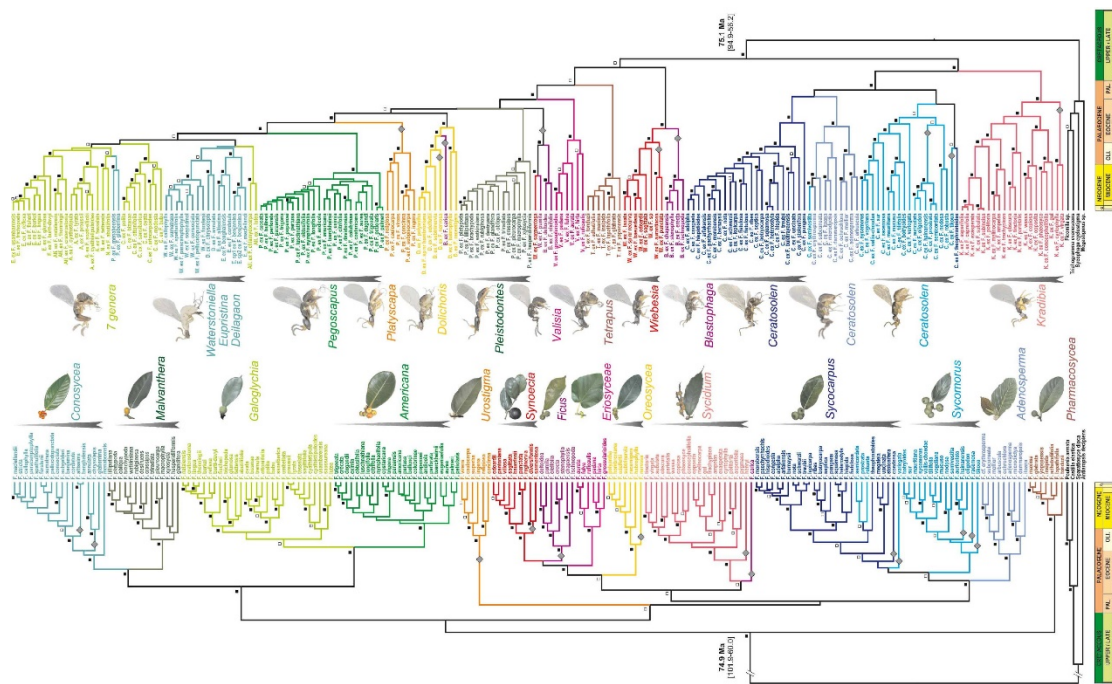


Figure 5. Chronograms of the evolutionary history of figs and fig wasps. Phylogenetic trees of figs and fig wasps (based for figs on 3 genes, wasps on 5 genes) were reconstructed using the maximum likelihood (ML) and Bayesian methods. Groups of figs and their associated genera of pollinators are represented using the same color. *Ficus* subgenera and Agaonidae subfamilies according to current classifications are delimited by colored rectangles. Gray rhombuses show clades of fig species from Continental Asia, whereas gray arrows indicate hypothesized southward migration of clades. (from Cruaud et al., 2012)

3.1.2. *Ficus carica* and *Blastophaga psenes*

The Mediterranean fig tree, *Ficus carica* (Moraceae), is one of the emblematic trees of the Mediterranean region and was domesticated around 5500 years ago (Zohary and Spiegel-Roy, 1975). Like other *Ficus* spp., trees of *F. carica* bear figs inflorescence (Figure 6), that are exclusively pollinated the agaonid *Blastophaga psenes* L. (Agaonidae) that develop exclusively in the figs of their host species. Once emerging from its natal fig, pollinators leave less than 24h. *Ficus carica* is dioecious tree (some fig trees can be monoecious). This means Male trees bear figs that produce pollen and in which the specific pollinator, the fig wasp develops and female trees bear figs in which only seeds develop, but not pollinators (Kjellberg et al., 1987) (Figure 7). Attraction of female *B. psenes* by VOCs emitted by receptive figs of male and female trees has already been well described (Proffit et al., 2020). Previous studies have shown that only four of the VOCs emitted by receptive figs (i.e. *S*-linalool, benzyl alcohol,

(*Z*)-linalool-oxide (furanoid), and (*E*)-linalool-oxide (furanoid)), within a narrow range of proportions, are responsible for attraction of the specific fig wasp pollinator and that even a very small change in their proportions disrupts pollinator attraction (Proffit et al., 2020).

In the Mediterranean region, a wide diversity of varieties of female fig trees (several hundred) have been selected. Comparatively, the classification of male fig trees into varieties is still debated. Traits of interest for human consumption in female figs that have been selected from wild figs are mainly based on fruit characteristics, including size, color, sweetness, softness, drying capacities linked to trade and storage, but also phenology, with early to late varieties in order to obtain fruits over the longest period possible.

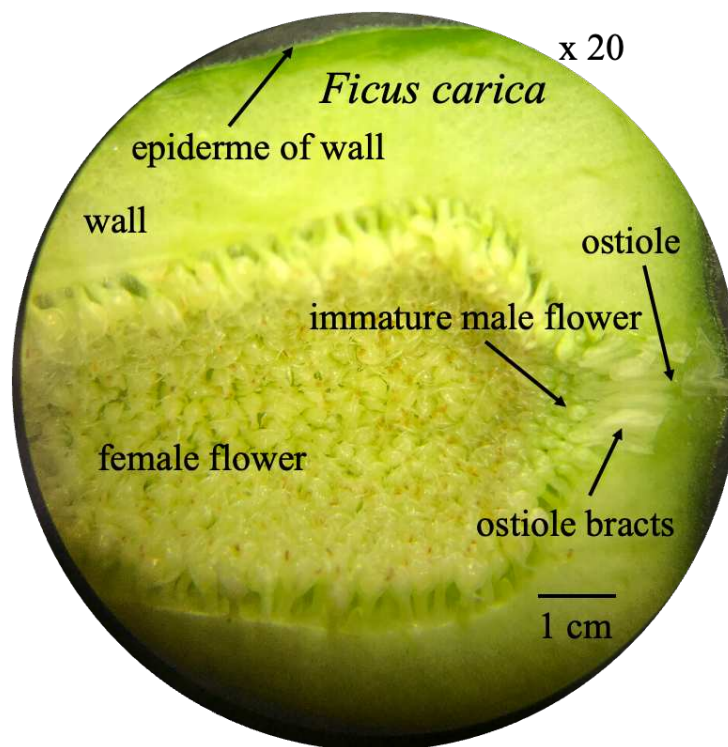


Figure 6. Longitudinal sections of a fig of *F. carica*. Scale bars: 1 cm, magnification : x 20.

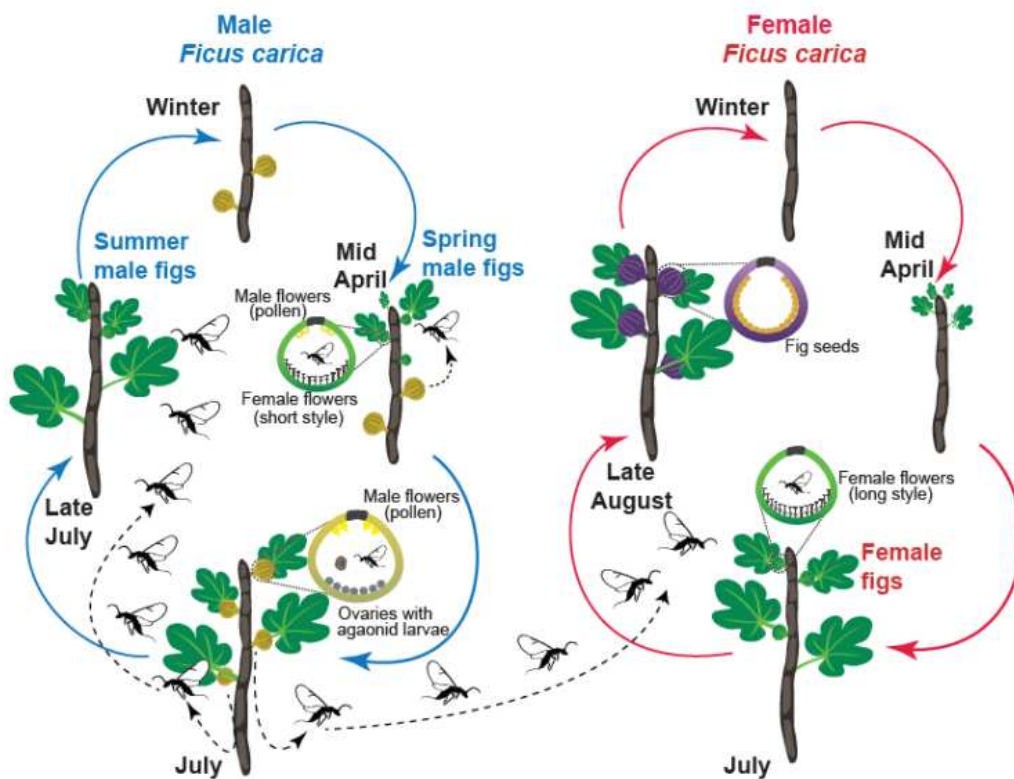


Figure 7. Life cycles of male and female tree of *Ficus carica* (respectively on the left and right side) and *Blastophaga psenes* in southern France. For each type of fig, receptive (green), ripe male (khaki) and ripe female (purple), a schematic representing wasps and flowers inside the fig is presented (from Proffit et al., 2020).

3.2. Specific objectives

My thesis is divided into four following chapters, in form of article, each with a specific focus (Figure 8):

- **Chapter 1: determining the factors shaping the composition of floral VOC along the evolutionary history in the genus *Ficus*.**

To do this, we supplemented a large, previously available dataset on the composition of receptive fig odors from several *Ficus* species, and tested the effects of factors that could potentially have shaped fig VOC composition over the evolutionary history of the genus.

> *Predictions: there is a strong phylogenetic signal in the VOCs emitted by receptive figs in Ficus, likely due to constraints in the biosynthetic pathways of volatile compounds.*

- Chapter 2: determining the effect of human-driven diversification on floral VOCs in *Ficus carica* and in particular on compounds responsible for pollinator attraction.

To do this, we studied the VOCs of receptive figs of several varieties of *F. carica* in Morocco, and tested whether VOC emission varied between varieties qualitatively but also quantitatively, in particular the VOCs responsible for pollinator attraction.

> Predictions: the proportions of VOCs responsible for pollinator attraction should be highly conserved between varieties at receptivity, in contrast to VOCs that play no role in pollinator attraction.

- Chapter 3: determining the effect of pollinator activity and abiotic factors on the temporal dynamics of floral VOC emission in *F. carica*.

To do this, we described the diurnal emission patterns of the four pollinator-attracting VOCs in *Ficus carica* and determined the effects of pollination and abiotic factors such as temperature, light intensity and humidity on their emissions.

*> Predictions: the four pollinator-attractive VOCs emitted by *F. carica* follow a rhythm of emission that varies during the day, with a peak of emission when pollinators are most active; they increase with temperature, decrease rapidly after pollination.*

- Chapter 4: characterizing the genes encoding the biosynthesis of VOCs involved in pollinator attraction in *F. carica* and on the production in the fig of these VOCs.

To do so, we characterized the function of several genes involved in the emission of pollinator-attractive VOCs in *F. carica* and located the tissue of the figs producing these VOCs.

> Predictions: expression of genes involved in pollinator attraction decrease after pollination.

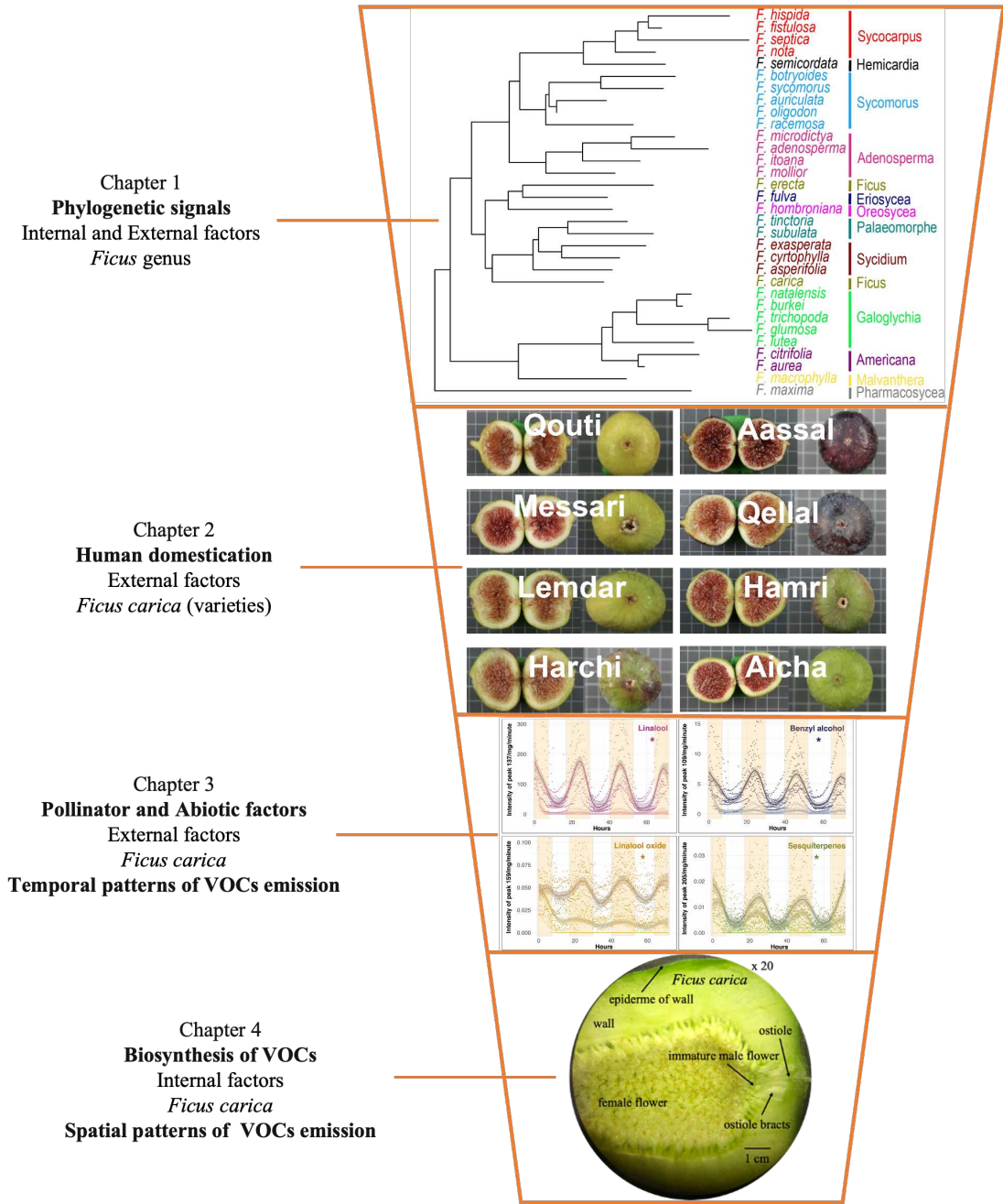
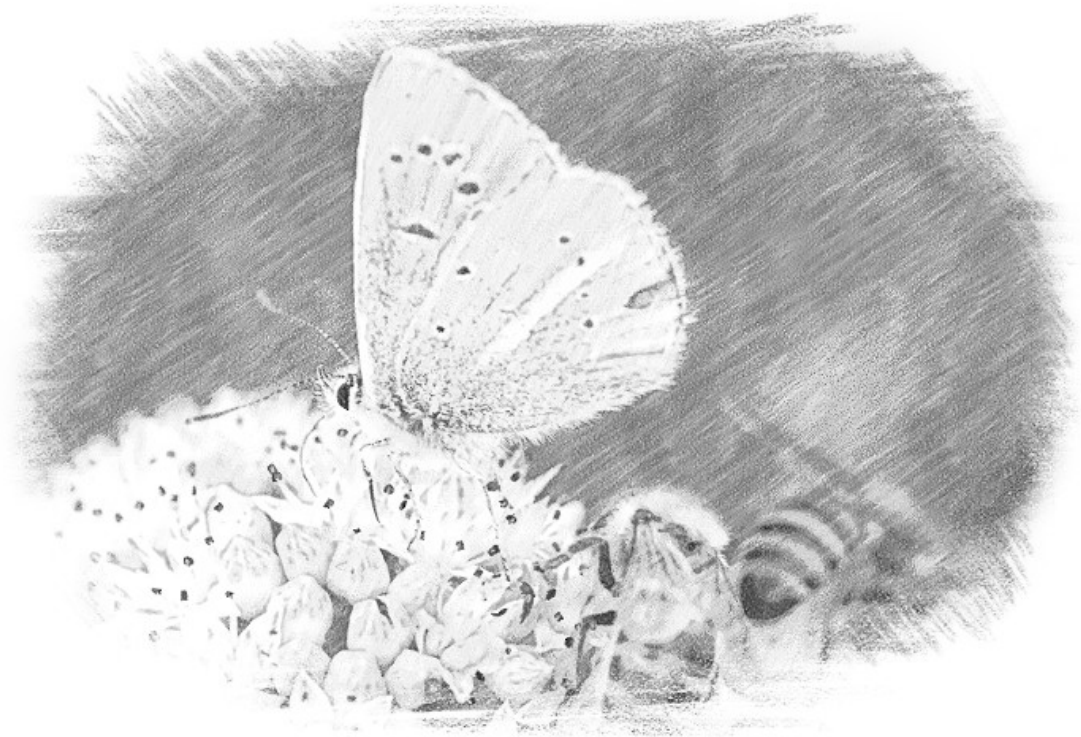


Figure 8. Diagram illustrating the objectives of the thesis.



Introduction générale

Les plantes, qui sont des organismes non mobiles et sessiles, doivent s'adapter au milieu environnant au cours de leur cycle de vie. Pour compenser leur immobilité, les plantes ont développé divers mécanismes d'interaction avec leur environnement biotique et abiotique. Parmi ceux-ci, la production et l'émission de mélanges complexes de composés organiques volatils (COVs) par différents organes permet aux plantes de transmettre des messages chimiques donnant des informations sur leur état. Certains de ces COVs, par exemple, constituent des signaux chimiques pour leurs pollinisateurs et affectent ainsi leur reproduction. Ces COVs vont également permettre aux plantes de former des relations symbiotiques avec des micro-organismes ou de se défendre contre leurs ennemis et de résister aux stress abiotiques.

1. Les Composés organiques volatils (COVs) des plantes

Les COVs végétaux sont des métabolites spécialisés qui jouent un rôle majeur dans les interactions entre les plantes et leur environnement biotique (Figure 1). Par exemple, ils jouent un rôle majeur dans l'attraction des pollinisateurs et des disperseurs de graines, ainsi que vers, d'autres organismes bénéfiques, mais également dans la défense des plantes contre les herbivores et les pathogènes (Agrawal, 2011, Beccera et al., 2009, Dudareva & Pichersky, 2008 ; Raguso, 2008 ; Junker & Parachnowitsch, 2015). Les COVs végétaux jouent également un rôle important dans la défense contre les facteurs abiotiques comme la température, les polluants, etc. En cas de stress abiotique, chez certaines espèces, l'émission d'isoprénoïdes est stimulée, soit par la neutralisation directe des espèces chimiques réactives de l'oxygène (ROS) (Loreto & Velikova, 2001), soit indirectement par la stabilisation des membranes cellulaires (Velikova et al., 2011) afin d'améliorer la résistance des plantes. Les COVs végétaux sont principalement libérés par les fleurs, mais aussi par les fruits, les feuilles, les tiges et même les racines (Farré-Armengol et al., 2016). À ce jour, plus de 1 700 COVs floraux ont été décrits (Knudsen et al., 2006). Ces COVs appartiennent à quatre grandes familles chimiques en fonction de leur origine métabolique : les terpénoïdes, les phénylpropanoïdes/benzénoïdes, les dérivés d'acides gras et d'acides aminés (Knudsen

et al., 2006, Maffei, 2010). Différentes voies de biosynthèse, situées dans différentes parties de la cellule végétale (cytosol ou plaste), sont responsables de la production de ces COVs (Pichersky et al., 2006, Maffei, 2010, Picazo-Aragonés et al., 2020). Une fois produits, ces COVs peuvent être libérés par différentes cellules végétales ou stockés dans une structure particulière comme les trichomes glandulaires, les canaux et cavités sécrétoires, les cellules sécrétoires des fleurs et des racines (Maffei, 2010).

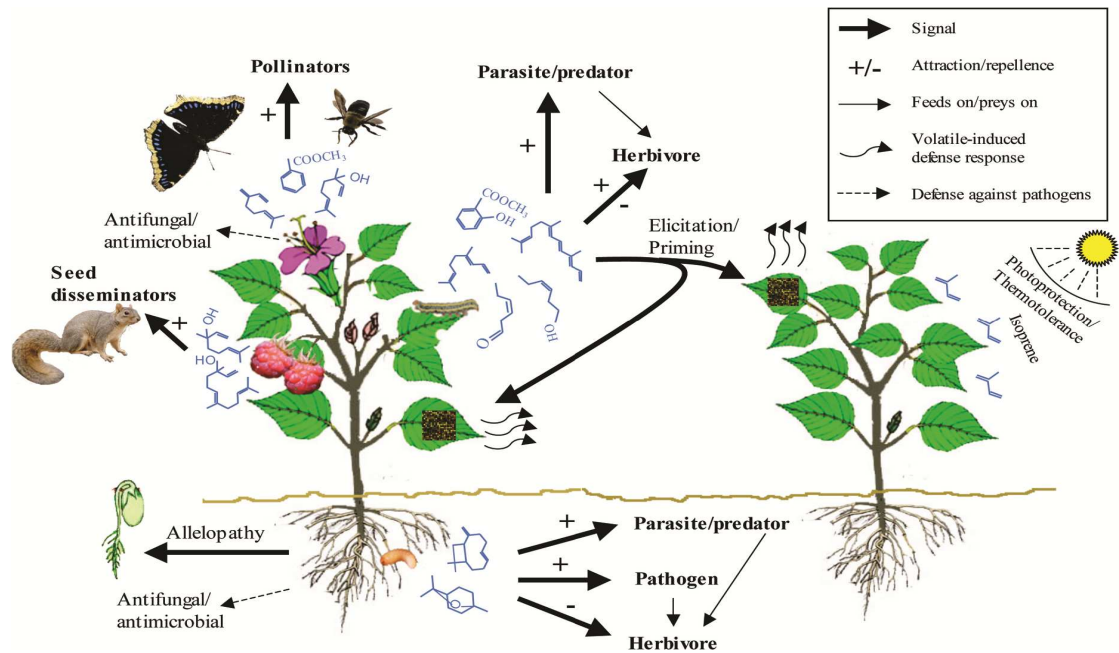


Figure 1. Vue d'ensemble des interactions entre les plantes et le milieu environnant par l'intermédiaire des substances volatiles. Les interactions plante-animal comprennent l'attraction des pollinisateurs et des disséminateurs de graines par les volatiles floraux et de fruits, l'attraction/la répulsion des herbivores et l'attraction des ennemis naturels des herbivores attaquant dans l'atmosphère et la rhizosphère. Les interactions plante-plante en surface comprennent l'élicitation ou l'amorçage des réponses de défense dans les feuilles saines et non endommagées de la même plante ou dans les plantes voisines non attaquées. Au niveau souterrain, ces interactions comprennent l'activité allélopathique sur la germination et la croissance des plantes voisines compétitives. Les volatiles libérés par les organes reproducteurs et les racines ont également une activité antimicrobienne, protégeant ainsi les plantes contre les attaques de pathogènes. En outre, l'isoprène, un volatil des feuilles, confère une photoprotection et une thermotolérance. (d'après Dudareva et al., 2006)

1.1. Principales voies biochimiques des COVs végétaux

Les COVs sont produits par de nombreuses synthèses et dans différentes parties de la cellule en fonction des classes chimiques (Figure 2) (Dudareva et al., 2013, 2006 ; Maffei, 2010 ; Picazo-Aragónés et al., 2020).

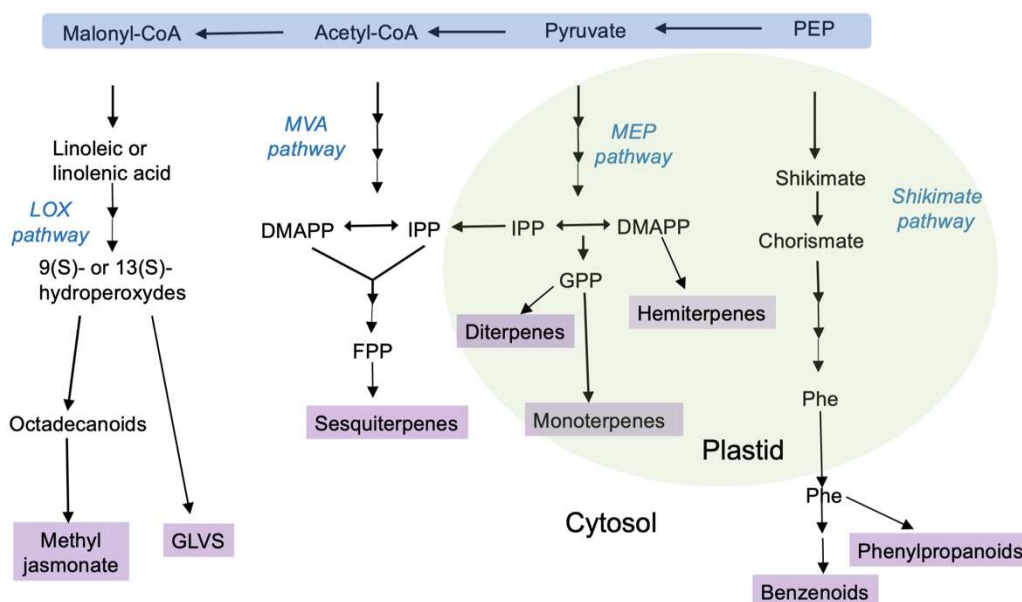


Figure 2. Vue d'ensemble des voies de biosynthèse conduisant à l'émission de COVs végétaux.

Les précurseurs des COVs végétaux proviennent du métabolisme central (représenté dans la boîte bleue). Les quatre principales voies de biosynthèse des COVs, à savoir le shikimate/phénylalanine, le méthylérythritol phosphate (MEP), l'acide mévalonique (MVA) et la lipoxygénase (LOX), conduisent respectivement à l'émission de benzénoïdes/phénylpropanoïdes, de monoterpènes, de sesquiterpènes et de méthyljasmonate/volatils de feuilles vertes. Les noms des voies sont en italique, les COVs sont mis en évidence avec une boîte violette. Les flèches empilées illustrent l'implication de plusieurs réactions enzymatiques. Abréviations : DMAPP, diméthylallyl pyrophosphate ; FPP, farnesyl pyrophosphate ; GLVs, green leaf volatiles ; GPP, geranyl pyrophosphate ; IPP, isopentenyl pyrophosphate ; PEP, *phosphoenolpyruvate* ; Phe, phénylalanine. (Modifié d'après Dudareva et al., 2006 ; Dudareva et al., 2013)

Terpénoïdes

Tous les terpénoïdes proviennent de deux précurseurs communs à cinq carbones, le diphosphate d'isopentényle (IPP) et ses isomères, le diphosphate de diméthylallyle (DMAPP) (McGarvey & Croteau, 1995), qui sont synthétisés à partir de deux voies indépendantes et séparées par des compartiments : la voie de l'acide mévalonique (MVA) et la voie du phosphate de méthylérythritol (MEP). Dans les plastides, la voie MEP fournit les précurseurs des hémiterpènes (C5), des monoterpènes (C10) et des diterpènes (C20) (Hsieh, et al., 2008), tandis que dans le réticulum endoplasmique et

les peroxysomes, la voie MVA produit les précurseurs des sesquiterpènes (C₁₅) (Simkin, et al., 2011 ; Pulido, et al., 2012).

Les terpénoïdes constituent la plus grande classe de volatils floraux (Knudsen et al, 2006). Le génome des plantes contient de nombreux gènes de terpènes synthases, codant pour des enzymes aux fonctions différentes. La propriété la plus exceptionnelle de ces enzymes est la capacité de nombre d'entre elles à synthétiser plusieurs produits à partir d'un seul précurseur de disphosphate de prényle (Degenhardt, et al., Gershenzon 2009). Par exemple, seules deux sesquiterpènes synthases sont responsables de la quasi-totalité des 20 sesquiterpènes émis par les fleurs d'*Arabidopsis* (Tholl et al., 2005). Certaines terpène synthases (TPS) peuvent également utiliser plus d'un substrat (Tholl 2006 ; Bleeker et al., 2011). Dans ce cas, leur localisation subcellulaire et la disponibilité d'un substrat particulier déterminent le type de produit formé (Nagegowda et al., 2008).

Dérivés de l'acide shikimique

Les dérivés de l'acide shikimique sont des composés contenant un cycle aromatique et constituent la deuxième classe de composés volatils dans les plantes. Le shikimate conduit à la phénylalanine, puis aux composés phénylpropanoïdes et benzénoïdes (Maffei, 2010 ; Dudareva et al., 2013). Ces composés peuvent généralement être divisés en trois classes en fonction de la longueur de leurs chaînes latérales : les phénylpropanoïdes (qui ont une chaîne latérale de trois carbones et sont donc appelés composés C₆ -C₃), les composés benzénoïdes dérivés du Phe (C₆ -C₁), ainsi que les composés C₆ -C₂ apparentés aux phénylpropanoïdes (Knudsen et al., 1993).

Les voies complètes de formation des phénylpropènes (C₆ -C₃) ont maintenant été élucidées chez *Clarkia breweri* (Koeduka et al., 2008), *Petunia hybrida* (Koeduka et al., 2006, 2008) et *Ocimum basilicum* (Koeduka et al., 2006 ; Vassão et al., 2006). La formation des benzénoïdes (C₆ -C₁) se fait par deux voies différentes. Une voie β -oxydative dépendante du CoA a récemment été entièrement élucidée chez *Petunia hybrida* et *Arabidopsis thaliana*. Une autre voie non β -oxydative a été signalée dans le genre *Capsella*, *Petunia axillaris*, *Arabidopsis* et le mufler (Sas et al., 2016 ; Amrad et

al., 2016 ; Ibdah et al., 2009 ; Long et al., 2009), bien que les étapes biochimiques menant à la formation de l'alcool benzylique soient encore en suspens. La voie de formation du phénylacétaldéhyde (C₆-C₃) a été signalée dans le pétunia et les pétales de rose (Kaminaga et al., 2006).

Dérivés d'acides gras

Les dérivés d'acides gras sont produits à partir d'acides gras insaturés en C18, qui sont soumis à la désoxygénation par l'enzyme lipoxygénase (LOX) (Feussner & Wasternack, 2002). Il existe deux branches différentes de la voie LOX : l'allène oxyde synthase (AOS) et l'hydroperoxyde lyase (HPL). La voie AOS : Le produit de l'acide jasmonique (JA) de l'AOS est converti en jasmonate de méthyle (Seo et al., 2001 ; Song et al., 1993). La voie HPL : Les aldéhydes et alcools à chaînes courtes en C6 et C9, connus sous le nom de "green leaf volatiles" (GLV), sont des constituants importants du bouquet floral volatile des plantes (Schade et al., 2001 ; Suchet et al., 2011).

Les différentes iso-enzymes LOX peuvent être classées en deux types en fonction de leur stéréospécificité. La plupart du temps, l'isoenzyme LOX oriente la voie LOX complète de la plante, comme la 9-LOX (oxygénation du neuvième carbone de l'acide gras) chez le concombre, la 13-LOX (oxygénation du treizième carbone de l'acide gras) chez la pastèque (Gigot et al., 2010).

1.2. Sites de production et de stockage des COVs.

Après leur production dans les différents organes de la plante, les COVs sont généralement libérés par des cellules sécrétrices à l'extérieur de la plante ou dans des espaces intercellulaires spécialisés (Fahn, 1988 ; Maffei, 2010). À des concentrations élevées, les COVs peuvent être toxiques pour les plantes elles-mêmes. Chez certaines espèces, la stratégie développée par les plantes pour surmonter ce problème consiste à stocker les COVs dans des compartiments extracellulaires, tels que les trichomes glandulaires, les canaux et cavités sécrétoires, les cellules sécrétrices (par exemple, la tomate, les agrumes, le pin, le vétiver et le muflier). (Maffei, 2010 ; Tissier, 2012). Les trichomes glandulaires (Figure 3A-1 et Figure 3B-6) peuvent sécréter une large gamme de métabolites pour se défendre contre diverses attaques biotiques et abiotiques. Les

trichomes non glandulaires, en revanche, sont incapables de produire ou de stocker des métabolites (Figure 3A-2 et Figure 3B-3). Chez certaines plantes, les COVs sont stockés dans les canaux résinifères (Figure 3C) et les cavités sécrétoires (Figure 3D) et représentent une défense constitutive prête à être délivrée en cas de rupture des tissus. Chez d'autres espèces, les COVs sont présents dans les cellules sécrétoires des racines (Figure 3E) et des fleurs (Figure 3F). Chez certaines espèces, les fleurs produisent leurs COVs attractifs dans des osmophores ou des cellules coniques situées sur les pétales (Kolossova et al., 2001).

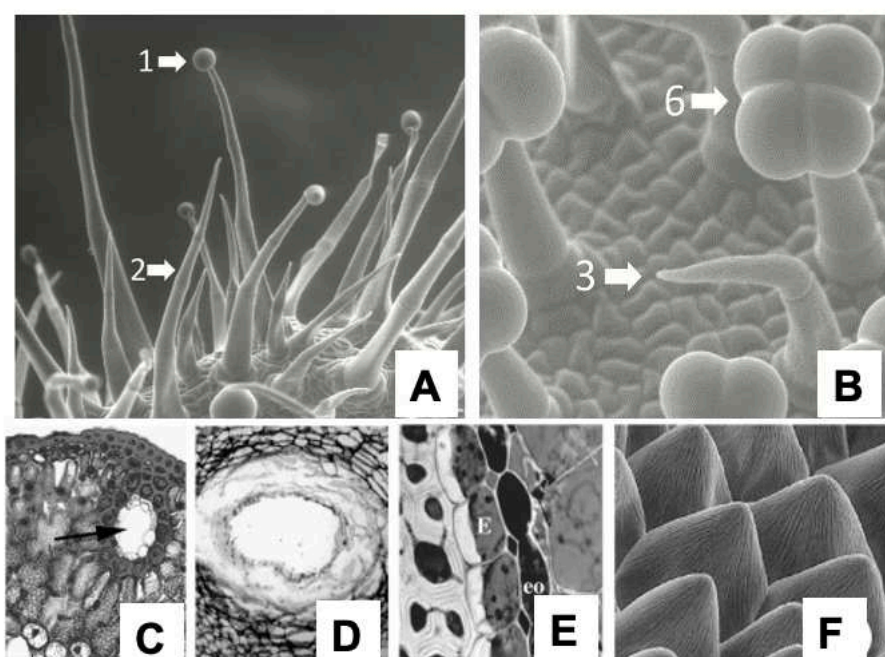


Figure 3. Structures sécrétoires produisant des COVs dans les plantes. (A) Image au microscopie électronique à balayage (MEB) d'une jeune feuille de tomate sauvage (*Solanum habrochaites*, Solanaceae, accession LA2409). Cette image illustre la complexité du paysage des trichomes de la feuille. On peut observer au moins cinq types de trichomes, numérotés selon Luckwill (1943) : Type 1, trichomes glandulaires hauts avec une seule cellule sécrétrice ; Type 2, trichomes non glandulaires hauts. (B) Détail d'une feuille de tomate (*Solanum lycopersicum* LA 4024) montrant : Type 3, trichome non glandulaire court et crochu ; Type 6, trichomes glandulaires à quatre cellules de tête. (C) Les aiguilles de gymnospermes montrent la présence de canaux résinifères ; la flèche indique la cavité où la résine est sécrétée. (D) La peau des agrumes montre la présence de cavités sécrétrices d'origine lysigène. (E) Coupe transversale d'une racine de *Vetiveria zizanioides* colorée au noir du Soudan B, montrant des cellules productrices d'huile dans la dernière couche du parenchyme cortical. (F) MEB de cellules coniques de l'épiderme interne de pétales de fleurs d'*Antirrhinum*. E = endoderme ; eo = cellules productrices d'huile essentielle. (Modifié d'après Maffei, 2010 ; Tissier, 2012)

2. Facteurs influençant les émissions de COVs

Les taux d'émission des COVs végétaux sont déterminés d'une part par leurs taux de biosynthèse et d'autre part par leurs caractéristiques physico-chimiques (solubilité, volatilité et diffusivité). La biosynthèse des COVs végétaux est modulée par des facteurs internes et externes (Peñuelas & Staudt, 2001). Les facteurs internes sont principalement des facteurs génétiques et biochimiques. Les facteurs externes peuvent être des facteurs biotiques tels que les attaques d'herbivores ou de ravageurs, la pression de sélection exercée par l'attraction des pollinisateurs, la domestication humaine, ou des facteurs abiotiques tels que la température, la lumière, la sécheresse, la pollution de l'environnement (Figure 4). Si de nombreuses études, ont testé l'effet de ces différents facteurs, souvent individuellement, sur l'émission des COVs, les études sur les odeurs florales restent limitées et un certain nombre de points restent encore à explorer.

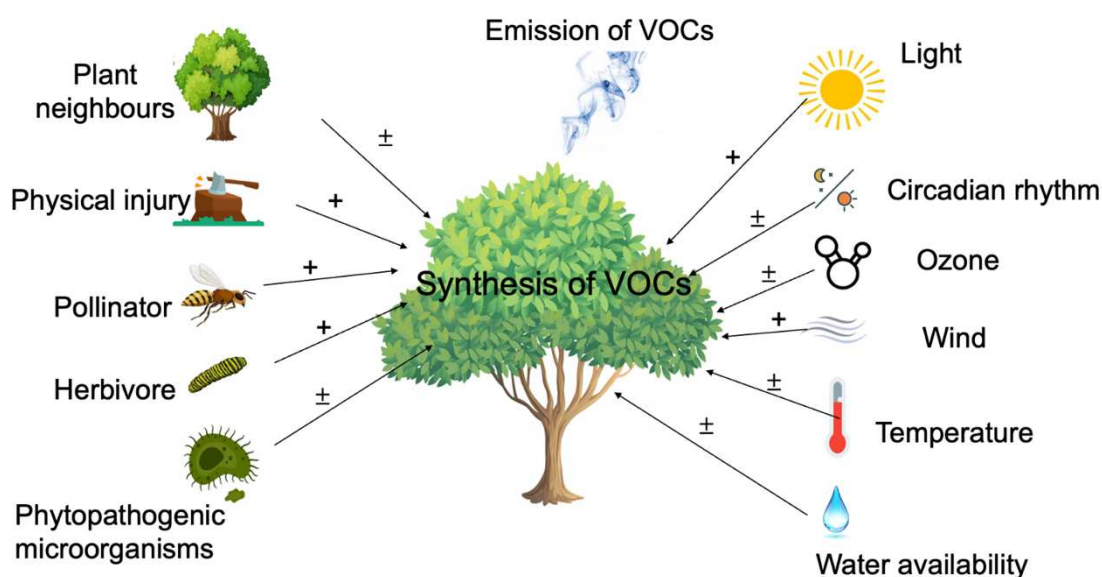


Figure 4. Facteurs affectant l'émission de COVs végétaux. Les symboles "+" indiquent une nette augmentation globale des émissions de COVs, les symboles "±" indiquent à la fois des effets d'augmentation et de diminution. (Modifié d'après Peñuelas & Staudt, 2001)

2.1 Facteurs internes : facteurs génétiques et biochimiques

La libération de COVs par les plantes est généralement régulée par le stade de développement (Picazo-Aragónés et al., 2020) et suit un rythme diurne souvent lié à l'horloge biologique endogène, c'est-à-dire le rythme circadien (Zeng et al., 2017). La

libération rythmique de COVs sous contrôle circadien a été démontrée chez de nombreuses espèces végétales (Zeng et al., 2017). Cette libération circadienne de volatils végétaux peut être liée à la rythmicité de l'expression des gènes codant pour des enzymes clés ou finales dans les voies de biosynthèse ou à la rythmicité de l'activité enzymatique. Par exemple, dans les fleurs de muflier, il a été démontré que la rythmicité de l'émission de terpénoïdes est contrôlée par l'horloge endogène via la voie du phosphate de méthylérythritol (MEP) (Figure 2) (enzyme clé) (Dudareva et al., 2005). Chez une autre espèce, *Artemisia annua*, cultivée dans des conditions constantes de lumière ou d'obscurité, les niveaux de transcription de QH6, un gène de β -pinène synthase (enzyme finale) continuent également à fluctuer avec les rythmes circadiens, ce qui entraîne des schémas diurnes de contenu et d'émission de β -pinène (Lu et al., 2002). Enfin, pour les fleurs de *Stephanotis*, les oscillations de l'activité enzymatique de l'acide salicylique carboxyl méthyltransférase coïncident avec l'émission nocturne de salicylate de méthyle (Pott et al., 2003).

En outre, l'efficacité de l'émission de substances volatiles peut dépendre de la disponibilité du substrat, mais aussi de facteurs de transcription (Fenske et al., 2015). Par exemple, le substrat de l'acide benzoïque régule la production et l'émission rythmiques de VPBs (volatile phenylpropanoïdes/benzenoïdes) tels que le benzoate de méthyle dans les fleurs de pétunia (Fenske et al., 2015). En outre, les facteurs de transcription PhLHY régulent la production de volatiles floraux dans le pétunia (Fenske et al., 2015).

2.2 Facteurs externes : facteurs biotiques et abiotiques

De nombreux facteurs externes tels que les facteurs biotiques (pollinisateurs, herbivores, micro-organismes et humains) et les facteurs abiotiques (lumière, température, humidité et pollution de l'air) influencent l'émission de substances volatiles par les plantes.

Attraction des pollinisateurs

Les pressions de sélection exercées par l'attraction des pollinisateurs ont des effets majeurs sur la composition des COVs floraux et leurs taux d'émission (Farré-Armengol

et al., 2020). Certaines études ont montré une évolution convergente de la composition des COVs floraux chez des espèces éloignées mais dont la reproduction dépend du même groupe de pollinisateurs (Majetic et al., 2009). Par exemple, le disulfure de diméthyle (DMDS) est un composant odorant important dans la plupart des espèces végétales pollinisées par les chauves-souris (Knudsen & Tollsten, 1995 ; Helversen et al., 2000), et le benzaldéhyde, l'aldéhyde de lilas et le phénylacétaldéhyde sont des constituants typiques des fleurs pollinisées par les papillons de nuit (Dudareva et al., 2006 ; Dötterl, et al., 2006 ; Plepys et al., 2002, Dobson, 2006). Ces cas de convergence ont été démontrés sur un petit nombre d'espèces, pollinisées par des groupes d'animaux très différents. Cependant, lorsqu'une lignée de plantes est pollinisée par des pollinisateurs phylogénétiquement très proches, la présence d'un signal phylogénétique est attendue pour un caractère attractif donné. **Toutefois, la présence de signaux phylogénétiques dans l'évolution des odeurs florales a rarement été démontrée.**

En outre, le calendrier des activités des pollinisateurs influe sur les émissions de COVs. Il a été signalé que les espèces végétales à pollinisation nocturne émettent de fortes odeurs pendant la nuit et celles à pollinisation diurne pendant la journée (Dötterl et al., 2006). Par exemple, les fleurs de *Petunia axillaris* (Oyama-Okubo et al., 2005) et de tabac sauvage, *Nicotiana attenuata* (Kessler et al., 2008 ; Yon et al., 2016), émettent des composés volatils la nuit pour attirer les pollinisateurs nocturnes (Fenske et al., 2015 ; Yon et al., 2016). En outre, au cours du développement, l'émission d'odeurs florales augmente généralement lorsque les pollinisateurs sont les plus actifs (Friberg et al., 2014 ; Jürgens et al., 2014). On s'attend également à ce qu'après une pollinisation réussie, l'émission de COVs floraux soit réduite pour limiter les coûts physiologiques associés à leur biosynthèse et aussi pour maximiser leur succès reproductif en redirigeant les pollinisateurs vers des fleurs non pollinisées de la même plante (Chen et al., 2009 ; Proffit et al., 2018). Ainsi, il a été rapporté chez plusieurs espèces qu'une réduction significative des odeurs florales est observée après 24 heures (Dötterl et al., 2005) à plusieurs jours (Schiestl & Ayasse, 2001) après la pollinisation. **Cependant**

aucune étude n'a testé à notre connaissance si ces changements peuvent avoir lieu sur un pas de temps plus court que 24h.

Défense contre les stress biotiques

Les herbivores et les micro-organismes pathogènes peuvent également influencer sur les émissions de COVs. Il a été démontré que les COVs peuvent jouer un rôle dans la défense directe ou indirecte des plantes contre les insectes herbivores (Ferry et al., 2007 ; Kim & Felton, 2013) et les pathogènes (Ameye et al., 2015). Par exemple, les plantes peuvent se protéger directement des herbivores en sécrétant des COVs répulsifs (Turlings et al., 1990 ; Ferry et al., 2007). Mais elles peuvent aussi jouer un rôle de défense indirecte en libérant des COVs qui attirent les ennemis naturels des herbivores (Turlings et al., 1990 ; Ferry et al., 2007). En outre, les GLV (green leaf volatiles) ont une activité antimicrobienne contre les bactéries. Par exemple, les composés C6-aroma des GLV ont une puissante action antibactérienne qui agit à la fois sur les bactéries Gram-négatives et Gram-positives (Nakamura et al., 2015).

Effets des pratiques humaines

La diversité induite par l'homme peut avoir un impact sur les émissions de COVs. En effet, la domestication pourrait affecter l'évolution de la composition des COVs, par la sélection directe de variétés ayant une composition chimique particulière (par exemple les roses chez les horticulteurs), par les effets indirects de la sélection de variétés ayant d'autres caractéristiques que les COVs (par exemple la couleur des fleurs) ou des caractéristiques d'autres organes (par exemple, l'impact sur les COVs floraux de caractéristiques liées aux fruits), ou simplement par la dérive génétique. En fait, la sélection directe de variétés ayant une composition particulière a agi sur les COVs dans de nombreuses plantes aromatiques cultivées pour l'industrie du parfum. Par exemple, l'huile essentielle de lavande vraie utilisée pour les parfums, *Lavandula angustifolia*, contient moins de camphre que l'huile essentielle de lavande sauvage, *Lavandula latifolia*, et que celle du lavandin hybride, *Lavandula x intermedia* (Guitton et al., 2018, Pokajewicz et al., 2022). D'autre part, dans les programmes de sélection commerciale, l'odeur florale étant rarement ciblée, contrairement à des caractères tels que la résistance

au froid et aux maladies, la forme des fleurs ou la durée de vie en vase, l'odeur florale est alors souvent absente des variétés modernes (Vainstein et al., 2001). En outre, au cours de la domestication des cultures fruitières, la sélection de certains caractères liés à une saveur, une couleur ou une odeur particulière a probablement entraîné l'ajout, l'élimination et/ou la modification de diverses voies métaboliques spécialisées, ce qui s'est traduit par le gain ou la perte de métabolites spécialisés (Dar et al., 2021). Ces métabolites spécialisés, tels que les glycosides ou l'éthylène, mais aussi des enzymes spécifiques, jouent un rôle dans la production de COVs (Mostafa et al., 2022). **Cependant à ce jour, aucune étude ne s'est intéressée à l'effet de la domestication sur le signal émis par les plantes pour attirer leurs pollinisateurs.**

Effets des facteurs abiotiques

L'émission de COVs peut être influencée par des facteurs abiotiques qui affectent directement la biosynthèse des COVs, ou indirectement en affectant la physiologie de la plante, comme la photosynthèse, ou encore leur volatilité (Loreto et al., 1996 ; Niinemets et al., 2002 ; Rasulov et al., 2009).

En général, l'augmentation de la température entraîne une augmentation des émissions de COVs des feuilles. Cette augmentation est due à la dépendance à la température des propriétés physicochimiques des COVs et des activités enzymatiques des terpènes synthases (Holopainen & Gershenzon, 2010). Ce type de schéma d'émission a été démontré pour les activités des enzymes clés de la voie de biosynthèse et les expressions de plusieurs gènes liés aux enzymes responsables de la biosynthèse des COVs à différentes températures chez *Jasminum auriculatum* Vahl (Barman & Mitra, 2021). Mais chez plusieurs espèces méditerranéennes, il a été démontré que les émissions florales augmentaient avec la température jusqu'à un optimum et diminuaient ensuite (Farré-Armengol et al., 2014).

L'émission de COVs augmente avec l'humidité en raison de l'augmentation de la conductance stomatique (Lange et al., 1971).

L'intensité lumineuse augmente l'émission de COVs, en particulier des monoterpènes. Ainsi que la voie de biosynthèse des monoterpènes, la voie du méthylérythritol

phosphate (MEP), est partiellement située dans les chloroplastes et nécessite un substrat carboné et des cofacteurs provenant de la photosynthèse (Loreto et al., 1996 ; Niinemets et al., 2002 ; Rasulov et al., 2009), alors que la biosynthèse des sesquiterpènes, qui se produit principalement dans le cytoplasme, est indépendante de la lumière (Lynch et al., 2020). Les réponses à la lumière des émissions de sesquiterpènes ne sont pas exactement connues, bien que des preuves de dépendance à la lumière aient été rapportées dans certaines études (Duhl et al., 2008 ; Geron & Arnsts, 2010).

La pollution de l'air est également un facteur qui affecte l'émission des COVs. Un certain nombre de polluants, tels que le CO₂ ou l'ozone (O₃), induisent la production et/ou l'émission de COVs à partir des feuilles dans les tissus végétaux et dans l'atmosphère. Par exemple, des augmentations et des diminutions des émissions de COVs ont été décrites en présence de fortes concentrations de CO₂ (Peñuelas & Llusà, 1997 ; Daussy, 2020).

Par rapport aux COVs de feuilles, l'effet des facteurs abiotiques sur l'émission de COVs floraux a été beaucoup moins étudié.

3. Objectifs de la thèse et du système étudié

Ma thèse propose de combler certaines des lacunes que nous avons sur les effets des facteurs internes (génétiques et biochimiques) et externes (biotiques-pollinisateurs, pratiques humaines et abiotiques-température, lumière, humidité) sur l'émission des COVs floraux, en particulier sur les COVs responsables de l'attraction des pollinisateurs. Pour cela, nous avons choisi de travailler sur une plante dont la reproduction ne dépend que d'une seule espèce de pollinisateur, pour laquelle la phylogénie de son genre est bien connue et dont certaines espèces ont été domestiqués depuis longtemps.

3.1. Modèle d'étude

3.1.1. Le genre *Ficus*

Le genre *Ficus* (Moraceae) est très diversifié, avec plus de 800 interactions spécifiques entre figes et guêpes, réparties sur tous les continents. Les espèces de *Ficus* portent des figes qui sont des inflorescences fermées appelées "sycones", qui se

caractérisent par une paroi fermée avec une entrée unique, l'ostiole, avec des centaines de fleurs minuscules qui tapissent l'intérieur d'une cavité centrale. Chaque espèce de *Ficus* est généralement pollinisée par un pollinisateur spécifique de la famille des Agaonidae (Hymenoptera), qui sont de minuscules guêpes qui se développent exclusivement dans les figes de leur espèce hôte et utilisent les COVs émis par les figes réceptives (c'est-à-dire au stade de développement où elles sont prêtes à être pollinisées) et ce pour localiser leur plante-hôte (Gibernau *et al.*, 1997 ; Grison-Pigé *et al.*, 2002 ; Proffit *et al.*, 2008). Le genre *Ficus* est apparu il y a des millions d'années (fin du Crétacé supérieur, ca. 75 MA [Cruaud *et al.*, 2012]), et les deux partenaires partagent donc une longue histoire coévolutive façonnant les traits floraux et les capacités de reconnaissance des signaux des insectes. Le genre *Ficus* est composé de 19 sections et la famille des Agaonidae comprend environ 20 genres, ce qui offre une taille d'échantillon adéquate pour les analyses basées sur la phylogénie. La comparaison des phylogénies des *Ficus* et des Agaonidae qui leur sont associés a révélé une co-diversification entre plantes et pollinisateurs : en général, les espèces de *Ficus* appartenant à différentes sections sont associées à différents genres de pollinisateurs (Figure 5) (Cruaud *et al.*, 2012). De nombreuses études ont montré que la rencontre des partenaires est uniquement médiée par l'émission de COVs floraux émis par les figes réceptives et que la spécificité de l'espèce est maintenue par l'émission par les figes, et la reconnaissance par les guêpes associées, de signaux volatils spécifiques à l'espèce (Ware *et al.*, 1993 ; Proffit *et al.*, 2008 ; Chen *et al.*, 2009 ; Wang *et al.*, 2013).

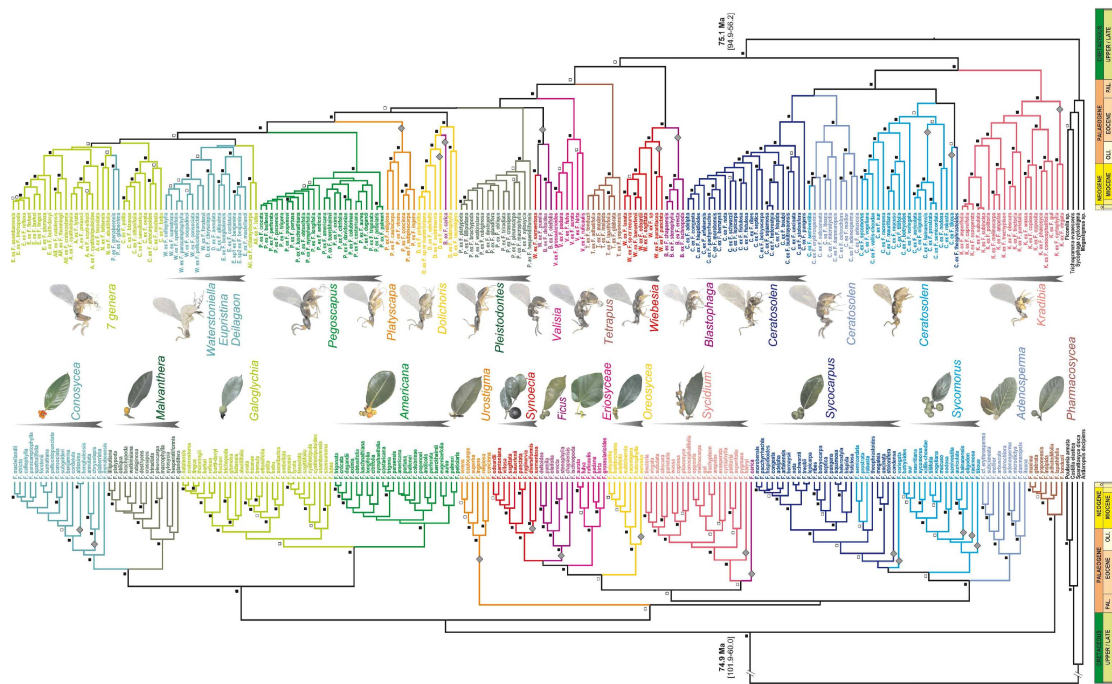


Figure 5. Chronogrammes de l'histoire évolutive des figuiers et de leurs guêpes. Les arbres phylogénétiques des figuiers et des guêpes du figuier (basés pour les figuiers sur 3 gènes, pour les guêpes sur 5 gènes) ont été reconstruits en utilisant les méthodes du maximum de vraisemblance (ML) et Bayésienne. Les groupes de figuiers et les genres de pollinisateurs qui leur sont associés sont représentés par la même couleur. Les sous-genres de *Ficus* et les sous-familles d'Agaonidae, selon les classifications actuelles, sont délimités par des rectangles de couleur. Les losanges gris montrent les clades d'espèces de figuiers d'Asie continentale, tandis que les flèches grises indiquent une migration hypothétique des clades vers le sud. (d'après Cruaud et al., 2012)

3.1.2. *Ficus carica* et *Blastophaga psenes*

Le figuier méditerranéen, *Ficus carica* (Moraceae), est l'un des arbres emblématiques de la région méditerranéenne et a été domestiqué il y a environ 5500 ans (Zohary & Spiegel-Roy, 1975). Comme d'autres *Ficus*, *F. carica* portent des inflorescences de figes (Figure 6), qui sont exclusivement pollinisées par l'agaonide *Blastophaga psenes* L. (Agaonidae) qui se développe exclusivement dans les figes de leur espèce hôte. Une fois sorti de sa fige natale, le pollinisateur la quitte en moins de 24 heures. *Ficus carica* est un arbre dioïque (à la différence d'autres figuiers qui peuvent être monoïques). C'est-à-dire dans ce cas que les arbres mâles portent des figes qui produisent du pollen et dans lesquelles se développe le pollinisateur spécifique, la guêpe du figuier. Les arbres femelles portent des figes dans lesquelles seules les graines se développent, mais pas les pollinisateurs (Kjellberg et al., 1987) (Figure 7). L'attraction des femelles

de *B. psenes* par les COVs émis par les figes réceptives des arbres mâles et femelles a déjà été bien décrite (Proffit et al., 2020). Des études antérieures ont montré que seuls quatre des COVs émis par les figes réceptives (c'est-à-dire le *S*-linalol, l'alcool benzylique, le (*Z*)-linalool-oxyde (furanolide) et le (*E*)-linalool-oxyde (furanolide)), dans une gamme étroite de proportions, sont responsables de l'attraction de la guêpe pollinisatrice spécifique du figuier et que même une très petite modification de leurs proportions perturbe l'attraction du pollinisateur (Proffit et al., 2020).

Dans la région méditerranéenne, une grande diversité de variétés de figuiers femelles (plusieurs centaines) a été sélectionnée. En comparaison, la classification des figuiers mâles en variétés est encore débattue. Les caractères d'intérêt pour l'alimentation humaine des figes femelles qui ont été sélectionnées à partir de figes sauvages sont principalement basés sur les caractéristiques des fruits, notamment la taille, la couleur, la douceur, le moelleux, les capacités de séchage liées au commerce et au stockage, mais aussi la phénologie, avec des variétés précoces à tardives afin d'obtenir des fruits sur la période la plus longue possible.

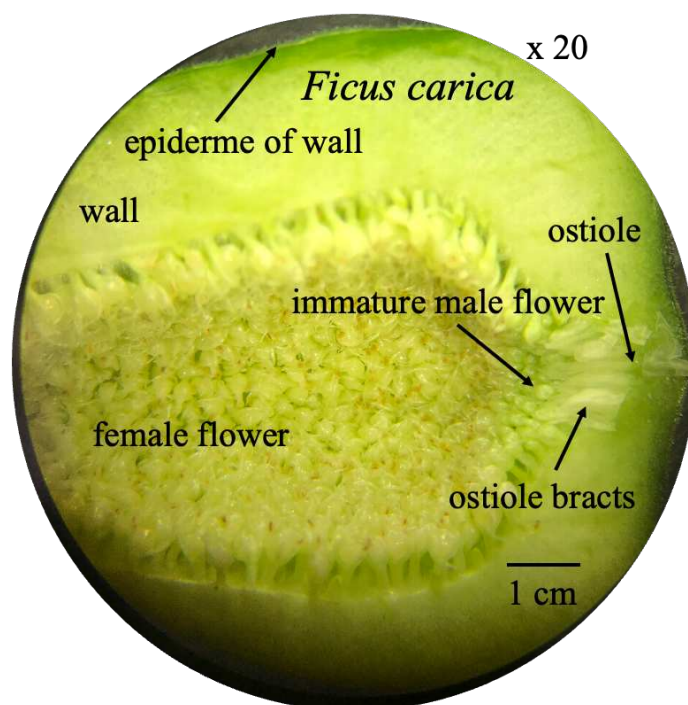


Figure 6. Coupes longitudinales de figes de *F. carica*. Barres d'échelle : 1 cm, grossissement : 20x.

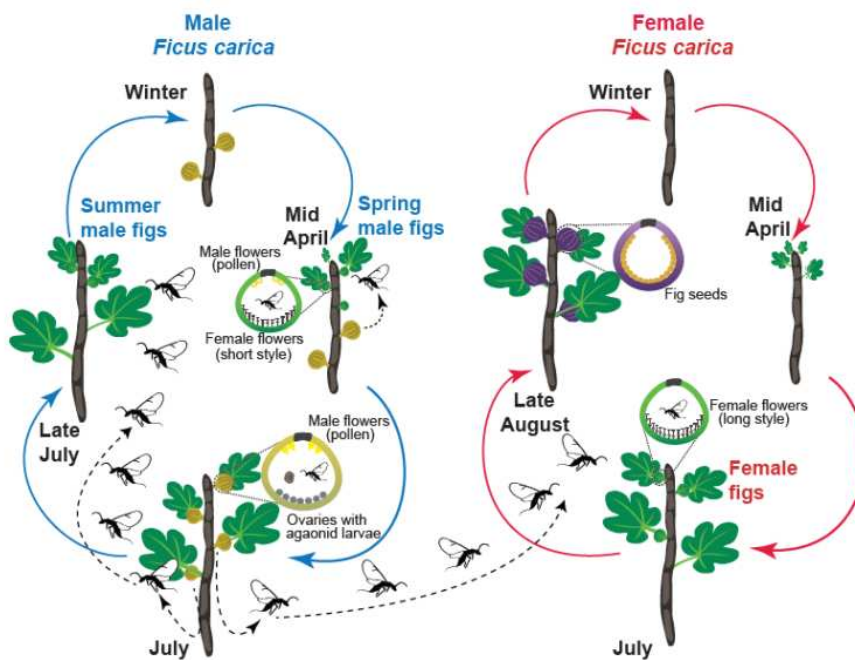


Figure 7. Cycles de vie des mâles et des femelles de *Ficus carica* (respectivement à gauche et à droite) et de *Blastophaga psenes* dans le sud de la France. Pour chaque type de figue, réceptive (vert), mâle mûr (kaki) et femelle mûre (violet), un schéma représentant les guêpes et les fleurs à l'intérieur de la figue est présenté (d'après Proffit et al., 2020).

3.2. Objectifs spécifiques

Ma thèse sera divisée par la suite en quatre chapitres sous forme d'article, chacun ayant un objectif spécifique (figure 8) :

- Chapitre 1 : Détermination des facteurs déterminant la composition des COVs floraux au cours de l'histoire de l'évolution du genre *Ficus*.

Pour ce faire, nous avons complété un vaste ensemble de données précédemment disponibles sur la composition des odeurs de figues réceptives provenant de plusieurs espèces de *Ficus*, et nous avons testé les effets des facteurs qui pourraient potentiellement avoir façonné la composition des COVs des figues au cours de l'histoire évolutive du genre.

> *Prédictions* : il existe un signal phylogénétique fort dans les COVs émis par les figues réceptives de *Ficus*, probablement dû à des contraintes dans les voies de biosynthèse des composés volatils.

- Chapitre 2 : Détermination de l'effet de la diversification induite par l'homme sur les COVs floraux de *Ficus carica* et en particulier sur les composés responsables de l'attraction des pollinisateurs.

Pour ce faire, nous avons étudié les COVs des figes réceptives de plusieurs variétés de *F. carica* au Maroc, et testé si l'émission de COVs variait entre les variétés qualitativement mais aussi quantitativement, en particulier les COVs responsables de l'attraction des pollinisateurs.

> Prédiction : les proportions de COVs responsables de l'attraction des pollinisateurs devraient être fortement conservées entre les variétés au niveau de la réceptivité, contrairement aux COVs qui ne jouent aucun rôle dans l'attraction des pollinisateurs.

- Chapitre 3 : Détermination de l'effet de l'activité des pollinisateurs et des facteurs abiotiques sur la dynamique temporelle de l'émission florale de COVs chez *F. carica*.

Pour ce faire, nous avons analysé les schémas d'émission diurne des quatre COVs attirant les pollinisateurs chez *Ficus carica* et déterminé les effets de la pollinisation et de facteurs abiotiques tels que la température, l'intensité lumineuse et l'humidité sur leurs émissions.

*> Prédiction : les quatre COVs attractifs pour les pollinisateurs émis par *F. carica* suivent un rythme d'émission qui varie au cours de la journée, avec un pic d'émission lorsque les pollinisateurs sont les plus actifs ; ils augmentent avec la température, et diminuent rapidement après la pollinisation.*

- Chapitre 4 : Caractérisation des gènes codant pour la biosynthèse des COVs impliqués dans l'attraction des pollinisateurs chez *F. carica* et sur la production dans la fige de ces COVs.

Pour ce faire, nous avons caractérisé la fonction de plusieurs gènes impliqués dans l'émission de COVs attractifs pour les pollinisateurs chez *F. carica* et localisé les tissus des figes produisant ces COVs.

> *Prédictions : l'expression des gènes impliqués dans l'attraction des pollinisateurs diminue après la pollinisation.*

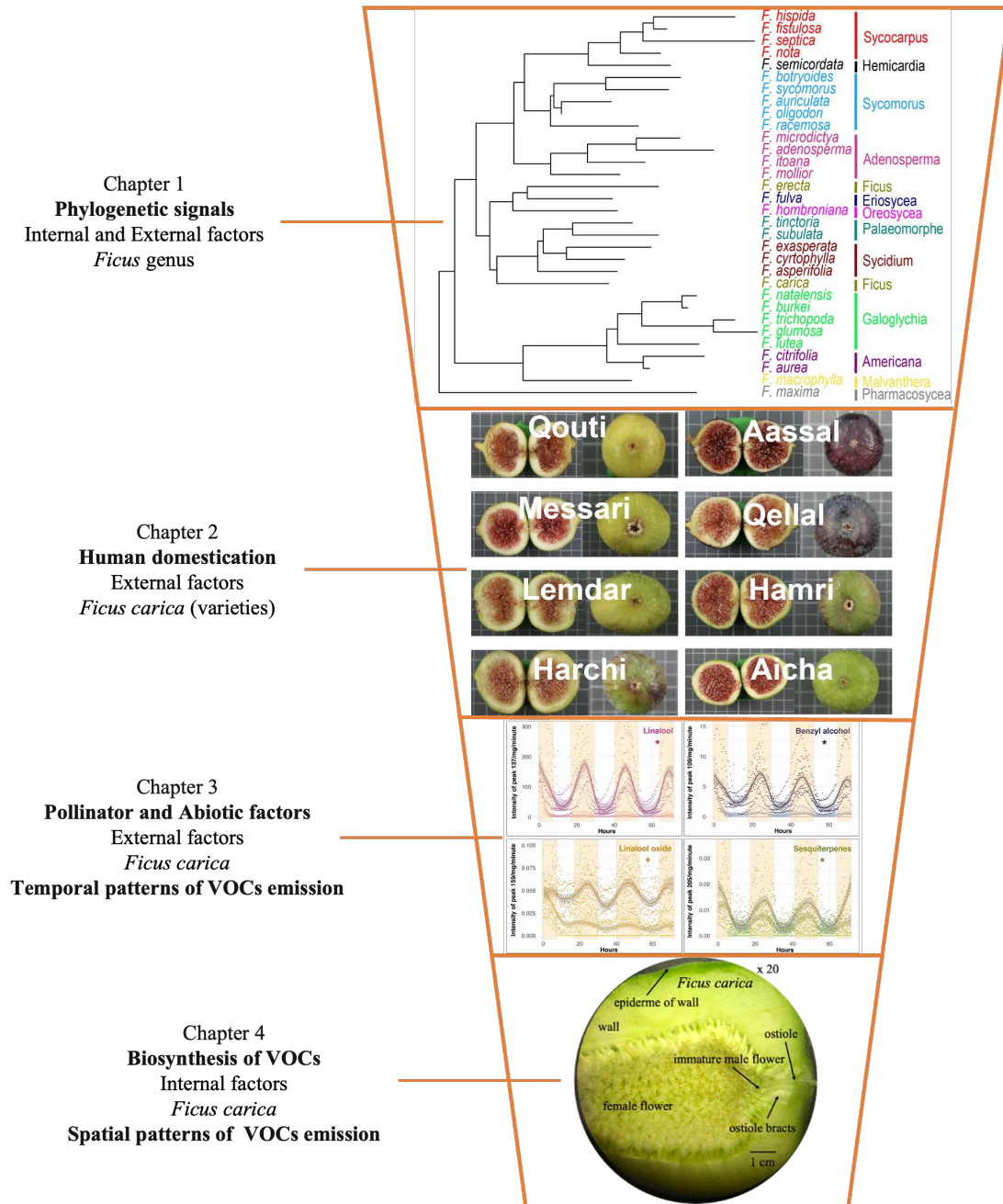
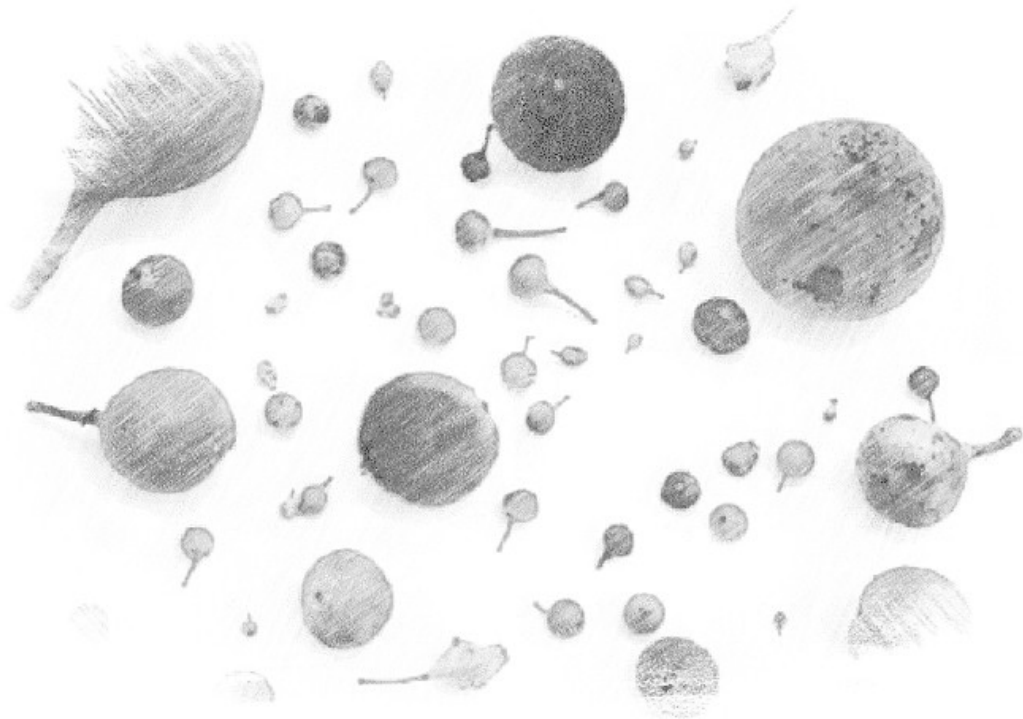


Figure 8. Schéma illustrant les objectifs de la thèse.



Chapter 1. Evolutionary history shapes the composition of floral scents in a specialized pollination mutualism

L'histoire évolutive façonne la composition des odeurs florales dans un mutualisme de pollinisation spécialisé

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Résumé

La plupart des études sur la médiation chimique entre les plantes et les pollinisateurs mettent l'accent sur l'impact direct de la sélection par les pollinisateurs sur la composition des odeurs florales. Néanmoins, la phylogénie peut contraindre la composition des odeurs et donc l'évolution du signal émis. En utilisant un système modèle pour les interactions de pollinisation obligatoire, comme celui entre les figes et leurs guêpes pollinisatrices spécifiques à l'espèce, nous avons étudié si l'histoire phylogénétique contraint la composition des signaux chimiques des plantes médiant les interactions avec les pollinisateurs. Dans ce "mutualisme de pollinisation", appelé « nursery pollination », les pollinisateurs ne peuvent se reproduire que dans les figes réceptives de leur arbre hôte, qui dépend à son tour de la guêpe en tant qu'unique pollinisateur. La rencontre entre le pollinisateur et la fige réceptive est médiée par les composés organiques volatils (COV) émis par les figes. Nous avons recueilli des

odeurs florales de figes réceptives en utilisant l'extraction *in situ* d'odeurs de 32 espèces de plusieurs sections de *Ficus* provenant de différentes régions tropicales et subtropicales, et nous avons analysé leur composition chimique par chromatographie en phase gazeuse couplée avec la spectrométrie de masse (GC-MS). En utilisant les phylogénies disponibles pour les *Ficus*, nous avons analysé le signal phylogénétique par des modèles semi-quantitatifs des données sur les odeurs florales en utilisant l'analyse phylogénétique en composantes principales et plusieurs indices multivariés du signal phylogénétique. Nos résultats ont révélé un signal phylogénétique fort dans les COVs émis par les figes réceptives, probablement dû à des contraintes dans les voies de biosynthèse des composés volatils. Cependant, en utilisant la même analyse, nous n'avons trouvé aucun effet avec la phylogénie du pollinisateur. Ces résultats constituent l'une des premières démonstrations, à grande échelle, comme quoi les contraintes phylogénétiques jouent un rôle significatif dans la diversification des signaux chimiques volatiles émis par les fleurs réceptives.

Mots clefs : Odeurs florales, mutualisme, pollinisation, phylogénie, médiation chimique

Evolutionary history shapes the composition of floral scents in a specialized pollination mutualism

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Abstract

Most studies of chemical mediation between plants and pollinators stress the direct impact of selection by pollinators on the composition of floral scents. Nevertheless, phylogeny may constrain scent composition and thereby the evolution of the emitted signal. Using a model system for obligate pollination interactions, those between figs and their species-specific pollinating fig wasps, we studied whether phylogenetic history constrains the composition of plant chemical signals mediating interactions with pollinators. In this 'nursery pollination mutualism', the pollinators can breed only in receptive figs of their host tree, which depends in turn on the wasp as its sole pollinator. The encounter of the pollinator and the receptive fig is mediated by volatile organic compounds (VOCs) emitted by figs. We collected floral scents from receptive figs using in situ headspace extraction of odours from 32 species of several sections of *Ficus*

from different tropical and subtropical regions, and analyzed their chemical composition by gas chromatography mass spectrometry (GC-MS). Using phylogenies available for *Ficus*, we analyzed the phylogenetic signal in semi-quantitative patterns of floral-scent data using phylogenetic principal component analysis and several multivariate indices of phylogenetic signal. Our results revealed a strong phylogenetic signal in the VOCs emitted by receptive figs, probably due to constraints in the biosynthetic pathways of volatile compounds. However, using the same analysis, we found no effect of the pollinator phylogeny. These findings constitute one of the first demonstrations, on a wide scale, that phylogenetic constraints play a significant role in the diversification of VOCs signals emitted by receptive flowers.

Keywords: Floral scents, mutualism, pollination, phylogeny, chemical mediation

Introduction

Most angiosperms rely on animals for pollination. Ollerton *et al.* (2011) estimated that globally 90% of angiosperms are animal-pollinated. The success of biotic pollination depends on the production of cues by plants and recognition of signals by animals to facilitate partner encounter (Chitka & Johnson, 2001; Majetic *et al.*, 2009). Although it was long under-investigated, the predominant modality of signaling is the emission of volatile organic compounds (VOCs) by plants and their olfactory detection by animals (Raguso, 2008; Hossaert-McKey *et al.*, 2010; Byers, 2021). Floral scents are usually mixtures of numerous VOCs, produced by several biosynthetic pathways in different compartments of the cell such as cytosol or plastids (Pichersky *et al.*, 2006; Muhlemann *et al.*, 2014; Picazo-Aragónés *et al.*, 2020), resulting in more or less complex floral phenotypes (Raguso *et al.*, 2015). An amazing diversity of VOCs has already been documented and more than 1700 floral VOCs have now been identified (Knudsen *et al.*, 2006; Knudsen & Gershenzon, 2020). They belong to three main biochemical classes, terpenoids, benzenoids and phenylpropanoids, and derivatives of fatty acids (Maffei, 2010; Muhlemann *et al.*, 2014). Floral scents vary among species

in their qualitative composition, in the proportions of different compounds in the blend, and in their overall concentrations; all these kinds of variation affect the attractiveness of scents to different pollinators (Raguso, 2001; Majetic *et al.*, 2009; Schiestl & Johnson, 2013) as well as the specificity of the interactions (Hossaert-McKey *et al.* 2010). However, our understanding of the evolution of these complex floral traits is still limited (Raguso *et al.*, 2015; Delle-Vedove *et al.*, 2017; Farré-Armengol *et al.*, 2020).

Variation in floral scents is thought to be driven by balancing selection exerted by pollinators and other agents but is also affected by phylogenetic constraints associated with biosynthesis of VOCs (Raguso 2001; Schäffler *et al.* 2012). The evolution of the composition of floral scents is under strong selective pressures (Schiestl & Johnson, 2013; Friberg *et al.*, 2019), including pollinator-mediated selection (Darwin, 1877; Junker & Parachnowitsch, 2015; Schiestl *et al.*, 2018; Moré *et al.*, 2021), considered to be the major force acting on the evolution of floral scents (Majetic *et al.*, 2009). Selection exerted by pollinators often drives convergence in scent composition among unrelated species sharing the same guild of pollinators (Knudsen & Tollsten, 1995). Floral VOCs are thus among the traits involved in the convergent evolution of pollination syndromes. For instance, dimethyl disulfide is a prominent scent constituent in most bat-pollinated species (Knudsen & Tollsten, 1995; Helversen *et al.*, 2000), and benzaldehyde, lilac aldehyde and phenylacetaldehyde are typical constituents of moth-pollinated flowers (Dotterl *et al.*, 2006; Dudareva *et al.*, 2006; Moré *et al.*, 2021). Floral VOCs involved in pollinator attraction can also be subjected to other biotic selection pressures, for example, those exerted by herbivorous insects (Segar *et al.*, 2019). Considering plant chemistry as a whole, the evolution of volatile compounds to attract mutualistic partners such as pollinators, and their patterns of expression in plants, are not independent of the production of other compounds that function in plant defense against antagonistic insects (Burkle & Runyon, 2016; Rusman *et al.*, 2018). However, phylogenetic constraint is also a potentially important constraint on pollinator-mediated selection of scent compounds. For example, a study of the evolution of floral scents of oil-secreting

orchids found phylogenetic constraint to be more important than pollinator-mediated selection in this system (Steiner et al., 2011). Most studies have investigated related plants pollinated by very different guilds of pollinators, where selection pressures favoring evolutionary divergence in floral odors are expected to be strong (Friberg *et al.*, 2019; Farré-Armengol *et al.*, 2020). Pollinator effects might thus be so strong that they mask or swamp the effects of phylogeny (Steiner *et al.*, 2011). As noted by Peakall *et al.* (2010), assessing phylogenetic signal in the evolution of floral scents might be easiest in a system in which closely related plants are pollinated by specific pollinators from a single guild.

We examine these questions using a system with specific pollinators from a single guild, the fig/fig wasp pollination mutualism. Species of the genus *Ficus* (Moraceae) represent one of the best biological models to explore the extent of phylogenetic signal in VOC odor profiles, because, like the orchid-wasp system studied by Peakall *et al.* (2010), it involves closely related plants that are pollinated by specific pollinators from a single guild. Each species of *Ficus* is generally pollinated by one species-specific pollinator of the family Agaonidae (Hymenoptera), tiny wasps that develop exclusively in the figs of their host species and use VOCs emitted by receptive figs (i.e. in the developmental stage where they are ready to be pollinated) to locate the host (Gibernau *et al.*, 1997; Grison-Pigé *et al.*, 2002; Proffit *et al.*, 2008). In addition, this genus is quite diverse, with more than 800 specific fig/fig wasp interactions distributed in all continents. The genus *Ficus* appeared a long time ago (Late Cretaceous, ca. 75 MY [Cruaud *et al.*, 2012]) and the two partners share a long history of coevolution shaping floral traits and the insects' capacities of signal recognition. The genus is composed of 19 sections and the family Agaonidae includes about 20 genera, offering an adequate sample size for phylogeny-based analyses. Comparisons of the phylogenies of *Ficus* and their associated Agaonidae have revealed co-diversification between plants and pollinators: in general, species of *Ficus* belonging to different sections are associated with different pollinator genera (Cruaud *et al.*, 2012). In addition, some section, and even subgenera, are pollinated only by one pollinator genera (e. g. section sycomorus)

and others are pollinated by several genera of pollinator (eg. Section Galoglychia). Numerous studies have shown that partner encounter is mediated solely by the emission of floral VOCs by receptive figs and that species-specificity is maintained by the emission by figs, and the recognition by the associated fig wasps, of species-specific volatile signals (Ware *et al.*, 1993; Proffit *et al.*, 2008; Chen *et al.*, 2009; Wang *et al.*, 2013; Souto *et al.*, 2018, Proffit *et al.*, 2020). However, in the case of closely related species, living in sympatry some pollinator sharing can occur at a low frequency (Cornille *et al.*, 2012; Wang *et al.*, 2016).

Several features of the fig/wasp system make it especially well-suited for examining questions about phylogenetic signal and phylogenetic constraints on pollinator-mediated selection. First, in this coevolved species-specific system, there are no competing pollinators and no competition between plant species for shared pollinators. There is thus little scope for pollinator-mediated selection. Furthermore, whereas shifts in pollinators or host shifts by pollinators could also drive selection for modification of VOCs composition, pollinator shifts in figs and host shifts in fig wasps are much smaller phenotypic “jumps” than, for example, shifts between moth and bee pollination in *Lithophragma* (Friberg *et al.*, 2019). In the fig/wasp system, host shifts of pollinators are from one *Ficus* sp. to another, and pollinator shifts of the plants are from one agaonid to another. The limited phylogenetic scope of host switches limits any pollinator-mediated selection they could drive. Second, although fig species and their associated wasps often have large distributions, geographic variation in the odour of their receptive figs is usually limited (Soler *et al.*, 2011), except for a few particular cases (e.g., *F. septica* VOCs among different islands in the Philippines, Rodriguez *et al.*, 2017). However, phylogenetic signal in the evolution of floral scents has never been assessed in fig/fig wasp systems.

In this study, we take advantage of a large dataset from 32 fig species including new data for half of these species, many with highly overlapping distributions, in order to examine the effects of factors that could potentially have shaped the composition of fig VOCs over the evolutionary history of the genus. We address the following questions:

(1) Does the volatile-chemical profile of receptive figs of each of these species differ from those of all the other species? (2) Is there a phylogenetic signal in the evolution of *Ficus* floral VOC profiles? For example, do different *Ficus* sections produce different classes of volatile compounds? (3) Do phylogenetic constraints help explain the phylogenetic signal? For example, do the differences in the chemical profiles of receptive figs of species belonging to different sections result from intrinsic constraints associated with VOC biosynthesis? And finally, (4) Does phylogeny of the pollinating wasps have an effect on the composition of VOCs emitted by receptive figs of the fig species with which they are associated? This might be expected if the attractiveness of different VOC profiles varies across wasp phylogeny. Departure from this “expected course of evolution” could indicate some kind of constraint on pollinator-mediated selection.

Materials and Methods

Studied species

Volatile Organic Compounds (VOCs) emitted by receptive figs (i.e., figs ready to be pollinated) were collected from 3 to 33 individuals per species from 32 different *Ficus* species (242 individuals in total) distributed mainly in the tropical regions of five continents (Table 1). These species (15 monoecious and 17 dioecious) were selected in order to represent the different clades of *Ficus* (13 of the 19 sections from the five subgenera), the phylogenetic relationships of which have already been published (Cruaud *et al.*, 2012). Our approach was to acquire a big data set and collect chemical samples on a maximum of species with adequate replication. The two main limiting factors in our floral VOCs collection were 1) to find representative species among the different subgenera and 2) to find trees at the right stage, i.e. receptive trees (individual fig trees in general flower once a year).

In situ collection of floral VOCs

Collection of VOCs was conducted in situ (from figs on trees in the field) or ex situ (from figs detached from the tree and taken to a laboratory nearby) using dynamic headspace with two different collection methods based on two different traps, containing either 30 mg of Alltech Super Q adsorbent (ARS Inc., Gainesville, FL, USA) (until 2009) or 3 mg of a 1:1 mixture of Tenax-TA 60–80 and Carbotrap 20–40 (from 2010). Comparative studies have shown that type of trap does not affect results (Hossaert-McKey *et al.*, 2016), and we used both methods of volatile collection indifferently in this study. Also, previous tests on several *Ficus* species found no significant differences in the volatile blends emitted by figs on trees in the field and figs detached from trees and transported to a nearby laboratory (Proffit *et al.*, 2008). Our data include some previously published data, as indicated in table 1.

Analysis of floral VOCs

Traps were stored in the dark at -20°C until analysis. Samples were analyzed at the “Platform for Chemical Analyses in Ecology” (PACE), technical facilities of the LabEx CeMEB (Centre Méditerranéen pour l’Environnement et la Biodiversité, Montpellier, France), using a gas chromatograph (GC, Trace™ 1310, Thermo Scientific™ Milan, Italy) coupled to a mass spectrometer (ISQ™ QD Single Quadrupole, Thermo Scientific™ Milan, Italy). Samples were analyzed using always the same methods as described in previous papers (see Cornille *et al.* [2012], Hossaert-McKey *et al.* [2016], and Souto-Vilarós *et al.* [2018] for details). Compound identification was based on computer matching of the mass spectra with NIST MS library, on retention indices reported in the literature (Adams, 2007), and obtained by injection of reference compounds, when these were available. By comparing the spectrum of each sample with that of the respective control sample (empty bag, same day and conditions of collection), putative contaminant compounds were subtracted from our chromatograms.

Table 1. Sampled species, reproductive systems, years of collection, sampling localities and number of samples collected for each species.

| Taxa/Species | Reproductive system | Year of collection | Sampling locality (country, region) | Sample size | References |
|--------------------------------------------|---------------------|--------------------|-------------------------------------|-------------|----------------------------------------|
| Subgen. Pharmacosycea | | | | | |
| <u>Sect. Pharmacosycea</u> | | | | | |
| <i>F. maxima</i> Mill. | M | 2009 | France (French Guiana) | 3 | This study |
| <u>Sect. Oreosycea</u> | | | | | |
| <i>F. hombroniana</i> Corner | D | 2016 | Papua New Guinea, Madang | 3 | This study |
| Subgen. Urostigma | | | | | |
| <u>Sect. Malvanthera</u> | | | | | |
| <i>F. macrophylla</i> Desf. ex Pers. | M | 2009 | USA, Hawaii | 4 | This study |
| <u>Sect. Americana</u> | | | | | |
| <i>F. aurea</i> Nutt. | M | 2009 | USA, southern Florida | 3 | This study |
| <i>F. citrifolia</i> Mill. | M | 2011 | USA, southern Florida | 7 | This study |
| <u>Sect. Galoglychia</u> | | | | | |
| <i>F. lutea</i> Vahl | M | 2008 | South Africa, Kwazulu-Natal | 4 | This study |
| <i>F. trichopoda</i> Baker | M | 2008 | South Africa, Kwazulu-Natal | 3 | This study |
| <i>F. burkei</i> (Miq.) Miq. | M | 2008 | South Africa, Kwazulu-Natal | 5 | Cornille <i>et al.</i> , 2012 |
| <i>F. natalensis</i> Hochst. | M | 2008 | South Africa, Kwazulu-Natal | 15 | Cornille <i>et al.</i> , 2012 |
| Subgen. Ficus | | | | | |
| <u>Sect. Ficus</u> | | | | | |
| <i>F. carica</i> L. | D | 2020 | France, Occitanie | 6 | This study |
| <i>F. erecta</i> Thunb. | D | 2010 | China, Taiwan | 7 | This study |
| <u>Sect. Eriosycea</u> | | | | | |
| <i>F. fulva</i> Reinw. ex Blume | D | 2002 | Brunei, North Borneo | 6 | Hossaert-McKey <i>et al.</i> , 2016 |
| Subgen. Sycidium | | | | | |
| <u>Sect. Sycidium</u> | | | | | |
| <i>F. asperifolia</i> Miq. | D | 2007 | UK, Greenhouse Univ. Leeds | 4 | This study |
| <i>F. cyrtophylla</i> (Wall. ex Miq.) Miq. | D | 2009 | China, Yunnan | 3 | This study |
| <i>F. exasperata</i> Vahl | D | 2003-2005 | India, Western Ghats | 33 | Borges <i>et al.</i> , 2008 |
| <u>Sect. Palaeomorphe</u> | | | | | |
| <i>F. subulata</i> Blume | D | 2009 | China, Yunnan | 4 | This study |
| <i>F. tinctoria</i> G. Forst. | D | 2005, 2010 | China, Yunnan, Taiwan | 7 | This study, Proffit <i>et al.</i> 2009 |
| Subgen. Sycomoros | | | | | |
| <u>Sect. Adenosperma</u> | | | | | |
| <i>F. itoana</i> Diels | D | 2016 | Papua New Guinea, Madang | 11 | Souto <i>et al.</i> , 2018 |
| <i>F. adenosperma</i> Miq. | D | 2016 | Papua New Guinea, Madang | 3 | Souto <i>et al.</i> , 2018 |
| <i>F. microdictya</i> Diels | M | 2016 | Papua New Guinea, Madang | 10 | Souto <i>et al.</i> , 2018 |
| <i>F. mollior</i> F. Muell. ex Benth. | D | 2016 | Papua New Guinea, Madang | 4 | This study |
| <u>Sect. Sycomoros</u> | | | | | |
| <i>F. racemosa</i> L. | M | 2005-2006 | China, Yunnan | 5 | Soler <i>et al.</i> , 2011 |
| <i>F. oligodon</i> Miq. | D | 2008 | China, Yunnan | 3 | This study |
| <i>F. auriculata</i> Lour. | D | 2008 | China, Yunnan | 5 | Hossaert-McKey <i>et al.</i> , 2016 |
| <i>F. sycomoros</i> L. | M | 2008 | South Africa, Kwazulu-Natal | 4 | Proffit and Johnson 2009 |
| <i>F. botryoides</i> Baker | M | 2007 | Madagascar | 3 | This study |
| <u>Sect. Hemicardia</u> | | | | | |
| <i>F. semicordata</i> Buch.Ham. ex Sm. | D | 2006-2007 | China, Yunnan | 10 | Chen <i>et al.</i> , 2009 |
| <u>Sect. Sycocarpus</u> | | | | | |
| <i>F. fistulosa</i> Reinw. ex Blume | D | 2006 | Brunei, Northern Borneo | 11 | Hossaert-McKey <i>et al.</i> , 2016 |
| <i>F. hispida</i> L.f. | D | 2003 | India | 31 | Hossaert-McKey <i>et al.</i> , 2016 |
| <i>F. septica</i> Burm.f. | M | 2013 | Philippines | 10 | Conchou <i>et al.</i> , 2014 |
| <i>F. nota</i> (Blanco) Merr. | M | 2013 | Philippines | 10 | Conchou <i>et al.</i> , 2014 |

Subgen.: Subgenus, Sect.: Section, M: Monoecious, D: Dioecious.

Data set on floral VOCs from receptive figs

For each compound, the percentage of the total peak area in each sample was calculated as an estimate of relative amount (semi-quantitative data) based on the concentrations of internal standards, and used for subsequent analyses. Only

compounds that were present in at least 50 % of the individuals of at least one species were considered. These comprised a total of 223 compounds.

The relative proportions of all the compounds emitted by each of the 242 individuals of the 32 *Ficus* species were compiled in a dataset to facilitate the comparison of VOCs composition among different *Ficus* species.

The number of VOCs emitted by each of the 242 individuals was also tabulated.

As only one genetic dataset was available for each species, mean values of the replicates available for scent composition were calculated for each species. Two datasets were built to test for phylogenetic signal (Kmult tests) and for the multivariate analysis of compound diversity among species (pPCA: Phylogenetic principal component analysis; see below for explanation of methods) with: i) major compounds only (i.e., compounds representing at least 5% of the blend in at least one species, i.e. 48 VOCs); ii) all compounds: major and minor compounds (i.e., 223 VOCs).

Molecular phylogenetic dataset

In order to reconstruct phylogenetic trees for the species of *Ficus* and Agaonidae included in this study, we used the published tree reconstructed on 200 *Ficus* and associated Agaonidae species (Cruaud *et al.*, 2012) using the same maximum likelihood (ML) and Bayesian methods. In that study, *Ficus* phylogeny was reconstructed using 5 genes: ITS (891 bp), ETS (528 bp), glyceraldehyde 3-phosphate dehydrogenase (G3pdh, 769 bp), chloroplast expressed glutamine synthetase region (ncpGS, 1630 bp), and granule-bound starch synthase (waxy region, 1734 bp). To infer phylogenetic relationships between Agaonidae species, Cruaud *et al.* (2012) combined two nuclear protein-coding genes [F2 copy of elongation factor-1a (EF1a, 516 bp), Wingless (Wg, 403 bp)]; two mitochondrial protein-coding genes [cytochrome c oxidase subunit I (COI, 1536 bp), cytochrome b (Cyt b, 749 bp)], and two ribosomal genes [28S rRNA (D2-D3 and D4-D5 expansion regions, 1520 bp), 18S rRNA (variable regions V3-5, 787 bp)].

Data analysis

All data analyses were performed in R (v. 4.0.2; R Development Core Team; <http://www.R-project.org>).

For the comparison of volatile profiles among different *Ficus* species, we performed a permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) using the function “adonis” in the package Vegan (Oksanen *et al.*, 2022) on the dataset of relative amounts of the VOCs of each individual per species from 32 species. PERMANOVA was run on the Bray–Curtis distance index with 9999 permutations per analysis and data were standardized prior to the analysis.

To compare patterns of VOC composition among 8 *Ficus* sections and 5 *Ficus* subgenera with more than one species sampled, we performed a PERMANOVA on the dataset of relative amounts of the VOCs belonging to the different chemical classes for each of sections and subgenera. PERMANOVAs were run on the Euclidean distance index with 9999 permutations per analysis.

We tested the presence of a phylogenetic signal in the VOCs emitted by figs using the Kmult test, which is an extension of the K statistic of Blomberg *et al.* (2003) for multivariate traits (Adams, 2014). The multivariate Blomberg’s K (Kmult; Blomberg *et al.*, 2003) was used to test for a significant phylogenetic signal in the multivariate dataset of VOCs. This multivariate analysis is able to detect phylogenetic signal in multidimensional traits whose dimensionality exceeds the number of species examined. It indicates evolution of traits by Brownian motion with an expected value of 1.0 under Brownian motion that remains consistent across levels of trait variation and across numbers of trait dimensions (Adams 2014). Prior to the analysis, the all-compounds dataset was centred log ratio (clr) transformed. Phylogenetic principal component analysis (pPCA), a recently developed method, controls for phylogenetic covariance and creates a PCA-like ordination (Revell, 2009). In the pPCA analysis, the phylogenetic resemblance in a complex set of continuous variables (semi-quantitative dataset) is graphically summarized in two principal components (PCs) with the highest (large variance and strong positive autocorrelation) and the lowest (high variance and

strong negative autocorrelation) eigenvalues (Jombart *et al.*, 2010). The first PC denotes the ‘global structure’ and reveals the VOCs that are more similar in related than in distant species. The ‘local structure’ is depicted in the last PC, which reveals the VOCs that create dissimilarities among closely related species. In the pPCAs, we used the measure of phylogenetic proximity underlying the test of Abouheif (1999) because of its goodness in detecting phylogenetic signal (Pavoine *et al.*, 2008). pPCA was implemented in the adephylo package (Jombart & Dray, 2008). In addition, in order to track possible effects of pollinator-mediated selection on the pattern of VOCs emitted by receptive figs, we conducted the same analyses, Kmult and pPCA, on the chemical dataset using the phylogeny of the 32 associated agaonid wasp species. Phylogenetic signal is most easily detected when a large number of taxa are available. The 32 fig species and their associated pollinators included in this study offered a more than adequate sample size for phylogeny-based analyses.

Results

1. Specificity of the signal of VOCs in *Ficus*

Volatile compounds emitted by receptive figs of the 32 species studied were highly diverse. In total, we detected more than 380 VOCs, but only 223 volatile compounds were considered as representative of the VOCs profile of these species (i.e. present in at least 50% of individuals of at least one species) and kept for the statistical analysis. Receptive figs tend to emit floral scents mainly composed of four different chemical classes: monoterpenes, sesquiterpenes, fatty acid derivatives and shikimic acid derivatives (Figure 1). Among these, some compounds widespread in floral VOCs were found in the floral bouquet of almost all the species studied, such as the sesquiterpene *E*- β -caryophyllene (>90%) or α -copaene and α -humulene and some monoterpenes, e.g., *E*- β -ocimene (>75%), linalool. In contrast, only one species produced a compound rare among *Ficus* floral VOCs, i.e., 4-methyl anisole found in the scent of *F. semicordata* (see Supplementary Table S1 for more details).

The blend of compounds was generally dominated by one or a few common compounds, the identity of which differed among the species. As a result, each of the 32 fig species we analyzed was found to have its own bouquet. This specificity of the floral VOCs is confirmed by the PERMANOVA showing that the volatile chemical profiles of the receptive figs are significantly different among the 32 species ($F_{31,241} = 10.59, p = 0.001$).

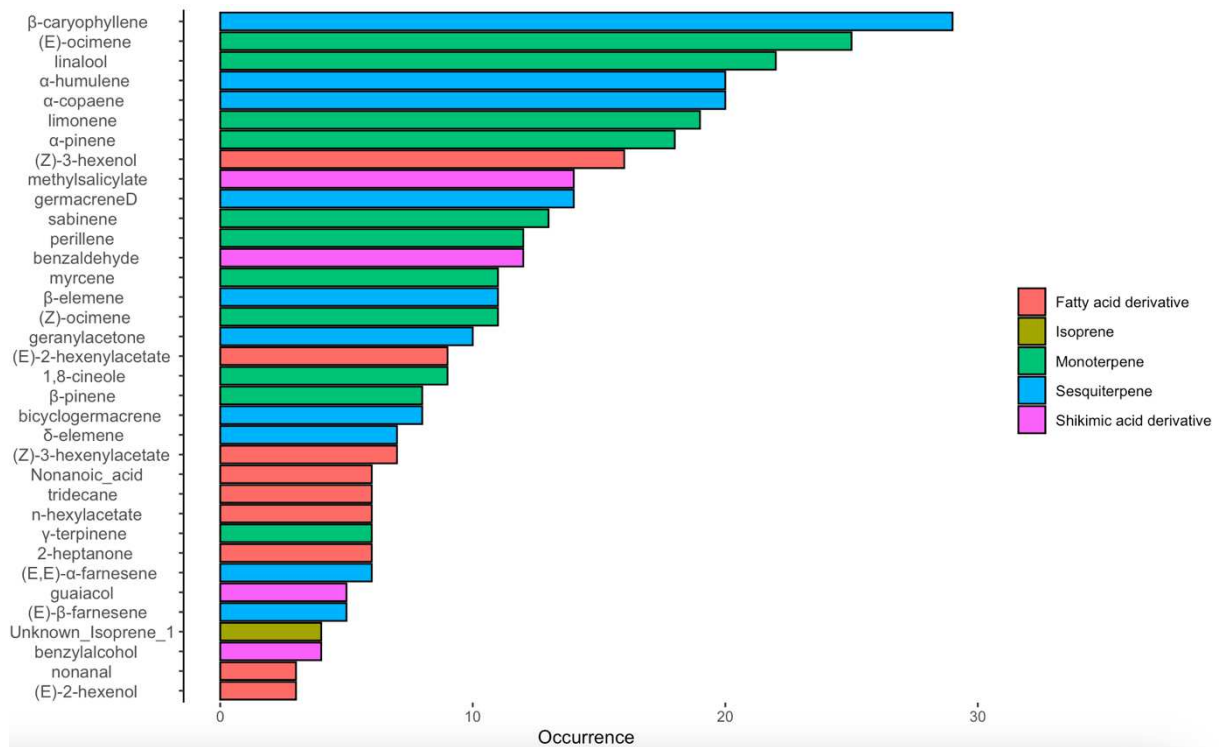


Figure 1. Occurrence of the 35 most abundant (found in ≥ 3 species) volatile compounds detected, colour-coded by their biochemical/elemental group.

2. Phylogenetic signal in the emission of volatile compounds by receptive figs of different species

Our analysis indicated a strong phylogenetic signal on VOCs emission with significant values for tests on the datasets on both major compounds (48 VOCs; $K_{mult} = 0.513, p = 0.006$) and all compounds (223 VOCs; $K_{mult} = 0.422, p = 0.001$). These results suggest a correlation between phylogenetic and chemical distances of floral scent in *Ficus*.

Our first analysis concerning only the major compounds, the global distribution of the compounds on the two main axes of the pPCA, PC1 (strong positive autocorrelation) and PC31 (strong negative autocorrelation), is presented in Figure 2A. These two components, PC1 and PC31, explain respectively 92.15 % and 46.84 % of the variation. The global distribution of compounds on the pPCA is strongly correlated with the different biosynthetic pathways. For example, PC1 (VOCs shared between related species) opposes monoterpenoids (positive loading) to sesquiterpenoids (negative loading) and PC31 (VOCs that are different between related species) opposes terpenoids (negative loadings for both mono- and sesquiterpenoids) to fatty acid-derived compounds (positive loadings).

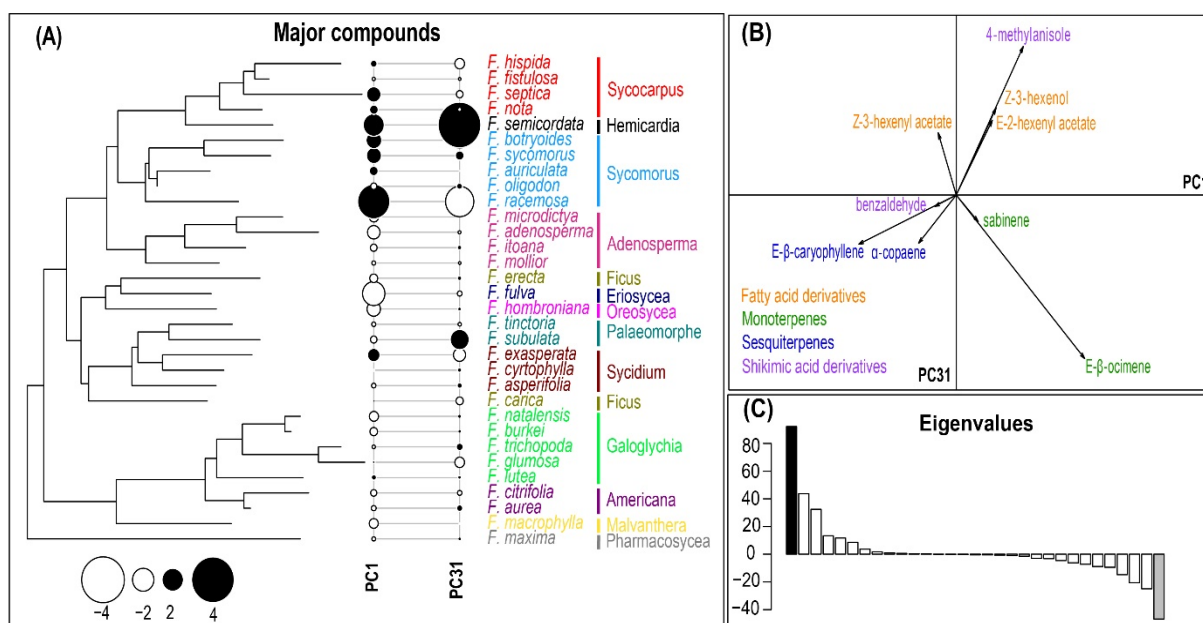


Figure 2. (A) Phylogeny of the *Ficus* species studied and results of the pPCA for the major VOCs emitted by receptive figs. Positive and negative scores on PC1 (more similar VOC composition among related species) and PC31 (dissimilar VOC composition among related species) are indicated by black and white circles, respectively. Symbol size is proportional to absolute values. The sections and species for each *Ficus* are indicated with different colors. **(B) Loading of the main compounds for the global and local principal components (analyses including only the major compounds).** Only the compounds with highest loadings (net value higher than 0.1) on the PCs are represented. VOCs belonging to different chemical classes are indicated with different colors. **(C) Bar plot displaying the corresponding eigenvalues of PC1 (black) and PC31 (gray), analyses including only the major compounds.**

At a more detailed level, reflecting this strong phylogenetic signal, receptive flowers of species belonging to the closely related sections, *Sycomorus* and *Syccarpus*,

released high proportions of the same monoterpene, E- β -ocimene (positive loadings on PC1, Figure 2B). In the same way, species of the related sections *Eriosyce* and *Oreosyce* had in common the emission of a high proportion of the sesquiterpene E- β -caryophyllene (negative loading on PC1, Figure 2B). In addition, species of the sections *Galoglychia*, *Malvanthera* and *Americana* released high proportions of the shikimic acid derivative benzaldehyde and of the sesquiterpene E- β -caryophyllene (negative loading on PC1, Figure 2B).

In contrast, PC31 underlined some differences between related species in which flowers emitted contrasting amounts of volatile compounds and therefore showed quite different scores on PC 31. This is, for example, the case of *Ficus racemose* (section *Sycomorus*) (negative score on PC31, Figure 2B), where floral volatiles were composed of high relative amounts of the monoterpene, E- β -ocimene, whereas its closest relative (same section) among the species sampled, *F. oligodon* (positive score on PC31, Figure 2) did not release this monoterpene. *Ficus semicordata* (section *Hemicardia*) (positive score on PC31, Figure 2), where floral volatiles were composed of high relative amounts of the shikimic acid derivative, 4-methyl anisole, whereas its closest relative section sampled, *F. hispida*, *F. fisfulosa*, *F. septica* and *F. nota* (section *Sycocarpus*) (negative score on PC31, Figure 2) did not release this compound.

In the second analysis, including all the 223 compounds emitted by flowers of the different species, the effect of the phylogenetic signal is more diffuse. The two components PC1 and PC31 explained only 10.26% and 6.32% of the variation in positive and negative autocorrelation, respectively, and PC2 and PC30 explained only 7.97% and 4.09% of the variation (Figure 3C). For this reason, we kept the four principal components in the analysis, in order to explain as much variation as possible in the diversity of floral VOCs (Figure 3A). However, this analysis underlined additional similitudes in VOCs emission among some sections. As in our first analysis, the pPCA showed that species of the related sections *Sycomorus* and *Sycocarpus* released high amounts of the monoterpene E- β -ocimene (negative loadings on PC2, Figure 3B). Whereas species within section *Sycocarpus* also released high proportions

of other monoterpenes such as α -pinene, limonene, and sabinene (negative loadings on PC1, Figure 3B). Species of different sections such as *Sycidium*, *Malvanthera*, and *Galoglychia* emit high amounts of the monoterpene E- β -ocimene and the sesquiterpene E- β -caryophyllene (positive loading on PC1, Figure 3B). In addition, species from sections *Adenosperma*, *Eriosycea*, *Ficus*, and *Oreosycea* share in common the production of floral VOCs with high proportions of E- β -caryophyllene (positive loading on PC1, Figure 3B). In addition to producing high amounts of E- β -caryophyllene, species in sections *Malvanthera* and *Americana* also released high amounts of the shikimic acid derivative benzaldehyde (positive loading on PC1, Figure 3B).

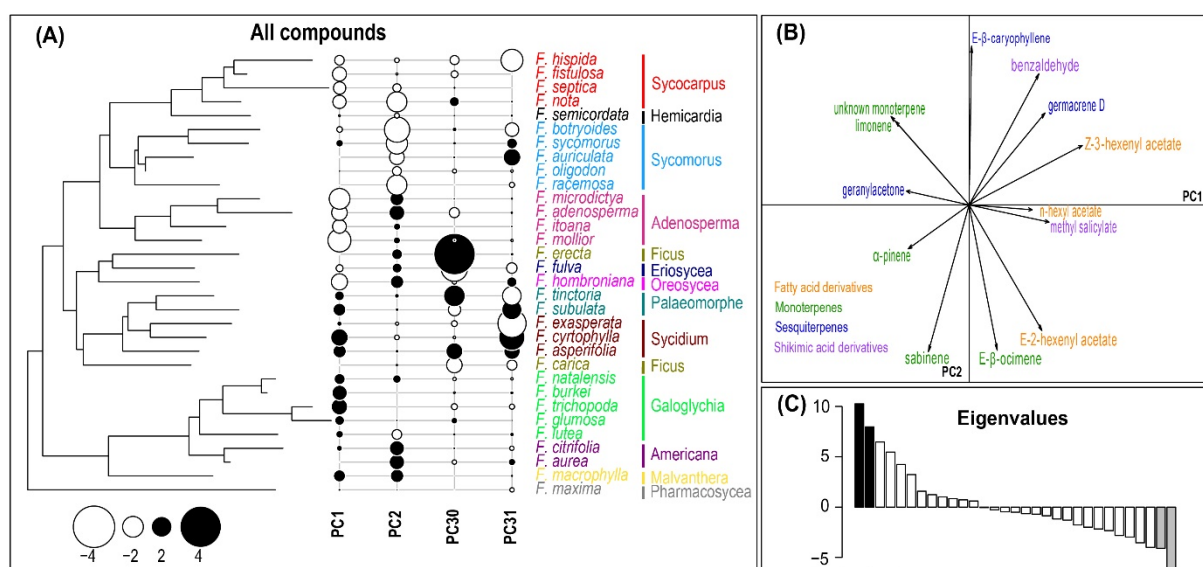


Figure 3. (A) Phylogeny of the *Ficus* species studied and results of the pPCA for all VOCs emitted by receptive figs. Positive and negative scores on PC1-PC2 (similar VOC composition in related species) and PC30-PC31 (dissimilarities among related species) are indicated by black and white circles, respectively. Symbol size is proportional to absolute values. The sections and species for each *Ficus* are indicated with different colors. (B) Loading of the main compounds for the global and local principal components with all the compounds. Only the compounds with highest loadings (net value higher than 0.2) on the PCs are represented. The main chemical classes for each VOC are indicated with different colors. (C) Barplot displays the corresponding eigenvalues of PC1-PC2 (black) and PC30-PC31 (gray) with all the compounds.

However, within sections, some closely related species emitted contrasting relative amounts of compounds and therefore showed quite different scores on PC30 and PC31. For example, *Ficus tinctoria* from section *Palaeomorpha* (positive score in PC30, Figure 3) emitted high relative amounts of the sesquiterpene E, E- α -farnesene, whereas

its closest relative among the species sampled, *F. subulata* (negative score, Figure 3A) did not release this compound. *Ficus exasperata* of section *Sycidium* (negative score in PC31, Figure 3A) emitted high relative amounts of the monoterpene α -pinene, whereas its closest relatives among the species sampled, *F. asperifolia* and *F. cyrtophylla* (positive scores, Figure 3A), did not release this compound but released high relative amounts of the fatty acid derivative 2-heptanone.

3. Phylogenetic signal of the pollinator species on volatile emission by figs

In contrast with the results of our analysis of phylogenetic signals for the fig species, tests for signals of the phylogeny of the 32 associated agaonid wasp species on floral scents of their hosts were not significant in either analysis (major compounds only: $K_{mult} = 0.39, p = 0.571$; all compounds: $K_{mult} = 0.34, p = 0.933$; Figure 4).

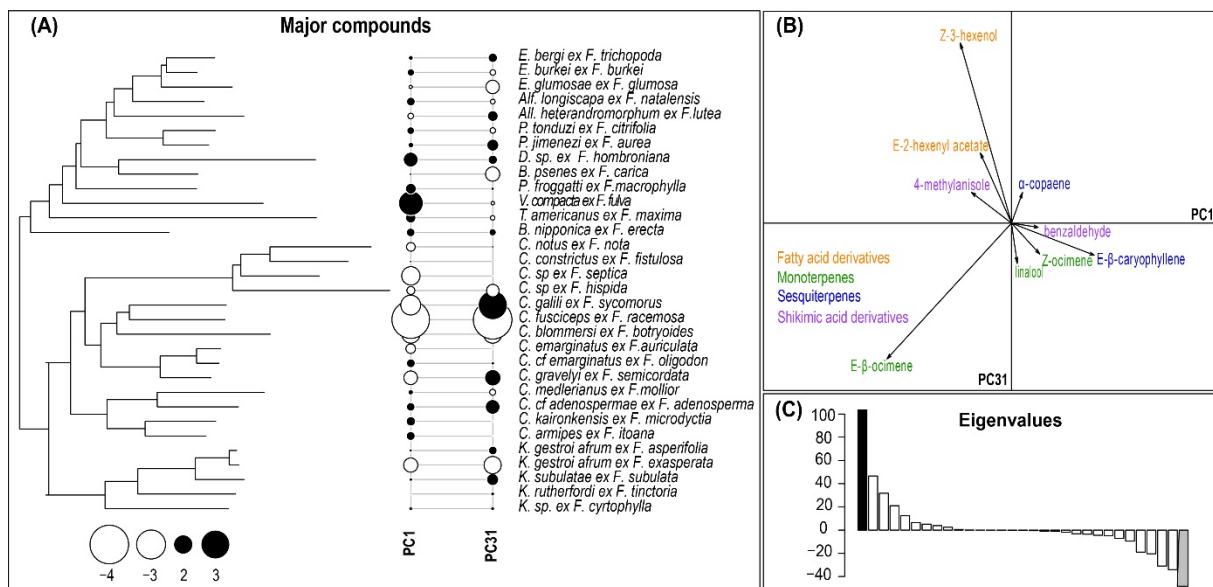


Figure 4. (A) Phylogeny of the associated pollinators of the *Ficus* species studied and results of the pPCA for major VOCs emitted by receptive figs. Positive and negative scores on PC1 (more similar VOCs) and PC31 (dissimilarities among related species) are indicated by black and white circles, respectively. Symbol size is proportional to absolute value. (B) Loading of the main compounds for the global and local principal components with all the compounds. Only the compounds with highest loadings (net value higher than 0.2) on the PCs are represented. The chemical classes to which VOCs belong are indicated with different colors. (C) Barplot displaying the corresponding eigenvalues of PC1 (black) and PC31 (gray) with all the compounds. (*Alf.* *Alfonsiella*, *All.* *Allotriozoon*, *B.* *Blastophaga*, *C.* *Ceratosolen*, *D.* *Dolichoris*, *E.* *Elisabethiella*, *K.* *Kradibia*, *P.* *forggatti*, *Pleistodontes forggatti*, *P. jimenezi* *Pegoscapus jimenezi*, *P. tonduzi* *Pegoscapus tonduzi*, *T.* *Tetrapus*, *V.* *Valisia*.)

Discussion

In this study, we highlight the remarkable diversity of floral scents among *Ficus*: species with more than 220 compounds emitted by receptive figs of the 32 focal species, with a mean number of 23.7 ± 12.5 compounds per species. Moreover, our large data set demonstrates a high similarity of floral scent within a species but a strong diversification among the 32 species. As shown in previous studies (Grison *et al.*, 1999; Proffit & Johnson, 2009; Soler *et al.*, 2010, Hossaert-McKey *et al.*, 2016, Proffit *et al.*, 2020), the message emitted by receptive figs is species-specific, a crucial trait acting as a prezygotic barrier avoiding host identification mistakes in *Ficus* species. The fact that numerous species often occur sympatrically and may produce receptive figs at the same time forces fig species to elaborate quite complex and specific blends in order to attract their specific pollinators (Hossaert-McKey *et al.*, 2010; Okamoto & Sun, 2001).

Among the emitted floral VOCs, terpenoids were dominant. But surprisingly, in this highly specific mutualistic interaction, the flower signal is made of a mixture of ubiquitous compounds (Grison-Pigé *et al.*, 2002; Farré-Amengol *et al.*, 2020) under their different forms (enantiomers, oxides) such as ocimene, linalool and caryophyllene, already found respectively in 71, 70 and 52 % of the floral VOCs (Knudsen *et al.*, 2006). Among the 32 species, only one species, *Ficus semicordata*, emits one rare compound (more than 90% of 4-methyl anisole), acting as a private channel (Chen *et al.* 2009). Nevertheless, the blend of floral VOCs has to be made of a very precise proportion of some major compounds as the corresponding pollinating insect is only attracted by this exact composition (Proffit *et al.*, 2020).

Our results reveal a strong phylogenetic signal in shaping the VOCs composition emitted by receptive figs, probably due to constraints in the biosynthetic pathways of volatile compounds. A same compound may be both repellent for herbivores and attractant for pollinators (Dobson, 1994; Euler & Baldwin, 1996). Moreover, many of the pathways involved in the biosynthesis of herbivore defense compounds also synthesize compounds that play roles in insect attraction. There are phylogenetic constraints placed on the herbivore traits that are associated with host use (Coley *et al.*,

2018). Herbivore host choice is determined by plant defensive traits. Divergence in defensive traits appears to be driven by herbivore pressure (Coley et al., 2018). Whether the natural selection (pollinator selection) have an impact on VOCs emitted by receptive figs? Using phylogenies available for wasps and the same analysis, we found no significant effect of pollinators on floral scents. The diversification of VOCs (the qualitative and quantitative composition of a floral scent varies within and between species) is probably as a product of phylogenetic constraints and balancing selection due to pollinator and florivore attraction (Raguso, 2001). Maybe it needs use pollinator attractive VOCs and phylogenies available for wasps to do analysis.

In the future, we need to expand the dataset to include multiple species in all *Ficus* sections to improve the sensitivity of our analyses. And perform similar analyses using only the compounds that are recognized by fig wasps, considering the balance between selection pressures imposed by fig wasps and phylogenetic constraints.

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Supplementary data

Table S1. 223 VOCs emitted from 32 *Ficus* species of receptive figs. Compounds detected are divided into four groups based on general biosynthetic origin. Yellow, blue, green, orange and white background represents fatty acid derivatives, monoterpenes, sesquiterpenes, shikimic acid derivatives and unknown compounds, respectively. For each species, the mean (\pm standard errors) of percentages of the VOCs per individual are presented.

| Compounds Species | (E)_2_heptenal | (E)_2_hexenal | (E)_2_hexenol | (Z)_3_hexenol | (Z)_3_hexenyl acetate | 1_hepten_3_one | 1_hexanol | 2_heptanol | 2_heptanone | 2_heptyl acetate | 2_nonanone | 2_octanone | 2_undecenyl acetate | 3_decanol |
|-----------------------|-----------------|-----------------|------------------|-------------------|-----------------------|-----------------|------------------|-------------------|-------------------|------------------|-----------------|-----------------|---------------------|-----------------|
| <i>F. Adenosperma</i> | 0 | 0 | 0 | 6.29 \pm 3.86 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Asperifolia</i> | 5.61 \pm 4.58 | 9.39 \pm 4.31 | 2.03 \pm 1.67 | 0 | 0 | 1.05 \pm 1.24 | 0 | 30.41 \pm 24.04 | 1.34 \pm 1.82 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 9.24 \pm 12.97 | 0 | 0 | 8.59 \pm 12.05 | 0 | 10.86 \pm 9.47 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 0 | 8.6 \pm 8.64 | 0 | 0 | 0 | 0 | 16.7 \pm 23.93 | 0 | 3.72 \pm 5.96 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 19.73 \pm 11.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0 | 0 | 0 | 0 | 0.54 \pm 0.67 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Citrifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0 | 1.01 \pm 1.02 | 19.59 \pm 15.35 | 0 | 0 | 0 | 16.79 \pm 14.55 | 0 | 0 | 0 | 0.16 \pm 0.06 | 0 |
| <i>F. Erecta</i> | 0 | 0 | 0 | 4.32 \pm 5.57 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Exasperata</i> | 0 | 0 | 0 | 0 | 0.95 \pm 1.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fistulosa</i> | 0 | 0 | 0 | 1.64 \pm 2.12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fulva</i> | 0 | 0 | 0 | 0.51 \pm 0.73 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.14 \pm 1.35 |
| <i>F. Hispida</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hombroniana</i> | 0 | 0 | 0 | 13.93 \pm 15.75 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 16.74 \pm 17.24 | 0 | 0 | 0 | 0 | 13.17 \pm 16.82 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Macrophylla</i> | 0 | 0 | 0 | 5.82 \pm 8.86 | 5.95 \pm 4.53 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Maxima</i> | 0 | 0 | 0 | 0.81 \pm 0.71 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.46 \pm 2.66 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Mollior</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 19.53 \pm 21.81 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 7.39 \pm 12.6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Oligodon</i> | 0 | 0 | 11.98 \pm 9.88 | 0 | 0 | 0 | 0 | 0 | 0 | 12.45 \pm 7.98 | 0 | 2.51 \pm 3.51 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 22.96 \pm 21.47 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 7.94 \pm 8.27 | 8.81 \pm 12.4 | 67.41 \pm 21.02 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0 | 0 | 0 | 40.87 \pm 43.58 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Tinctoria</i> | 0 | 0 | 0 | 2.42 \pm 6.01 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Trichopoda</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| Compounds Species | (Z)_3_hexenyl_butyrate | 3_octanone | 3_octanal | (E)_2_hexenyl acetate | unknow_fatty acid derivative 1 | unknow_fatty acid derivative 2 | 2_methylundecane | Z_jasmone | decanal | decane | dodecane | dodecanal |
|-----------------------|------------------------|------------|-----------|-----------------------|--------------------------------|--------------------------------|------------------|-----------|-----------|-----------|-----------|-----------|
| <i>F. Adenosperma</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Asperifolia</i> | 0 | 0 | 0.4±0.47 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 0 | 10.93±4.92 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 7.89±5.21 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.68±3.53 | 0 | 0 | 0 |
| <i>F. Citrifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0 | 1.06±1.78 | 0 | 0 | 0 | 0.16±0.14 | 0 | 0 | 0 | 1.27±1.59 |
| <i>F. Erecta</i> | 0 | 0 | 3.14±8.3 | 0 | 0 | 0 | 0.65±1.32 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Exasperata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.36±1.01 | 0 | 0 |
| <i>F. Fistulosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.64±1.19 | 0 | 0 | 0 |
| <i>F. Fulva</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hombroniana</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 6.17±5.38 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Macrophylla</i> | 0 | 0 | 0 | 1.29±1.41 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Maxima</i> | 0 | 0 | 0.5±0.78 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Mollior</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 14.7±12.84 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.07±0.09 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.32±0.42 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0.02±0.03 | 0 | 0 | 18.94±18.96 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Tinctoria</i> | 0 | 0.22±0.4 | 2.17±3.67 | 1.18±2.12 | 0.26±0.46 | 0.16±0.28 | 0.37±0.52 | 0 | 0.36±0.72 | 0.63±0.69 | 0 | 0 |
| <i>F. Trichopoda</i> | 0 | 0 | 0 | 19.23±21.12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| Compounds Species | heptanal | hexadecane | hexanal | methyl octanoate | unknow_fatty acid derivative 3 | n_hexyl acetate | nonanal | nonyl acetate | octen_3_ol | pentadecane | tetradecane | tetradecene | tetradecanol | tridecane | tridecanol |
|-----------------------|----------|------------|-----------|------------------|--------------------------------|-----------------|-----------|---------------|------------|-------------|-------------|-------------|--------------|-------------|------------|
| <i>F. Adenosperma</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Asperifolia</i> | 0 | 0 | 4.25±2.63 | 0 | 0 | 0 | 0 | 0 | 2.57±2.6 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 0 | 0 | 0 | 0.75±0.75 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0.65±0.94 | 0 | 0 | 0.08±0.05 | 0 | 0 | 0 | 0.09±0.05 | 0 |
| <i>F. Citrifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0 | 0 | 0 | 2.45±1.87 | 0 | 3.06±2.27 | 0 | 0.03±0.04 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Erecta</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.34±0.42 | 0 | 0 | 0.2±0.37 | 0 |
| <i>F. Exasperata</i> | 0 | 0.07±0.13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.12±0.31 | 0.47±0.7 | 0 | 0 | 0.66±1.82 | 0 |
| <i>F. Fistulosa</i> | 0 | 0 | 0 | 0.37±0.53 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fulva</i> | 0 | 0 | 0 | 0.13±0.16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 6.53±7.75 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.29±0.43 | 0 | 0 | 0 | 0 |
| <i>F. Hombroniana</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 0 | 0 | 9.75±13.12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Macrophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.26±2.22 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Maxima</i> | 0 | 0 | 0 | 0 | 0 | 0 | 2.69±3.1 | 0 | 0 | 0 | 2.17±1.46 | 0 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Mollior</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.39±0.37 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04±0.05 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 1.77±3.31 | 8.9±2.27 | 0 | 0 | 0 | 1.11±2.11 | 0.33±0.61 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0 | 1.12±0.8 | 0 | 0 | 0 | 0.28±0.32 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Tinctoria</i> | 1±1.73 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.36±0.54 | 0.25±0.38 | 0.56±0.78 | 0 | 0.12±0.26 | 0.42±0.59 |
| <i>F. Trichopoda</i> | 0 | 0 | 0 | 0 | 0 | 15.01±15.72 | 0 | 0 | 0 | 0 | 0 | 0 | 6.52±11.2 | 15.03±25.43 | 0 |

| Compounds Species | tridecene | undecane | undecanal | (E)_4,8_dimethyl_1,3,7_Nonatriene | Nonanoic_acid | (Z)_3_hexenyl_2_methyl_butanoate | (Z)_3_hexenyl_3_methyl_butanoate | 3_ethyl_4_methyl_pentan_1_ol | Neryl Acetone |
|-----------------------|-----------|-----------|-----------|-----------------------------------|---------------|----------------------------------|----------------------------------|------------------------------|---------------|
| <i>F. Adenosperma</i> | 0 | 0 | 0 | 1.44±0.67 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Asperifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 1.67±2.38 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0 | 0 | 0.11±0.18 | 0 | 0 | 0 | 0.02±0.05 | 0 | 0.05±0.06 |
| <i>F. Citrifolia</i> | 0 | 0 | 0 | 9.89±8.28 | 0 | 1.35±1.37 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Erecta</i> | 0 | 0 | 0.65±0.99 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Exasperata</i> | 0 | 0.96±1.95 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fistulosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fulva</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hombroniana</i> | 0 | 0 | 0 | 12.78±5.94 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Macrophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Maxima</i> | 0 | 0.79±0.75 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Mollior</i> | 0 | 0 | 0 | 22.62±13.2 | 1.34±1.62 | 0 | 0 | 0 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 0.44±0.81 | 0 | 0 | 0 | 16.03±21.44 | 0 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 2.06±4.21 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Tinctoria</i> | 0 | 0.63±1.16 | 0.11±0.2 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Trichopoda</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| Compounds Species | n_octanal | 3Z_Hexenyl tiglate | (E)_ocimene | (Z)_ocimene | 1,8_cineole | myrcenone | 6_methyl_5_hepten_2_ol | (E,Z)_alloocimene | (E,E)_alloocimene | α _pinene | α _terpinene | α _thujene | β _pinene | β _phellandrene |
|-----------------------|-----------|--------------------|-------------|-------------|-------------|-----------|------------------------|-------------------|-------------------|------------------|---------------------|-------------------|-----------------|-----------------------|
| <i>F. Adenosperma</i> | 0 | 0 | 1.37±0.36 | 0 | 1.12±0.65 | 0 | 0 | 0 | 0 | 0.33±0.22 | 0 | 0 | 0 | 0 |
| <i>F. Asperifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 20.65±18.01 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 38.96±18.67 | 1.77±2 | 10.43±17.33 | 0 | 0 | 0 | 0.96±0.95 | 1.87±1.63 | 0 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 14.51±8.96 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0.88±0.91 | 0.04±0.05 | 18.04±12.38 | 9.5±7.3 | 0.02±0.02 | 0 | 0.11±0.17 | 2.51±1.9 | 0.05±0.04 | 0 | 0 | 0 | 2.02±1.86 | 0 |
| <i>F. Citrifolia</i> | 0 | 0 | 12.08±18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 12.59±11.69 | 0 | 0 | 0 | 0 | 0.93±0.61 | 0 | 0 | 0 | 0 | 0 | 0.43±0.38 |
| <i>F. Erecta</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.6±2.1 | 0 | 0 | 2.44±5.07 | 0 |
| <i>F. Exasperata</i> | 0 | 0 | 34.86±19.07 | 1.72±1.21 | 0 | 0 | 0 | 0 | 0 | 5.67±11.26 | 0.24±0.57 | 0.22±0.24 | 1.94±2.18 | 0 |
| <i>F. Fistulosa</i> | 0 | 0 | 9.43±10.22 | 0 | 3.98±2.58 | 0 | 0 | 0 | 0 | 1.15±2.73 | 0 | 0 | 0 | 0 |
| <i>F. Fulva</i> | 0 | 0 | 3.11±4.21 | 1.27±0.73 | 0.22±0.17 | 0.03±0.02 | 0 | 0 | 0 | 3.64±4.23 | 0 | 0 | 1.98±2.38 | 0 |
| <i>F. Glumosa</i> | 0 | 0 | 25.2±34.28 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 0 | 27.41±21.15 | 1.47±1.29 | 0 | 0 | 0 | 0 | 0 | 7.67±12.94 | 0 | 0 | 2.48±3.86 | 0 |
| <i>F. Hombroniana</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 14.19±8.21 | 0.97±0.82 | 0.8±0.57 | 0 | 0 | 0.95±0.84 | 0 | 2.98±2.47 | 0 | 0 | 0.35±0.47 | 0 |
| <i>F. Macrophylla</i> | 0 | 0 | 5.59±6.66 | 0 | 0 | 0 | 0 | 0 | 0 | 4.53±8.59 | 0 | 0 | 8.18±15.61 | 0 |
| <i>F. Maxima</i> | 0 | 0 | 5.2±6.36 | 43.66±5.12 | 0 | 0 | 0 | 0 | 0 | 1.6±1.3 | 0.87±0.76 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 0 | 0 | 3.42±3.68 | 0 | 0 | 0 | 0 | 0 | 0 | 1.58±1.24 | 0 | 0 | 0 | 0 |
| <i>F. Mollior</i> | 0 | 0 | 10.27±10.29 | 0 | 6.29±11.79 | 0 | 0 | 0 | 0 | 1.95±3.05 | 0 | 0 | 0 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 11.48±11.78 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 17.69±19.57 | 1.09±1.45 | 5.22±4.62 | 0 | 0 | 0.25±0.34 | 0 | 2.86±1.71 | 0 | 0.52±0.65 | 0 | 0 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.26±3.32 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 72.54±15.51 | 7.21±3.98 | 0 | 0 | 0 | 0 | 0 | 0.67±0.81 | 0 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0.61±1.03 | 0 | 0 | 0 | 0 | 0 | 0 | 0.06±0.09 | 0 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 34.22±32.85 | 2.47±2.79 | 0 | 0 | 0 | 0.89±1.29 | 0 | 1.54±2.08 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0 | 0 | 20.41±22.33 | 0.75±0.88 | 0 | 0 | 0 | 0.29±0.17 | 0.49±0.74 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Tinctoria</i> | 0 | 0 | 9.38±18.29 | 0 | 0.17±0.29 | 0 | 0 | 0 | 0 | 1.89±3.19 | 0.21±0.38 | 0 | 0.23±0.41 | 0 |
| <i>F. Trichopoda</i> | 0 | 0 | 3.09±0.68 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| Compounds Species | camphene | Z_linalooloxide (furanoid) | Z_linalooloxide (pyranoid) | cyclolinalone | epoxy ocimene | γ _terpinene | limonene | linalool | 6_methyl_3,5_heptadien_2_one | 6_methyl_3_hepten_2_one | myrcene | ρ _cymene |
|-----------------------|-----------|----------------------------|----------------------------|---------------|---------------|---------------------|------------|-------------|------------------------------|-------------------------|-----------|----------------|
| <i>F. Adenosperma</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0.61±0.31 | 0.77±0.33 | 0 | 0 | 0 | 0 |
| <i>F. Asperifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 1.12±1.49 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.96±1.27 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0.66±0.84 | 0 | 0.1±0.1 | 1.39±1.21 | 0 | 0 | 1.02±1.7 | 0 | 0 | 1.2±1.56 | 4.62±4 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0 | 2.69±3.02 | 0.04±0.07 | 0 | 0 | 0 | 2.64±1.05 | 29.44±29.03 | 0 | 0 | 0 | 0.02±0.02 |
| <i>F. Citrifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0 | 0 | 0.14±0.07 | 0 | 0.36±0.28 | 0 | 5.1±4.57 | 1.48±0.58 | 0 | 0 |
| <i>F. Erecta</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0.11±0.29 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Exasperata</i> | 0 | 0 | 0 | 0 | 0 | 6.59±4.48 | 3.47±4.54 | 6.61±10.4 | 0 | 0 | 1.13±0.6 | 2.14±2.09 |
| <i>F. Fistulosa</i> | 0 | 2.5±5.43 | 0 | 0 | 0 | 0 | 1.28±1.3 | 2.81±3.22 | 0 | 0 | 0.54±0.84 | 0 |
| <i>F. Fulva</i> | 0.55±0.56 | 0.65±1.41 | 0 | 0 | 0 | 0 | 0.43±0.3 | 0.22±0.21 | 0 | 0 | 0.7±0.56 | 0 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5.66±3.9 | 0 | 2.15±2.04 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 1.3±1.4 | 0.09±0.15 | 2.01±2.33 | 0 | 0.72±1.43 | 4.7±7.97 | 3.82±5.24 | 0 | 0 | 0.48±0.61 | 0.65±1.22 |
| <i>F. Hombroniana</i> | 0 | 0 | 0 | 0 | 0 | 0 | 4.45±6.11 | 2.05±2.95 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 0 | 0 | 3.56±5.16 | 1.37±1.64 | 0.53±0.64 | 0 | 0.56±0.79 | 1.82±1.43 | 3.97±6.63 |
| <i>F. Macrophylla</i> | 0 | 0 | 0 | 0 | 0 | 0.64±0.88 | 0 | 0.27±0.52 | 0 | 0 | 0.9±1.22 | 0 |
| <i>F. Maxima</i> | 0 | 0 | 0 | 0 | 0 | 4.72±3.83 | 0.22±0.19 | 0 | 0 | 0 | 0.66±0.66 | 0 |
| <i>F. Microdyctia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 6.71±5.66 | 0.44±0.42 | 0 | 0 | 0 | 0 |
| <i>F. Mollior</i> | 0 | 0 | 0 | 0 | 0 | 0 | 3.54±2.75 | 1.84±2.92 | 0 | 0 | 0.95±1.11 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.96±2.77 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 0 | 0 | 0.38±0.47 | 0 | 0.23±0.32 | 0 | 0 | 0 | 0.52±0.74 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 0 | 0 | 9.56±13.72 | 11.18±16.28 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7.13±4.89 | 0 | 0 | 0.77±0.55 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 4.91±10.61 | 3.26±4.78 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0.4±0.69 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.06±2.41 | 0 | 0.29±0.2 | 0 | 0 |
| <i>F. Tinctoria</i> | 0 | 0 | 0 | 0 | 0 | 0 | 1.08±1.36 | 0.16±0.35 | 0 | 0 | 0 | 0 |
| <i>F. Trichopoda</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4.37±7.54 | 0 | 0 | 5.3±8.39 | 0 |

| Compounds Spices | ρ -cymenene | perillene | ρ -mentha_2,5_dienol Z | 1,3,4(8)_menthatriene | 1,4,8_menthatriene | 1(7),2,4_menthatriene | sabinene | terpinolene | E_linalooloxide (furanoid) | E_linalooloxide (pyranoid) |
|-----------------------|------------------|-----------|-----------------------------|-----------------------|--------------------|-----------------------|-------------|-------------|----------------------------|----------------------------|
| <i>F. Adenosperma</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Asperifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 1.13±1.09 | 0 | 0 | 0 | 0 | 2.36±3.21 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 0 | 0.69±0.81 | 0 | 3.84±1.2 | 7.81±6.66 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0.08±0.09 | 0 | 0 | 0.57±1.31 |
| <i>F. Citrifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0.12±0.16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Erecta</i> | 0 | 0 | 0 | 0 | 0 | 0 | 2.64±4.61 | 0.33±0.67 | 0 | 0 |
| <i>F. Exasperata</i> | 0 | 0.33±0.54 | 0 | 0 | 0 | 0 | 2.63±2.52 | 0.68±0.5 | 0 | 0 |
| <i>F. Fistulosa</i> | 0 | 6.32±8.71 | 0 | 0 | 0 | 0 | 1.73±1.29 | 0 | 1.17±1.69 | 0 |
| <i>F. Fulva</i> | 0 | 0.41±0.38 | 0 | 0 | 0 | 0 | 0.75±0.99 | 0 | 0.21±0.41 | 0 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 0.6±0.64 | 0 | 0 | 0 | 0 | 0.81±1.66 | 0 | 0.22±0.34 | 0.11±0.14 |
| <i>F. Hombroniana</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0.53±0.54 | 0 | 0 | 0 | 0 | 3.62±3.42 | 0 | 0 | 0 |
| <i>F. Macrophylla</i> | 1.38±1.59 | 0.08±0.06 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Maxima</i> | 4.72±1.6 | 3.93±2.66 | 0 | 0 | 4.06±3.94 | 0 | 0 | 2.42±1.78 | 2.74±3.08 | 0.97±1.37 |
| <i>F. Microdyctia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Mollior</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.97±1.19 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 0 | 0 | 0 | 13.04±11.25 | 0.18±0.21 | 0 | 0 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 2.77±3.24 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 0.29±0.32 | 0 | 0 | 0 | 0 | 0.15±0.3 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 2.86±4.04 | 0.4±1.19 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorua</i> | 0 | 0.07±0.12 | 0 | 0 | 0 | 0.93±1.08 | 0 | 0 | 0 | 0 |
| <i>F. Tinctoria</i> | 0 | 1.1±1.08 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Trichopoda</i> | 0 | 0.21±0.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| Compounds Species | unknown_monoterpene_derivative | 2,6_dimethyl_5_heptene | (E)_β_farnesene | (E,E)_α_farnesene | (E)_γ_bisabolene | 6_methyl_1,5_heptadiene | 6,10_dimethyl_5,9_undecadien_2_one | α_bulnesene | α_cedrene | α_copaene | α_cubebene |
|-----------------------|--------------------------------|------------------------|-----------------|-------------------|------------------|-------------------------|------------------------------------|-------------|-----------|-------------|------------|
| <i>F. Adenosperma</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 57.83±3.54 | 4.17±0.28 |
| <i>F. Asperifolia</i> | 0 | 0 | 0 | 0 | 0 | 2.17±1.98 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9.12±9.73 | 1.4±1.41 |
| <i>F. Auriculata</i> | 0 | 0 | 1.53±1.97 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 0 | 2.21±3.06 | 0 | 0 | 0 | 0 | 0.85±1.23 | 3.93±3.99 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0 | 0 | 0 | 2.17±1.86 | 0 | 0 | 0 | 0 | 0 | 0.03±0.06 | 0 |
| <i>F. Citrifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.67±2.86 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04±0.04 | 0 |
| <i>F. Erecta</i> | 0 | 0 | 10.84±13.08 | 0.49±0.98 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Exasperata</i> | 0 | 0 | 0 | 0.56±0.9 | 0 | 0 | 0 | 0 | 0 | 2.82±3.01 | 0 |
| <i>F. Fistulosa</i> | 0 | 0 | 2.97±3.78 | 0 | 0 | 0 | 0 | 0 | 0 | 14.67±11.89 | 0.54±0.5 |
| <i>F. Fulva</i> | 0 | 0.1±0.14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6.13±1.7 | 0.88±0.15 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0.75±0.73 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7.02±5.29 | 0 |
| <i>F. Hombroiana</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.44±2.84 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4.94±3.37 | 0 |
| <i>F. Macrophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.97±2.54 | 0.78±0.69 |
| <i>F. Maxima</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 15.54±13.04 | 0 |
| <i>F. Mollior</i> | 4.02±4.75 | 0 | 0 | 0 | 0 | 0 | 0 | 3.86±6.81 | 0 | 0 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6.57±5.88 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0.68±0.86 | 0 | 0 | 0 | 0 | 0 | 0 | 0.91±0.82 | 0 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 2.89±3.81 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0.81±0.93 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.28±0.42 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.59±4.36 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.97±2.29 | 0 |
| <i>F. Tinctoria</i> | 0 | 0 | 0.19±0.34 | 6.63±9.76 | 0 | 0 | 0 | 0 | 0 | 26.02±22.88 | 0.67±1.29 |
| <i>F. Trichopoda</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5.63±2.51 | 0 |

| Compounds Species | α _curcumene | α _gurjunene | α _humulene | alloaromadendrene | α _longipinene | α _muurolene | aristolene | aromadendrene | α _selinene | α _tr_bergamotene | α _ylangene | β _bergamotene | β _bisabolene | β _bourbonene |
|-----------------------|---------------------|---------------------|--------------------|-------------------|-----------------------|---------------------|------------|---------------|--------------------|--------------------------|--------------------|----------------------|---------------------|---------------------|
| <i>F. Adenosperma</i> | 0 | 1.25±0.62 | 1.59±1.19 | 2.91±0.56 | 0 | 1.25±0.1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Asperifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0.34±0.5 | 0 | 1.15±1.46 | 0 | 0 | 0 | 2.48±2.79 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.79±2.93 | 0 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.59±0.75 | 0 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0 | 0 | 0.16±0.1 | 0.02±0.02 | 0 | 0 | 0 | 0 | 0 | 0.32±0.69 | 0 | 0 | 0 | 0.77±0.54 |
| <i>F. Citrifolia</i> | 0 | 0 | 0.76±0.82 | 0.43±0.47 | 0 | 0 | 0 | 0 | 0 | 2.87±5.64 | 0 | 0 | 0 | 4.11±5.86 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Erecta</i> | 1.38±1.65 | 0 | 1.67±1.48 | 0 | 0 | 0 | 0 | 0.37±0.5 | 0 | 0 | 0 | 0.9±1.39 | 0.72±1.24 | 0 |
| <i>F. Exasperata</i> | 0 | 0 | 1.38±1.6 | 2.02±2.01 | 0 | 0 | 0 | 0 | 0 | 2.92±3.56 | 0 | 0 | 0 | 0 |
| <i>F. Fistulosa</i> | 0 | 0 | 1.46±1.36 | 2.68±4.23 | 0 | 0 | 0 | 4.93±3.98 | 0 | 0 | 0 | 0 | 0 | 1.6±1.78 |
| <i>F. Fulva</i> | 0 | 0 | 7.24±1.17 | 0.36±0.26 | 0 | 0.16±0.06 | 0 | 0.05±0.02 | 0 | 0 | 0 | 0 | 0 | 0.32±0.09 |
| <i>F. Glumosa</i> | 0 | 0 | 1.02±0.93 | 1.9±1.75 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 0 | 2.03±1.58 | 0.3±0.33 | 0 | 0.78±1.12 | 0 | 0.26±0.42 | 0 | 4.16±5.05 | 1.16±2.7 | 0 | 0 | 0 |
| <i>F. Hombroniana</i> | 0 | 0 | 5.73±1.45 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Macrophylla</i> | 0 | 0.11±0.1 | 1.38±1.36 | 0.17±0.24 | 0 | 0 | 0 | 0 | 0 | 0.34±0.24 | 0 | 0 | 0 | 0 |
| <i>F. Maxima</i> | 0 | 0 | 0.88±1.16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Microdactia</i> | 0 | 1.21±1.02 | 2.48±1.63 | 0 | 0 | 0 | 0 | 0 | 0 | 1.68±1.58 | 0 | 0 | 0 | 0 |
| <i>F. Mollior</i> | 0 | 0 | 0.55±0.69 | 0 | 0 | 0 | 0 | 0 | 0 | 1.25±0.65 | 0 | 0 | 0 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 5.06±5.54 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.38±5.51 | 0.24±0.35 | 0 | 0 | 0 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0.18±0.2 | 0.01±0.03 | 0 | 0.01±0.02 | 0 | 0 | 0 | 0 | 0.01±0.02 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0.68±0.91 | 0 | 0 | 0 | 0 | 0 | 0 | 0.26±0.44 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0.14±0.23 | 0 | 0 | 0 | 0 | 0 | 0.33±0.56 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0 | 0.51±0.69 | 0.34±0.63 | 0 | 0.57±0.86 | 0 | 0.14±0.16 | 0 | 0 | 0 | 3.22±5.22 | 0 | 0 | 0 |
| <i>F. Tinctoria</i> | 0.53±1.03 | 0 | 1.28±1.13 | 1.14±0.72 | 0.58±0.6 | 0.47±0.27 | 0 | 0.12±0.2 | 0 | 0.21±0.36 | 0.24±0.5 | 0 | 0 | 0 |
| <i>F. Trichopoda</i> | 0 | 1.06±1.01 | 0 | 1.8±2.45 | 0 | 0.72±0.9 | 0 | 0.49±0.57 | 0 | 0 | 0 | 0 | 0 | 0.71±0.97 |

| Compounds Species | β _caryophyllene | β _copaene | β _cubebene | β _elemene | β _gurjunene | bicyclgermacrene | β _selinene | β _sesquiphellandrene | caryophyllene oxide | Z_α_bergamotene | cyclosativene | daucene | δ_cadinene | δ_elemene |
|-----------------------|------------------------|------------------|-------------------|------------------|--------------------|------------------|-------------------|-----------------------------|---------------------|-----------------|---------------|-----------|------------|-------------|
| <i>F. Adenosperma</i> | 6.85±4.45 | 0 | 0 | 3.55±1.12 | 0 | 0 | 0 | 0 | 0 | 0 | 0.32±0.19 | 0 | 1.58±0.22 | 0 |
| <i>F. Asperifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 3.12±2.05 | 0 | 0 | 9.1±15.21 | 0 | 1.17±1.49 | 0 | 0 | 0 | 0 | 0 | 0 | 0.18±0.25 | 0 |
| <i>F. Auriculata</i> | 2.22±1.99 | 0 | 0 | 1.31±1.58 | 0 | 0 | 4.71±8.95 | 0 | 0 | 0 | 0 | 0 | 0 | 2.58±3.06 |
| <i>F. Botryoides</i> | 2.5±3.34 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.23±1.56 | 0 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 29.65±10.58 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 15.99±8.77 | 0.06±0.04 | 0 | 0.76±0.91 | 0 | 2.46±2.67 | 0 | 0 | 0.23±0.22 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Citrifolia</i> | 16.31±10.38 | 2.01±1.9 | 0 | 0.75±0.73 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0.16±0.18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Erecta</i> | 9.36±15.24 | 0 | 0 | 0 | 0 | 0 | 0 | 7.22±12.04 | 0 | 0.64±0.98 | 0 | 0 | 0 | 0 |
| <i>F. Exasperata</i> | 2.23±1.86 | 0 | 0 | 1.43±3.49 | 0 | 0.48±0.65 | 0.13±0.18 | 0 | 0 | 0.27±0.45 | 0 | 0 | 1.26±2.01 | 0 |
| <i>F. Fistulosa</i> | 6.65±5.08 | 0 | 0 | 2.98±3.71 | 0 | 2.02±1.68 | 1.19±1.55 | 0 | 0.53±0.65 | 1.28±0.89 | 0 | 0 | 0 | 2.93±2.45 |
| <i>F. Fulva</i> | 52.44±6.61 | 0 | 0 | 5.22±0.8 | 0 | 1.33±0.72 | 0.35±0.07 | 0 | 0.32±0.46 | 0 | 0 | 0 | 1.27±0.37 | 0.2±0.08 |
| <i>F. Glumosa</i> | 14.87±11.09 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 6.39±4.06 | 0 | 0 | 1.02±1.28 | 0 | 1.29±1.16 | 0.34±0.43 | 0 | 0 | 0 | 0 | 0.69±0.78 | 2.06±1.43 | 0 |
| <i>F. Hombroniana</i> | 29.18±3.02 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.65±1.59 |
| <i>F. Lutea</i> | 2.8±3.68 | 0 | 0 | 0 | 0.16±0.21 | 0 | 0 | 0 | 0 | 0 | 0 | 4.03±6.99 | 0 | 0 |
| <i>F. Macrophylla</i> | 18.25±18.48 | 0 | 0.46±0.37 | 0.43±0.47 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.93±1.36 |
| <i>F. Maxima</i> | 5.75±5.11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 11.88±8.29 | 0 | 0 | 0 | 0 | 8.81±7.46 | 0 | 0 | 0 | 0.58±0.7 | 3.49±1.35 | 0 | 0 | 15.36±11.57 |
| <i>F. Mollitor</i> | 12.11±16.38 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.69±2.13 | 0 | 0 | 0 | 0 |
| <i>F. Natalensis</i> | 23.6±19.65 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.53±0.82 | 0 |
| <i>F. Nota</i> | 0.57±0.89 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.3±3.74 | 0 | 0 | 0 | 0 |
| <i>F. Oligodon</i> | 16.84±21.17 | 0 | 0 | 0 | 0.86±0.82 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.18±0.2 | 0 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0.85±0.62 | 0 | 0 | 0 | 0 | 0 | 0.03±0.05 | 0 | 0 | 0 | 0 | 0 | 0.02±0.03 | 0 |
| <i>F. Septica</i> | 5.53±7.48 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.3±0.52 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0.59±1.13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.32±0.4 |
| <i>F. Sycomorus</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0.28±0.28 | 0 | 0 | 0 | 0 | 0 | 0.46±0.46 | 0 |
| <i>F. Tinctoria</i> | 5.21±3.94 | 0 | 0.48±0.87 | 0 | 0 | 0.12±0.2 | 0.21±0.39 | 0 | 0 | 1.16±1.43 | 1.85±4.14 | 0 | 1.19±1.14 | 0 |
| <i>F. Trichopoda</i> | 8.2±4.12 | 0 | 0 | 0.62±0.61 | 0 | 0 | 0 | 0 | 0 | 0.6±0.63 | 0 | 0 | 1.82±0.94 | 0 |

| Compounds Species | dendrolasine | 1,8_dimethyl_1,3_nonadiene | δ _guaiene | elixene | γ _cadinene | geranylacetone | germacrene A | germacrene D | γ _muurolene | isocaryophyllene | khusimene | nerolidol | sativene | selina_4,11_diene |
|-----------------------|--------------|----------------------------|-------------------|----------|--------------------|----------------|--------------|--------------|---------------------|------------------|-----------|-----------|-----------|-------------------|
| <i>F. Adenosperma</i> | 0 | 0 | 0 | 0 | 0 | 0.38±0.23 | 0 | 0.45±0.46 | 0.37±0.38 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Asperifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 0 | 0 | 0 | 2.06±3.11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 0 | 0 | 0 | 0.36±0.41 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9.48±2.1 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Citrifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6.77±8.64 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0.28±0.39 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.03±0.04 | 0 | 0 |
| <i>F. Erecta</i> | 1.02±1.89 | 0 | 0 | 0 | 0 | 0 | 0 | 0.25±0.67 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Exasperata</i> | 0 | 0 | 0 | 0.59±0.9 | 0.15±0.16 | 0 | 0.21±0.66 | 2.19±2.18 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fistulosa</i> | 0 | 0 | 0.41±0.63 | 0 | 0 | 0 | 0 | 12.56±10.42 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fulva</i> | 0.06±0.06 | 0 | 1.22±0.67 | 0 | 0 | 0 | 0.9±0.17 | 3.11±0.75 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 | 0.89±1.1 | 0 | 20.14±14.2 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 0 | 0 | 0 | 0.18±0.28 | 0 | 0.55±0.77 | 1.84±2.76 | 1.96±1.77 | 0.48±0.75 | 0 | 0 | 0 | 1.93±1.81 |
| <i>F. Hombroniana</i> | 0 | 0 | 0 | 0 | 0 | 10.55±8.84 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Macrophylla</i> | 0 | 0 | 0 | 0 | 0 | 0.25±0.1 | 0 | 1.37±1.2 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Maxima</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 0 | 0 | 0 | 0 | 0 | 2.45±1.75 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Mollior</i> | 0 | 0 | 0 | 0 | 0 | 5.62±3.75 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6.53±6.37 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 0 | 2.34±3.02 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.01±0.02 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorusc</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.56±3.02 | 0 | 0 | 0 |
| <i>F. Tinctoria</i> | 1.16±1.8 | 0 | 0 | 0 | 0.29±0.56 | 0 | 0 | 5.06±10.38 | 0±0.56 | 0 | 0 | 0 | 1.71±2.29 | 0 |
| <i>F. Trichopoda</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4.63±3.86 | 0 | 0 | 0 | 0 | 0 | 0 |

| Compounds Species | sesquifenchene | spathulenol | tetrahydrosesquiterpene | zizaene | Cycloseychellene | unknown_sesquiterpene_1 | alpha_Guaiene | unknown_sesquiterpene_04 | unknown_sesquiterpene_2 | Z_Calamene | E_Cadina_1,4_diene |
|-----------------------|----------------|-------------|-------------------------|-----------|------------------|-------------------------|---------------|--------------------------|-------------------------|------------|--------------------|
| <i>F. Adenosperma</i> | 0 | 0 | 0 | 0 | 0 | 1.05±0.97 | 0.93±1.11 | 0.11±0.14 | 0.4±0.08 | 0.69±0.72 | 0.19±0.01 |
| <i>F. Asperifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Citrifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Erecta</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Exasperata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fistulosa</i> | 0 | 0.34±0.72 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fulva</i> | 0 | 0.27±0.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0.28±0.46 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hombroniana</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.45±1.26 | 0.95±0.98 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Macrophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Maxima</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 0 | 0 | 0 | 0 | 0.81±0.82 | 0 | 0 | 0 | 2.23±1.54 | 0.23±0.28 | 0.07±0.06 |
| <i>F. Mollior</i> | 0 | 0 | 0 | 0 | 1.89±2.47 | 0 | 3.87±7.06 | 0 | 2.81±3.54 | 0 | 0.06±0.1 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 1.94±1.66 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Tinctoria</i> | 0 | 0 | 0.21±0.44 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Trichopoda</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| Compounds Species | alpha_Calacorene | beta_Calacorene | sesqui65 | guaia_6,9_diene | isomere_delta_clemene | 7_episesquithujene | sesquithujene | alpha_santalene | unknown_sesquiterpene_3 (selinene family) | epi_beta_santalene | farnesene isomer |
|-----------------------|------------------|-----------------|-----------|-----------------|-----------------------|--------------------|---------------|-----------------|-------------------------------------------|--------------------|------------------|
| <i>F. Adenosperma</i> | 0.37±0.2 | 0.13±0.06 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Asperifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Citrifolia</i> | 0 | 0 | 1.27±1.29 | 0.68±0.89 | 1.68±2.44 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Erecta</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Exasperata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fistulosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fulva</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hombroniana</i> | 0 | 0.8±1.05 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Macrophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Maxima</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 0.14±0.23 | 0.34±0.21 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Mollior</i> | 0 | 0.24±0.38 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 0 | 0 | 0.27±0.45 | 3.18±3.86 | 5.08±7.14 | 0.62±1.01 | 0.18±0.27 | 0.74±1.11 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Tinctoria</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Trichopoda</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

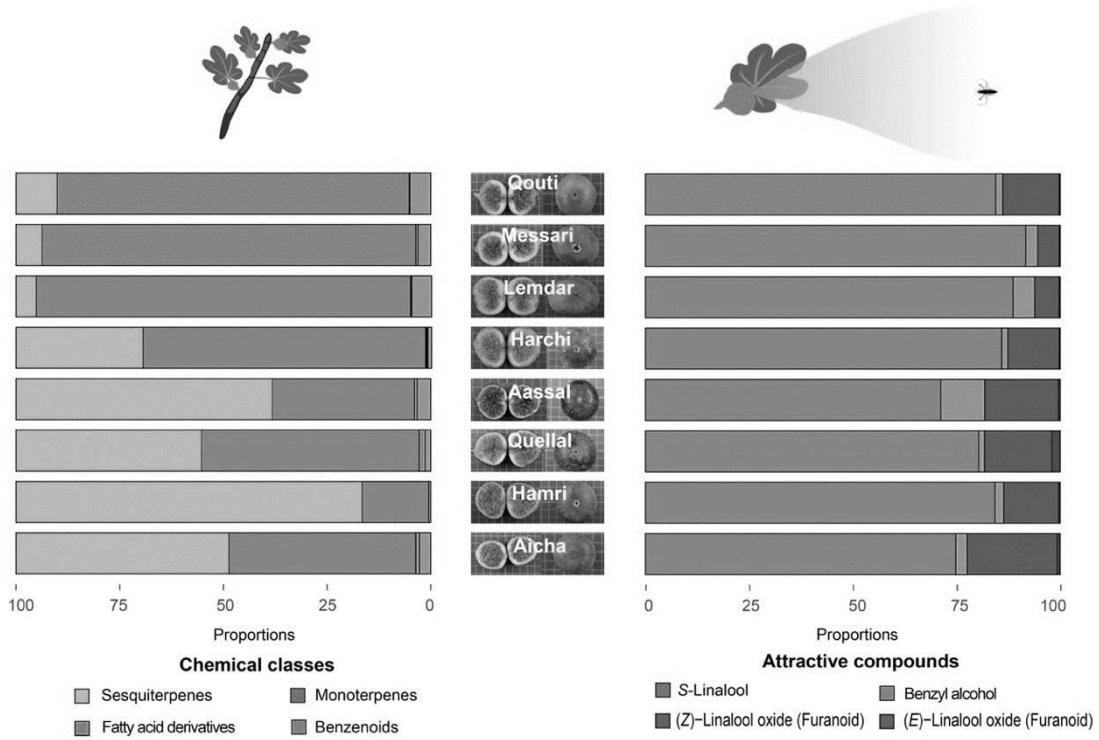
| Compounds Species | bicycloelemene | sesquiterpene2 | sesquiterpene3 | 4_ethylanisole | 4_methylanisole | 4_methyl_benzaldehyde | acetophenone | benzyl acetate | benzaldehyde | benzoic acid | benzyl alcohol | phenylacetonitrile | eugenol | guaiacol |
|-----------------------|----------------|----------------|----------------|----------------|-----------------|-----------------------|--------------|----------------|--------------|--------------|----------------|--------------------|----------|-----------|
| <i>F. Adenosperma</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Asperifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.45±1.39 | 0 | 7.06±9.21 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.41±0.12 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5.5±5.45 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 2.75±2.55 | 0.05±0.05 | 0.01±0.01 | 0 | 0 | 0 | 1.08±2.46 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Citrifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 18.11±23.03 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Erecta</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4.83±5.64 | 3.76±5.82 | 9.72±9.91 | 0 | 2.74±4.1 | 4.52±7.65 |
| <i>F. Exasperata</i> | 0 | 0 | 0 | 1.56±2.79 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fistulosa</i> | 0 | 0 | 0 | 0 | 0 | 0.24±0.35 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fulva</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.15±0.26 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.87±2.47 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hombroniana</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5.71±5.28 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Macrophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.95±3.39 | 21.16±16.67 | 0 | 3.06±2.24 | 0 | 0 | 0 |
| <i>F. Maxima</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.04±3.22 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Mollior</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.01±2.18 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.08±0.63 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0 | 0 | 95.96±3.06 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0 | 0 | 0 | 0 | 0 | 0.31±0.43 | 0 | 0 | 1.45±0.63 | 0 | 0 | 0 | 0 | 1.12±1.38 |
| <i>F. Tinctoria</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0.55±0.61 | 0 | 0.44±0.47 | 2.44±3.15 | 0 | 0 | 0 | 0.97±1.86 |
| <i>F. Trichopoda</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.51±0.51 | 0 | 0 | 0 | 0 | 0.46±0.51 |

| Compounds Species | (Z)_3_hexenyl benzoate | indole | methoxybenzene | methyl benzoate | p_methylbenzyl alcohol | methyl salicylate | o_methylacetophenone | 2_phenylethanol | styrene | veratrole | aromatic 1 | aromatic 5 | aromatic 6 |
|-----------------------|------------------------|-----------|----------------|-----------------|------------------------|-------------------|----------------------|-----------------|-----------|-----------|------------|------------|------------|
| <i>F. Adenosperma</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Asperifolia</i> | 0 | 0 | 0 | 0 | 0 | 8.91±3.45 | 0 | 11.25±11.84 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 | 0.81±0.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Citrifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0 | 0 | 0 | 1.03±0.43 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Erecta</i> | 0 | 0 | 0 | 0 | 0 | 4.45±8.15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Exasperata</i> | 0 | 0.32±0.69 | 0 | 0 | 0 | 0.22±0.29 | 0.64±0.72 | 0 | 0 | 0.43±0.8 | 0 | 0 | 0 |
| <i>F. Fistulosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fulva</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 | 1.09±1.25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 3.46±6.34 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hombroniana</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 0 | 0 | 0.32±0.51 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Macrophylla</i> | 0 | 0 | 1.07±1.37 | 5.49±8.03 | 0.24±0.32 | 0.38±0.55 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Maxima</i> | 0 | 0.66±0.76 | 0 | 0 | 1.68±1.82 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Mollior</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.75±0.9 | 0.94±1.28 | 0.37±0.56 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 0 | 2.37±2.7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 1.09±1.63 | 0 | 0 | 0 | 0 | 0.48±0.62 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 1.16±1.65 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1±1.41 | 1.52±1.9 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 0 | 1.53±1.66 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0 | 0 | 0 | 0 | 0 | 1.54±0.91 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Tinctoria</i> | 0 | 0.14±0.25 | 0 | 0 | 0 | 7.07±6.95 | 0 | 0 | 0.59±0.92 | 0 | 0 | 0 | 0 |
| <i>F. Trichopoda</i> | 0 | 0 | 0 | 0 | 0 | 0.59±0.9 | 0 | 0 | 0 | 1.56±0.81 | 0 | 0 | 0 |

| Compounds Species | carvarol,methyl ether | unknown_1 | unknown_2 | unknown_3 | unknown_4 | unknown_5 | unknown_6 | unknown_7 | unknown_8 | unknown_9 | unknown_10 | unknown_11 | unknown_12 | unknown_13 | unknown_14 |
|-----------------------|-----------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|------------|------------|------------|------------|------------|
| <i>F. Adenosperma</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Asperifolia</i> | 0 | 0.5±0.67 | 2.67±3.4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 0 | 1.98±2.5 | 1.35±2.09 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0.81±1.99 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Citrifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Erecta</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Exasperata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fistulosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0.47±0.94 | 0.87±1.43 | 0.35±0.75 | 0.14±0.25 | 0.5±0.52 | 1.44±1.21 | 0 | 0 | 0 |
| <i>F. Fulva</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.1±0.11 | 0.11±0.1 | 0.16±0.17 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hombroniana</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Macrophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Maxima</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Mollior</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Tinctoria</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Trichopoda</i> | 0 | 0 | 0 | 0 | 0 | 0.44±0.46 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| Compounds Speices | unknown_15 | unknown_16 | unknown_17 | unknown_18 | unknown_19 | unknown_20 | unknown_21 | unknown_22 | unknown_23 | unknown_24 | unknown_25 | unknown_26 | unknown_27 | unknown_28 | unknown_29 |
|-----------------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| <i>F. Adenosperma</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Asperifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Citrifolia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.09±0.08 | 0.23±0.29 | 1.3±0.96 |
| <i>F. Erecta</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Exasperata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fistulosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fulva</i> | 0.1±0.16 | 0.73±1.37 | 0.12±0.28 | 0.03±0.04 | 0.23±0.55 | 0.24±0.25 | 0.24±0.11 | 0.06±0.07 | 0.4±0.07 | 0.23±0.16 | 0.17±0.03 | 0.1±0.11 | 0 | 0 | 0 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hombroniana</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Macrophylla</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Maxima</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Mollior</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Tinctoria</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>F. Trichopoda</i> | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| Compounds Speices | unknown_30 | unknown_31 | unknown_32 | unknown_33 | unknown_34 |
|-----------------------|------------|------------|------------|------------|------------|
| <i>F. Adenosperma</i> | 0 | 0 | 0 | 0 | 0.24±0.22 |
| <i>F. Asperifolia</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Aurea</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Auriculata</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Botryoides</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Burkei</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Carica</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Citrifolia</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Cyrtophylla</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Erecta</i> | 0 | 8.37±10.63 | 0.05±0.1 | 0 | 0 |
| <i>F. Exasperata</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fistulosa</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Fulva</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Glumosa</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hispida</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Hombroniana</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Lutea</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Macrophylla</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Maxima</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Microdyctia</i> | 0 | 0 | 0 | 0 | 3.51±2.76 |
| <i>F. Mollior</i> | 0 | 0 | 0 | 0 | 3.97±3.73 |
| <i>F. Natalensis</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Nota</i> | 0 | 0 | 0 | 2.17±3.88 | 0 |
| <i>F. Oligodon</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Racemosa</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Semicordata</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Septica</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Subulata</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Sycomorus</i> | 0 | 0 | 0 | 0 | 0 |
| <i>F. Tinctoria</i> | 0.6±1.03 | 1.05±1.93 | 0 | 0 | 0 |
| <i>F. Trichopoda</i> | 0 | 0 | 0 | 0 | 0 |



Chapter 2. Floral scent of the Mediterranean fig tree: significant inter-varietal difference but strong conservation of the signal responsible for pollinator attraction

Parfum floral du figuier méditerranéen : une différence inter-variétale significative, mais une forte conservation du signal responsable de l'attraction des pollinisateurs

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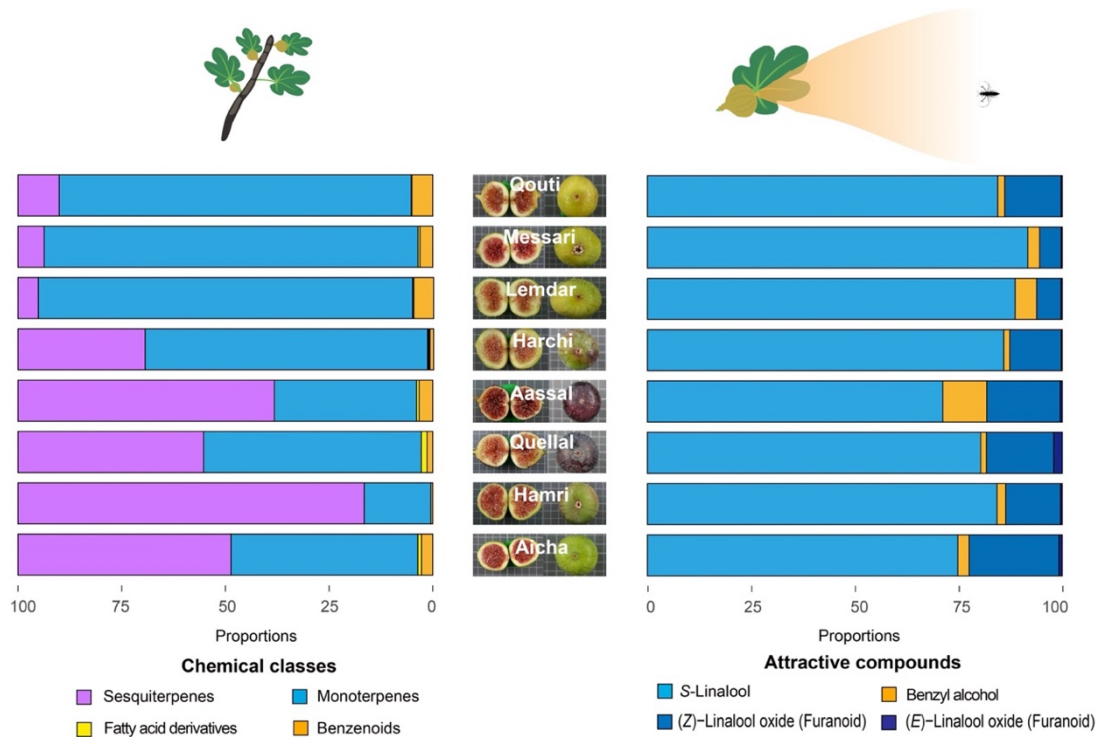
¹ These authors contributed equally to the manuscript

Résumé

Depuis des milliers d'années, l'homme a domestiqué différentes plantes en sélectionnant des caractères particuliers, affectant souvent des caractéristiques moins connues, notamment les composés organiques volatils (COVs) émis par ces plantes à des fins de défense ou de reproduction. Le figuier *Ficus carica* possède une très large gamme de variétés dans la région méditerranéenne et est sélectionné pour ses caractéristiques affectant les fruits, y compris la pollinisation, mais l'effet de la diversification induite par l'homme sur les COVs émis par les figes réceptives pour attirer leur pollinisateur (*Blastophaga psenes*) n'est pas connu. Dans la présente étude, les COVs de figes réceptives de huit variétés du nord du Maroc ont été collectés à différents moments de la période de pollinisation manuelle et analysés par chromatographie en phase gazeuse couplée à la spectrométrie de masse (GC-MS). Des

analyses génétiques utilisant des loci microsatellites ont été réalisées sur les mêmes variétés. Malgré de fortes différences inter-variétales dans la quantité et les proportions relatives de tous les COVs, les proportions relatives des quatre COVs attractifs ont montré une variation limitée entre les variétés. On ne trouve cependant pas de corrélation significative entre les marqueurs génétiques et les profils chimiques des différentes variétés. Alors que la diversification induite par l'homme a conduit à des différences entre les variétés dans les profils de COVs, cet article suggère que tout au long du processus de domestication et de diversification variétale, la sélection stabilisatrice a maintenu un signal fort en faveur de l'attraction des pollinisateurs.



Mots clefs : Figues, Composés Organiques Volatiles, Attraction du pollinisateur, Diversité inter-variétales, Domestication.





OPEN

Floral scent of the Mediterranean fig tree: significant inter-varietal difference but strong conservation of the signal responsible for pollinator attraction

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For thousands of years, humans have domesticated different plants by selecting for particular characters, often affecting less-known traits, including the volatile organic compounds (VOCs) emitted by these plants for defense or reproduction. The fig tree *Ficus carica* has a very wide range of varieties in the Mediterranean region and is selected for its traits affecting fruits, including pollination, but the effect of human-driven diversification on the VOCs emitted by the receptive figs to attract their pollinator (*Blastophaga psenes*) is not known. In the present study, VOCs from receptive figs of eight varieties in northern Morocco, were collected at different times within the manual pollination period and analyzed by gas chromatography-mass spectrometry. Genetic analyses using microsatellite loci were performed on the same varieties. Despite strong inter-varietal differences in the quantity and relative proportions of all VOCs, the relative proportions of the four pollinator-attractive VOCs showed limited variation among varieties. There was no significant correlation between genetic markers and chemical profiles of the different varieties. While diversification driven by humans has led to differences between varieties in VOC profiles, this paper suggests that throughout the process of domestication and varietal diversification, stabilizing selection has maintained a strong signal favoring pollinator attraction.

Domestication is a complex process of selecting and adapting wild plant and animal species for human use. Most of the crops of today were domesticated from their wild ancestors within the past 12,000 years¹. Human practices, through crop domestication and selection of traits, have altered the characteristics of wild ancestors to produce crop plants generally characterized by higher yield, more favorable organoleptic properties, and other characteristics such as their capacity for storage. While these traits have been selected for, others have been lost. For instance, cultivated varieties often have lower defenses and resistance to pests than their wild relatives^{2,3}. There also exist differences in defenses between cultivated varieties of some crop species, such as manioc⁴, sorghum⁵, and oca⁶. Compared to plant defense, inter-varietal comparisons of floral traits related to pollinator attraction are more limited^{7,8}. Nevertheless, generalist pollinators, bees in general, appear to be able to distinguish between crop varieties of the same species and it has been suggested that floral volatile organic compounds (VOCs) play a major role in this choice^{9,10}. As pollination is an essential ecosystem service to crops¹¹, improving our understanding of the evolutionary effects of human-driven varietal diversification on VOCs emitted by flowers involved in plant-pollinator interactions is crucial, particularly with regard to the biodiversity loss we are currently facing.

Domestication may have affected the evolution of the composition of floral VOCs through direct selection of a particular chemical composition, through the indirect effects of selection on other floral traits or on traits of other organs such as fruits, or by genetic drift. In fact, direct selection acts on floral VOCs in many aromatic

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plants cultivated for the perfume industry. For example, the essential oil from the true lavender, *Lavandula angustifolia*, used for perfume contains a lower camphor content than does essential oil from the wild spike lavender, *Lavandula latifolia*, and that from the hybrid lavandin, *Lavandula x intermedia*^{12,13}. On the other hand, in commercial breeding programs, since floral odor is rarely targeted, unlike characteristics such as cold and disease resistance, flower shape, or vase life, floral odor is often missing in modern varieties¹⁴. The cause of the lack of fragrance in these flowers is often unknown. However, there is strong evidence for the existence in some plants of pleiotropic relationships between VOCs and color pigments as they often derive from common biosynthetic precursors¹⁵. For instance, white flowers of some plant species emit higher levels of benzenoid/phenylpropanoid scent compounds than their colored counterparts¹⁶. Moreover, during the domestication of fruit crops, the selection of certain fruit traits related to a particular flavor, color, or odor, probably resulted in the addition, elimination, and/or modification of various specialized metabolic pathways, leading to gain or loss of specialized metabolites¹⁷. These specialized metabolites, such as glycosides or ethylene, but also particular enzymes, play roles in VOC production¹⁸. However, despite the fact that VOCs emitted by different varieties of the same species are generally very diverse in terms of constituents and relative proportion of floral VOCs^{19,20}, there are currently no studies characterizing the effect of human-driven diversification on the emission of VOCs responsible for pollinator attraction.

The Mediterranean fig tree, *Ficus carica* (Moraceae), represents one of the most appropriate biological models to explore this question. It is one of the emblematic trees of the Mediterranean region, and was domesticated around 5500 years ago²¹. Like other *Ficus* spp., trees of *F. carica* bear figs that are enclosed inflorescences termed 'syconia'. The species is dioecious. Male trees bear figs that produce pollen and in which the specific pollinator, the fig wasp *Blastophaga psenes* (Hymenoptera: Agaonidae), develops. Female trees bear figs in which only seeds develop, but not pollinators²². Attraction of female *B. psenes* by VOCs emitted by receptive figs of male and female trees has already been well described²³. In the Mediterranean region, a wide diversity of varieties of female fig trees (several hundred) have been selected. Comparatively, the classification of male fig trees into varieties is still debated. Traits of interest for human consumption in female figs that have been selected from wild figs are mainly based on fruit characteristics, including size, color, sweetness, softness, drying capacities linked to trade and storage, but also phenology, with early to late varieties in order to obtain fruits over the longest period possible. The traits selected over the centuries are also related to the type of fruit production: in some varieties the classical mutualistic interaction with the fig wasps has been maintained to produce fruit, but other varieties have been selected to produce parthenocarpic (seedless) figs that do not require pollination for fruit production²⁴. In addition, the dioecious nature of figs and their manual pollination, termed caprifigation, have both been long well-known by Mediterranean farmers²⁵. In fig cultivation, to ensure this manual pollination, male figs are traditionally either cultivated separately from the female figs (e.g. in Turkey, for the Smyrna figs) or are collected from wild trees, as in Morocco. Then, to facilitate pollination of female figs, farmers bring mature male figs, when the pollinators are ready to emerge, to the specific female fig varieties when they are receptive, ready to be pollinated. To date, no study has examined inter-varietal variations in VOCs emitted by the receptive figs.

In Morocco, the Rif region shows the highest level of varietal diversity of female fig trees^{26,27}. Farmers in this region differentiate more than a hundred fig varieties, propagated by cuttings of stems²⁷. Fig orchards are polyvarietal with a mean of six varieties per orchard (Hmimsa, unpublished data). In each orchard, the set of varieties present is based on complex trait preferences, with haphazard arrangements of varieties²⁷. Among these traits, farmers favor varieties with different but partially overlapping receptive phases²⁸. They use manual pollination to assist reproduction of female varieties that are not parthenocarpic. Even if individual tree of *F. carica* bears figs that are on the whole at the same stage of development, some small differences among figs on the same tree exist. Thus, manual pollination starts for each variety when the farmers consider that enough figs per tree are receptive, the most experienced ones referring to a change in odor as a signal for them to search for male figs²⁸, and takes place several times for each tree of each variety. However, the variation in VOC emission from receptive figs along this manual pollination period is not known.

In the present study, we investigated the VOCs of receptive figs of eight non-parthenocarpic fig varieties in the Rif region in northern Morocco, in order to determine if their emission varied qualitatively but also quantitatively, particularly those responsible for pollinator attraction. Previous studies have shown that only four of the VOCs emitted by receptive figs (i.e. S-linalool, benzyl alcohol, (Z)-linalool-oxide (furanoid), and (E)-linalool-oxide (furanoid)), within a narrow range of proportions, are responsible for attraction of the specific fig wasp pollinator and that even a very small change in their proportions disrupts pollinator attraction²³. Based on these results, we hypothesized that the proportions of the VOCs responsible for pollinator attraction should be highly conserved among the different varieties at receptivity, in contrast to the VOCs that play no role in pollinator attraction, as they are not affected by such stabilizing selection. As farmers decide to pollinate trees of different varieties at different times during the fig receptive season, we also hypothesized that the emission of the four attractive VOCs will show different patterns of variation between varieties during the manual pollination period, with each variety having a peak emission at the time it is pollinated. In order to test our hypothesis, we compared the VOCs collected from receptive figs of three (the first year) and six (the second year) individual trees belonging to each of eight varieties during two successive years. These varieties were among the most frequently planted in the region. In parallel, we performed a genetic comparison of the different varieties using 15 microsatellite loci to determine genetic distances. In addition, during the second year we collected and analyzed the VOCs of receptive figs of the same six trees per variety during the whole period of manual pollination at three different times during the manual pollination period: at the beginning, middle and end of this period (Fig. 1). We addressed the following questions: (1) Are there differences among varieties in the VOC composition of figs at receptive stage? (2) Is there a correlation between genetic and chemical distances in these eight varieties? (3) Over the entire manual pollination period of the figs, is there qualitative and quantitative variation in VOCs

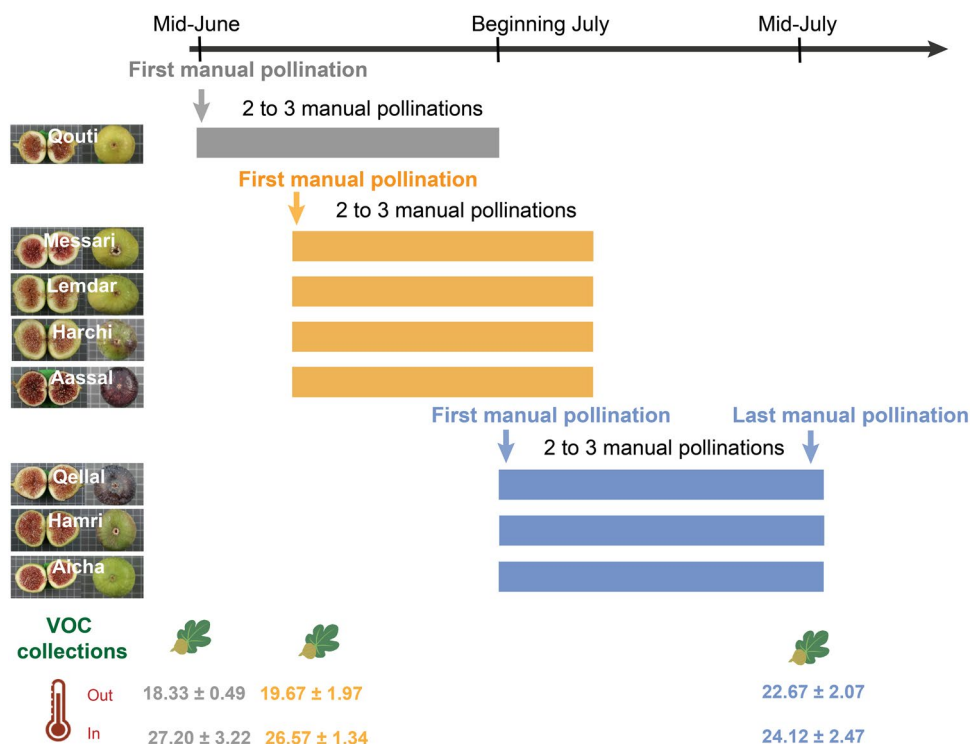


Figure 1. Diagram showing the timing of manual pollination and of collection of volatile organic compounds (VOCs) from receptive figs for the eight varieties of *Ficus carica* studied. The time of the year is indicated by the black arrow at the top of the diagram. For each variety, the first manual pollination time is indicated and each individual tree was pollinated two to three more times over a period of two to three weeks. Varieties with the same color (grey: Beginning; yellow: Middle; blue: End) experienced the first manual pollination at the same time. Three VOC collection sections of the same six individual trees per variety were made (indicated by the drawing of the figs). For each of these sections, the mean (\pm standard errors) per individual tree of the temperatures inside (in) and outside (out) the bag containing the figs are presented in degrees Celsius.

emitted by receptive figs? (4) Is the time of manual pollination of each variety associated with the peak emission of VOCs attractive to pollinators?

Results

In total, we detected 46 VOCs and identified 35 emitted by the receptive figs of the eight varieties and during the two successive years. Receptive figs emitted floral scents mainly composed of four different chemical classes: monoterpenes, sesquiterpenes, fatty acid derivatives and benzenoids (Table 1). The blend of compounds was always dominated by terpene compounds (> 90%) in all varieties. The varieties Qouti, Lemdar and Messari released high amounts of monoterpenes (> 80%), whereas varieties Aassal, Aicha, Hamri and Qellal emitted higher amounts of sesquiterpenes (> 40%), and the variety Harchi was intermediate (monoterpenes > 60%, sesquiterpenes > 30%) (Table 1, Supplementary Fig. S1). In all varieties, the form of linalool detected was exclusively S-linalool. The temperature outside and inside the bag showed limited variation in both years (Fig. 1) and in all analyses, bag temperature had no significant effect on the estimated quantity or relative proportions ($p > 0.05$ for all tests).

Comparison among varieties of the VOCs emitted by receptive figs. Variety, year and their interaction all had significant effects on the total estimated quantity of all VOCs (respectively $F_{7,70} = 4.63$, $p < 0.001$; $F_{1,70} = 11.19$, $p < 0.001$; $F_{7,70} = 2.25$, $p < 0.05$, Fig. 2a). Similarly, variety and its interaction with year had significant effects on the estimated quantity of the four attractive VOCs ($F_{7,70} = 8.49$, $p < 0.001$; $F_{7,70} = 2.73$, $p < 0.05$) (Fig. 2b), but year had no significant effect on these quantities ($F_{1,70} = 3.62$, $p = 0.06$, Fig. 2b). For all VOCs, receptive figs of Messari emitted significantly higher quantities of VOCs than did those of the other varieties (pairwise comparisons $p < 0.05$ for all tests) except Lemdar ($p = 0.21$), and Lemdar emitted significantly higher quantities of VOCs than Aicha (pairwise comparison $p < 0.05$) (Fig. 2a). For the dataset on the four pollinator-attractive VOCs, receptive figs of the varieties Aassal, Hamri, Qellal and Aicha emitted significantly lower quantities than those of Messari and Lemdar (pairwise comparisons $p < 0.05$ for all tests), and Harchi emitted similar quantities as Lemdar and Qouti but lower quantities than Messari (pairwise comparisons $p < 0.05$) (Fig. 2b).

In addition, the relative proportions of VOCs varied significantly among the varieties, between years, and with their interaction for all emitted VOCs (respectively $F_{7,70} = 8.87$, $p < 0.001$; $F_{1,70} = 5.93$, $p < 0.001$; $F_{7,70} = 2.02$,

| | RI | Qouti N=9 | Messari N=8 | Lemdar N=9 | Harchi N=9 | Aassal N=9 | Qellal N=9 | Hamri N=9 | Aicha N=9 |
|----------------------------------------|------|--------------|----------------|---------------|---------------|---------------|---------------|--------------|--------------|
| Benzenoids | | | | | | | | | |
| Benzaldehyde ^a | 964 | 3.66±3.07 | 0.57±0.83 | 0 | 0.11±0.33 | 0.01±0.02 | 0.5±0.84 | 0.12±0.17 | 1.6±1.75 |
| Benzyl alcohol* ^a | 1036 | 1.41±1.15 | 2.51±2.43 | 4.79±3.9 | 0.79±1.12 | 3.28±2.68 | 0.88±0.99 | 0.34±0.71 | 1.13±1.54 |
| Total percent | | 5.07 | 3.08 | 4.79 | 0.9 | 3.29 | 1.38 | 0.46 | 2.73 |
| Fatty acid derivatives | | | | | | | | | |
| (Z)-Hex-3-enol | 865 | 0.1±0.14 | 0.41±0.51 | 0.07±0.12 | 0.08±0.1 | 0.57±1.25 | 1.23±2.19 | 0 | 0.44±0.9 |
| (Z)-Hex-3-enyl acetate ^a | 1006 | 0.02±0.03 | 0.08±0.11 | 0.02±0.02 | 0.03±0.05 | 0.06±0.09 | 0.16±0.3 | 0 | 0.45±1.2 |
| Total percent | | 0.12 | 0.5 | 0.09 | 0.11 | 0.63 | 1.39 | 0.00 | 0.89 |
| Monoterpenes | | | | | | | | | |
| α-Pinene ^a | 933 | 0.01±0.01 | 0.01±0.03 | 0.01±0.01 | 0.05±0.04 | 0.02±0.03 | 0.17±0.34 | 0.1±0.11 | 0.1±0.1 |
| Sabinene | 986 | 0.06±0.06 | 0.02±0.03 | 0.03±0.06 | 0.15±0.16 | 0.05±0.12 | 0.08±0.1 | 0.18±0.21 | 0.01±0.04 |
| Myrcene ^a | 993 | 0.72±1.55 | 0.78±0.94 | 0.32±0.21 | 0.48±0.37 | 0.28±0.35 | 0.92±1.42 | 0.02±0.04 | 0.73±1.62 |
| Limonene ^a | 1030 | 1.2±1.48 | 0.77±0.74 | 0.49±0.42 | 1.57±1.44 | 1.13±0.59 | 3.12±2.98 | 3.14±3.7 | 3.18±1.56 |
| (Z)-β-Ocimene ^a | 1039 | 1.14±0.93 | 1.34±1.16 | 1.31±1.43 | 0.96±0.62 | 1.49±2.11 | 1.45±1.33 | 0.18±0.23 | 1.24±1.19 |
| (E)-β-Ocimene ^a | 1049 | 0.52±1.33 | 0.47±0.7 | 0.13±0.08 | 0.11±0.11 | 0.21±0.31 | 0.62±1.01 | 0.32±0.25 | 1.06±1.29 |
| γ-Terpinen ^a | 1061 | 0.12±0.14 | 0.04±0.06 | 0.01±0.02 | 0.2±0.14 | 0.21±0.12 | 0.25±0.48 | 0.12±0.12 | 0.16±0.15 |
| (Z)-Linalool oxide (Fur.) ^a | 1074 | 9.94±4.58 | 4.34±2.81 | 5.26±5.32 | 7.27±5.48 | 6.03±6.81 | 6.87±6.08 | 2.04±3.72 | 7.19±6.2 |
| (E)-Linalool oxide (Fur.) ^a | 1090 | 0.2±0.13 | 0.22±0.16 | 0.27±0.16 | 0.16±0.13 | 0.15±0.15 | 0.73±0.65 | 0.05±0.07 | 0.26±0.22 |
| S-Linalool ^a | 1100 | 69.26±18.97 | 81.08±10.99 | 81.44±10.93 | 55.71±27.16 | 23.54±24.18 | 36.81±24.46 | 9.67±7.31 | 30.21±25.05 |
| allo-Ocimene ^a | 1131 | 0.41±0.39 | 0.52±0.5 | 0.5±0.33 | 0.48±0.5 | 0.38±0.42 | 0.39±0.32 | 0.05±0.09 | 0.34±0.53 |
| neo-allo-Ocimene | 1144 | 0.05±0.13 | 0.02±0.04 | 0.01±0.01 | 0.02±0.03 | 0 | 0.01±0.02 | 0 | 0.01±0.04 |
| (Z)-Linalool oxide (Pyr.) | 1173 | 0.87±0.85 | 0.4±0.23 | 0.32±0.26 | 0.99±0.78 | 0.62±0.57 | 0.72±0.7 | 0.15±0.19 | 0.41±0.21 |
| (E)-Linalool oxide (Pyr.) | 1177 | 0.16±0.19 | 0.11±0.08 | 0.04±0.02 | 0.1±0.06 | 0.11±0.09 | 0.33±0.26 | 0.03±0.02 | 0.11±0.06 |
| Unknown 1 | 1213 | 0.22±0.19 | 0.01±0.03 | 0.06±0.13 | 0.07±0.1 | 0.08±0.09 | 0.04±0.12 | 0.01 | 0 |
| Total percent | | 84.88 | 90.14 | 90.2 | 68.32 | 34.29 | 52.49 | 16.07 | 45.02 |
| Sesquiterpenes | | | | | | | | | |
| δ-Elementene ^a | 1228 | 0 | 0±0.01 | 0.01±0.01 | 0.02±0.03 | 0.07±0.07 | 0.26±0.3 | 0.02±0.04 | 0.09±0.25 |
| Unknown 2 | 1345 | 0 | 0 | 0.02±0.03 | 0.02±0.05 | 0.69±0.47 | 2.01±2.23 | 0.01±0.03 | 0.12±0.36 |
| α-Cubebene | 1358 | 0 | 0 | 0.01±0.02 | 0.34±0.31 | 0.09±0.13 | 0.14±0.19 | 0.83±0.15 | 0.44±0.36 |
| Isoleudene | 1381 | 0.04±0.08 | 0.01 | 0.01±0.02 | 0.07±0.1 | 0.07±0.08 | 0 | 0.11±0.11 | 0.14±0.37 |
| α-Copaene ^a | 1386 | 1.02±0.93 | 1.02±1.17 | 0.25±0.25 | 7.5±6.05 | 2.6±1.33 | 5.03±3.2 | 13.05±2.15 | 11.67±7.56 |
| Daucene | 1389 | 0 | 0 | 0.03±0.05 | 0.01±0.03 | 0.04±0.08 | 0.1±0.2 | 0 | 0 |
| β-Bourbonene | 1395 | 0.29±0.33 | 0.17±0.21 | 0.29±0.34 | 1±1.06 | 1.89±1.17 | 0.67±0.57 | 2.03±0.68 | 1.84±1.05 |
| Unknown 3 | 1398 | 0.02±0.06 | 0.02±0.05 | 0.02±0.03 | 0.34±0.36 | 0.48±0.56 | 0 | 0.89±0.37 | 0.41±0.37 |
| Cycloseychellene | 1409 | 0.17±0.32 | 0.06±0.1 | 0 | 0.2±0.39 | 0.1±0.15 | 0.56±0.67 | 0.65±0.38 | 0.53±0.65 |
| Unknown 4 | 1420 | 1.35±2.11 | 0.53±0.59 | 0.18±0.19 | 1.51±1.64 | 1.17±0.76 | 4.88±3.81 | 4.37±0.89 | 4.27±3.11 |
| β-Caryophyllene ^a | 1429 | 0.49±0.68 | 0.31±0.36 | 1±1.05 | 2.41±2.41 | 11.61±7.41 | 1.32±1.31 | 6.77±1.46 | 2.18±1.42 |
| Unknown 5 | 1437 | 0.1±0.16 | 0.06±0.1 | 0 | 0.27±0.4 | 0.11±0.09 | 0.74±0.58 | 1.16±0.66 | 0.87±0.81 |
| Muurolo-3.5-diene | 1441 | 0.51±0.77 | 0.35±0.42 | 0.53±0.58 | 1.92±1.89 | 6.4±4.37 | 2.12±1.72 | 5.73±1.19 | 2.38±1.36 |
| Himachalene | 1444 | 0.18±0.2 | 0.14±0.13 | 0.18±0.23 | 0.3±0.3 | 0.77±0.58 | 0.51±0.61 | 0.78±0.24 | 0.42±0.45 |
| Geranyl acetone ^a | 1453 | 0.2±0.31 | 0.07±0.09 | 0.08±0.09 | 0.2±0.17 | 0.07±0.17 | 0.69±0.97 | 0.37±0.39 | 0.54±0.44 |
| (Z)-Cadina-1(6),4-diene | 1456 | 0.11±0.18 | 0.09±0.11 | 0.19±0.23 | 0.58±0.61 | 2.42±1.62 | 0.33±0.39 | 1.72±0.44 | 0.55±0.4 |
| Unknown 6 | 1465 | 3.72±4.79 | 2.29±2.72 | 0.3±0.52 | 6.39±7.74 | 9.07±5.02 | 17.38±13.9 | 22.93±4.96 | 13.14±7.86 |
| Unknown 7 | 1470 | 0.31±0.4 | 0.1±0.16 | 0 | 0.99±2.16 | 0.22±0.25 | 1.09±1.3 | 1.49±0.73 | 0.72±0.83 |
| γ-Muuroloene | 1486 | 0.06±0.16 | 0.04±0.08 | 0.03±0.05 | 0.12±0.19 | 0.31±0.23 | 0.21±0.42 | 0.46±0.4 | 0.53±0.96 |
| Germacrene D ^a | 1492 | 0.83±0.93 | 0.67±0.86 | 1.63±1.84 | 5.09±4.58 | 20.03±13.53 | 2.38±2.35 | 15.5±6.86 | 7.42±8.48 |
| Bicyclogerma-crene | 1508 | 0.06±0.14 | 0.07±0.11 | 0.1±0.17 | 0.53±0.65 | 2.48±1.85 | 2.21±2.21 | 1.47±0.88 | 0.47±0.55 |
| Unknown 8 | 1516 | 0.14±0.23 | 0.02±0.05 | 0.01±0.02 | 0.1±0.09 | 0.09±0.08 | 0.43±0.37 | 0.43±0.21 | 0.38±0.31 |
| Unknown 9 | 1522 | 0.01±0.04 | 0.01±0.03 | 0 | 0.03±0.05 | 0 | 0.15±0.33 | 0.45±0.93 | 0.13±0.21 |
| Unknown 10 | 1524 | 0.23±0.26 | 0.15±0.2 | 0±0.01 | 0.23±0.35 | 0.39±0.37 | 0.5±0.48 | 0.85±0.65 | 0.61±0.76 |
| Continued | | | | | | | | | |

| | RI | Qouti N=9 | Messari N=8 | Lemdar N=9 | Harchi N=9 | Aassal N=9 | Qellal N=9 | Hamri N=9 | Aicha N=9 |
|----------------------|------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| δ -Cadinene | 1532 | 0.01 \pm 0.02 | 0.03 \pm 0.03 | 0.02 \pm 0.04 | 0.38 \pm 0.35 | 0.53 \pm 0.44 | 0.92 \pm 1.07 | 1.32 \pm 0.81 | 1.06 \pm 1.09 |
| α -Calacorene | 1553 | 0.06 \pm 0.18 | 0.02 \pm 0.05 | 0.01 \pm 0.02 | 0.06 \pm 0.13 | 0.05 \pm 0.06 | 0 | 0.06 \pm 0.11 | 0.32 \pm 0.79 |
| β -Calacorene | 1568 | 0.02 \pm 0.02 | 0.03 \pm 0.06 | 0.01 \pm 0.03 | 0.06 \pm 0.09 | 0.04 \pm 0.08 | 0.1 \pm 0.18 | 0.02 \pm 0.04 | 0.15 \pm 0.18 |
| Total percent | | 9.93 | 6.28 | 4.92 | 30.67 | 61.8 | 44.74 | 83.47 | 51.37 |

Table 1. Relative proportions of all volatile organic compounds (VOCs) emitted by receptive figs of the eight varieties of *Ficus carica*. Compounds detected are divided into four groups based on general biosynthetic origin. Within each group, compounds are listed in order of their retention index (RI). For each variety, the mean (\pm standard errors) of percentages of the VOCs per individual are presented. N, number of individual trees used for scent extraction. *Pollinator-attractive VOCs. Fur. Furanoid, Pyr. Pyranoid. ^aidentified with synthetic reference. The total percentage of each chemical group for each variety is indicated in bold.

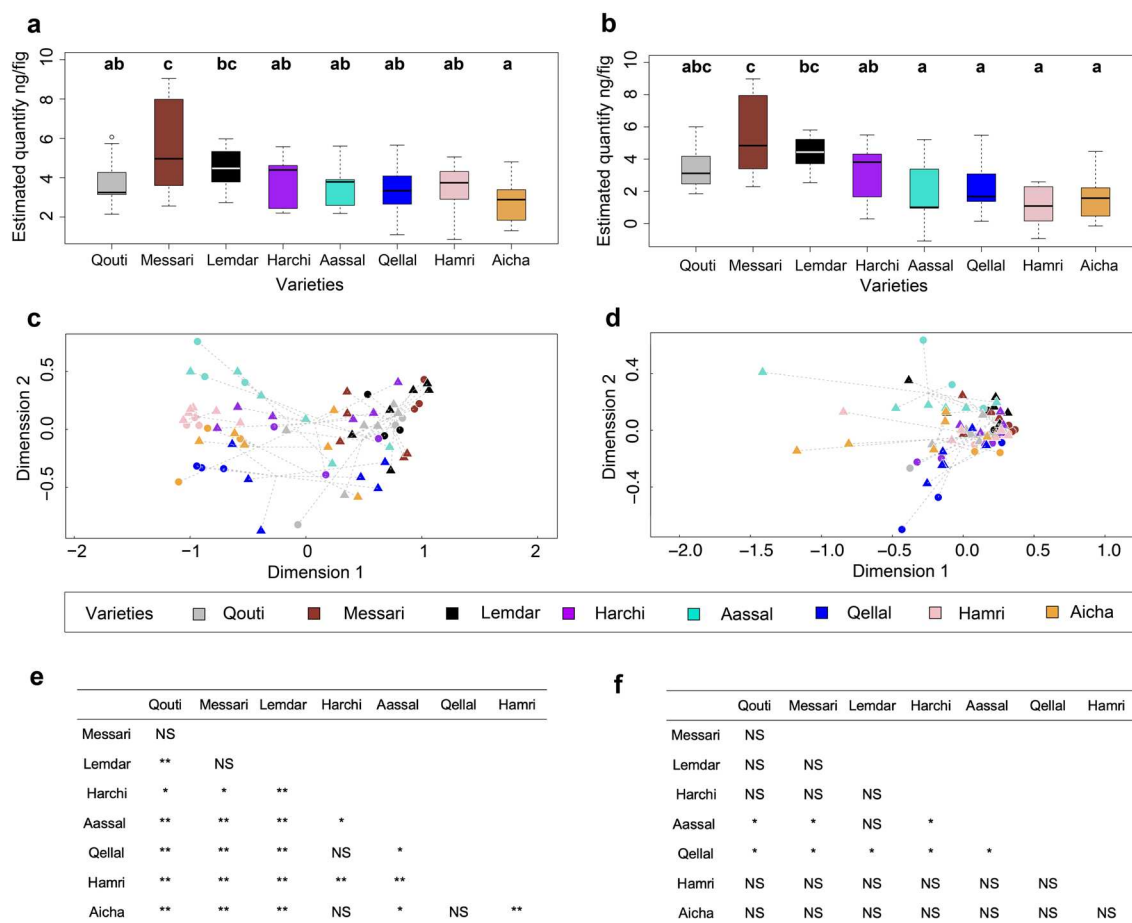


Figure 2. Comparison among the eight varieties of *Ficus carica* of the total estimated quantities and relative proportions of volatile organic compounds (VOCs) emitted by receptive figs. The box plot of estimated total quantities (log-transformed) of all VOCs (a) and of the four pollinator-attractive VOCs (b) are presented, together with the results of pairwise comparisons of estimated total quantities within each variety using least-squares means comparisons. Different letters indicate statistically significant differences. Non-metric multidimensional scaling ordination (NMSDS) of the relative proportions based on Bray–Curtis distance are presented for all VOCs (c, Stress=0.113) and for the four pollinator-attractive VOCs (d, Stress=0.066) (circle: 2017, triangle: 2018). In addition, results of pairwise comparisons of the relative proportions using PERMANOVA are presented for all VOCs (e) and for the four VOCs responsible for pollinator attraction (f). $p < 0.01$: ** $p < 0.05$: * $p > 0.05$: NS.

$p < 0.001$, Fig. 2c). For the four attractive VOCs, whereas relative proportions varied significantly among varieties and between years, there was no significant effect of the interaction of these two factors (respectively $F_{7,70} = 4.23$, $p < 0.001$; $F_{1,70} = 4.63$, $p < 0.05$; $F_{7,70} = 1.21$, $p = 0.28$, Fig. 2d). For all VOCs emitted, the volatile profiles of receptive figs were significantly different, except between: Aicha, Harchi and Qellal; Harchi and Qellal; and Messari,

Qouti and Lemdar (Fig. 2e). In contrast, for the four attractive VOCs, most varieties had a similar chemical profile, except for Aassal, whose profile was significantly different from those of half of the varieties; and Qellal, whose profile was different from those of all varieties except Hamri and Aicha (Fig. 2f) (Supplementary Fig. S2).

Comparison among varieties between chemical and genetic distances. Analysis of 80 fig trees (10 per variety) using 15 SSR loci revealed 62 distinct SSR profiles. A total of 53 alleles were detected, with an average of 3.5 alleles per locus (Supplementary Table S1). The highest number of alleles (seven alleles) was detected at the FSYC01 locus, while the lowest number (two alleles) was obtained at five loci (MFC11, MFC8, LMFC26, MFC4 and LMFC19). The size of the alleles varied from 96 bp at the MFC3 locus to 332 at the LMFC19 locus (Supplementary Table S1).

Regarding intra-variety genetic diversity (Table 2), the level of polymorphism revealed by the 15 SSR markers was relatively high, with an average polymorphism index (P) of the order of 0.66 ± 0.07 . Thus, the allelic richness per variety (A_p) 30 (Lemdar) with an average value of genotypes per variety (N_{GP}) of around 20.25 ± 2.66 and average alleles per locus (N_A) of the order of 1.83 ± 0.13 . Expected heterozygosity (H_E) varied from 0.25 (Hamri) to 0.35 (Qellal and Aassal), with an average value of 0.30 ± 0.04 . However, the observed heterozygosity (H_o) varied between 0.35 (Hamri) and 0.59 (Aassal), with an average value of 0.49 ± 0.1 . For all the varieties, the F_{is} value was negative, indicating an excess of heterozygotes. The genetic distances of Nei calculated from mean values of the allele frequencies were used to produce a hierarchical classification (Fig. 3). The dendrogram groups together six main groups at the threshold of 0.45. These results reflect a significant level of diversity within a relatively restricted geographical area.

The genetic and chemical dendrograms using respectively the allele frequencies at 15 microsatellite loci and the mean relative proportions of all VOCs (from 2017 and the middle of the manual pollination period of 2018) are presented in Fig. 3. We found no significant correlation (Mantel statistic $r = -0.0731$, $p = 0.609$) between genetic and chemical distances in the eight varieties in *F. carica*.

Variation of VOC emission along the manual pollination period in the different varieties. Despite the fact that an individual tree of *F. carica* bears figs that are on the whole at the same stage of development, there are small differences among figs on the same tree. This is why in the present study we

| | P | N_A | A_p | N_{GP} | H_E | H_O | F_{is} | p value |
|--------------------|----------|----------------------|----------------------|-----------------------|----------------------|----------------------|-----------------------|-------------------|
| Qouti | 0.67 | 1.87 | 28.00 | 18.00 | 0.32 | 0.55 | - 0.68 | < 0.001 |
| Messari | 0.53 | 1.60 | 24.00 | 18.00 | 0.26 | 0.45 | - 0.72 | < 0.001 |
| Lemdar | 0.73 | 2.00 | 30.00 | 25.00 | 0.27 | 0.36 | - 0.30 | < 0.001 |
| Harchi | 0.60 | 1.87 | 28.00 | 21.00 | 0.30 | 0.48 | - 0.57 | < 0.001 |
| Aassal | 0.67 | 1.87 | 28.00 | 18.00 | 0.35 | 0.59 | - 0.68 | < 0.001 |
| Qellal | 0.73 | 1.93 | 29.00 | 22.00 | 0.35 | 0.56 | - 0.61 | < 0.001 |
| Hamri | 0.65 | 1.74 | 26.00 | 22.00 | 0.25 | 0.35 | - 0.34 | < 0.001 |
| Aicha | 0.67 | 1.73 | 26.00 | 18.00 | 0.33 | 0.57 | - 0.70 | < 0.001 |
| Mean | 0.66 | 1.83 | 27.38 | 20.25 | 0.30 | 0.49 | - 0.58 | < 0.001 |
| Standard deviation | 0.07 | 0.13 | 1.92 | 2.66 | 0.04 | 0.10 | 0.17 | < 0.001 |

Table 2. Genetic diversity for the eight varieties of *Ficus carica* based on 15 microsatellite markers. Polymorphism information content (P), average number of alleles per locus (N_A), allelic richness (A_p), range size of alleles by variety, number of genotype (N_{GP}), expected heterozygosity (H_E), observed heterozygosity (H_o) and Wright's fixation indices (F_{is}) are presented. Significant p values ($p < 0.05$) are indicated in bold.

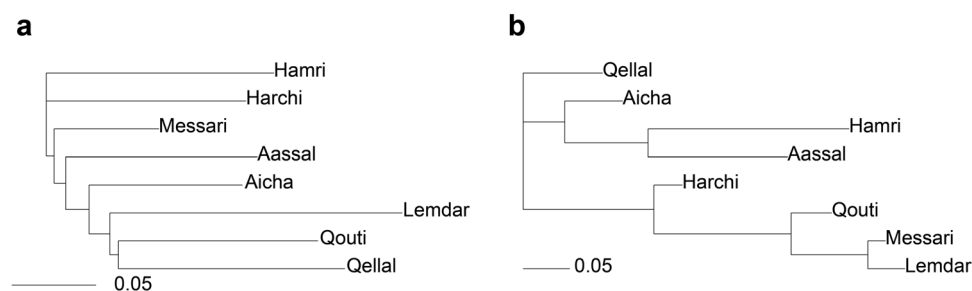


Figure 3. Genetic (a) and chemical (b) dendrograms. Independent matrices coding genetic distances between varieties were calculated from allele frequency data by neighbor-joining based on Nei distance. Chemical dendrogram was created by using neighbor joining based on the Bray-Curtis distance matrix for the averaged relative proportions of VOCs per variety.

managed to collect receptive figs for all varieties at three different times during the whole period of manual pollination (Fig. 1).

The estimated quantity of all VOCs varied significantly among the different times of manual pollination (Fig. 1) for the early and middle-pollinated varieties except Aassal ($p < 0.05$), but not for the three late-pollinated varieties (Table 3). For the four varieties with fluctuating quantities, in pairwise comparisons, the estimated quantity was smaller at the beginning compared to the middle and the end of the manual pollination (Fig. 4a). In addition, figs of the variety Messari released higher quantities of all VOCs at the end of the manual pollination period than at the beginning and the middle of the manual pollination period (Fig. 4a).

The total estimated quantity of the four attractive VOCs varied significantly among the different times of the manual pollination period for all varieties except Aassal (Table 3, $p < 0.05$). In pairwise comparisons, in most varieties, figs emitted lower quantities of these four VOCs at the beginning compared to the middle and end of pollination (Fig. 4b). For these two last times, the quantity emitted was similar in all varieties except for Hamri and Messari, for which the quantity at the end was higher than in the middle. For the varieties Qellal and Qouti, the quantity of VOCs emitted at the middle of the pollination period was higher than at the beginning and end.

Regarding the relative proportions of all VOCs, manual pollination time had a significant effect for all varieties (Table 3, PERMANOVA, $p < 0.05$). In pairwise comparisons, different patterns of variation among varieties were found but generally the relative proportions at the beginning of manual pollination were significantly different from those in the other times, except for Aassal, Qellal and Qouti (Supplementary Fig. S4). For Aassal, there was no significant difference among the three times. For Qellal, the middle period was only significantly different from the beginning. For Qouti, the middle period was significantly different from the two other periods.

Manual pollination time had a significant effect on the relative proportions of the four chemical classes (benzenoid, fatty acid, monoterpene and sesquiterpene biosynthetic pathways) for all varieties (Supplementary Table S2, $p < 0.05$), with pairwise comparisons showing different patterns of variation among varieties (Supplementary Fig. S5).

Relative proportions of the four attractive VOCs collected at different time during the manual pollination season were significantly different for all varieties except Aassal and Aicha (Table 3, $p < 0.05$, Fig. 5). For the six other varieties, pairwise comparisons showed that proportions of these four VOCs at the beginning of the manual pollination period were significantly different from those in the other times, except for Hamri, where proportions at the beginning were significantly different only from those at the end, and for Qouti, where proportions at the middle were significantly different from those in the other times (Fig. 5). For Harchi, differences in the proportions of these VOCs were different among all three times (Fig. 5d).

Discussion

In the present study, through the characterization of the volatile profiles of the receptive figs of eight *F. carica* varieties from northern Morocco, we showed substantial inter-varietal differences. Some varieties (Qouti, Lemdar and Messari) release high amounts of monoterpenes, while others (Aassal, Aicha, Hamri and Qellal) emit high amounts of sesquiterpenes, and one variety is intermediate (Harchi). In addition, we found inter-varietal variations in total amounts and relative proportions of VOCs, as well as in temporal patterns of VOC emission. However, despite these inter-varietal differences, the chemical signal responsible for pollinator attraction is highly conserved between varieties.

The dioecious Mediterranean fig tree, *F. carica*, has a particular phenology with generally male trees flowering twice a year, in spring and summer, and female trees flowering only once a year, in summer, and therefore partially synchronously with males in summer. Previous studies have described the VOCs emitted by receptive figs of wild female and male trees of *F. carica*^{23,29,30}, but not those emitted by cultivated varieties. These studies reported that the relative proportions of all VOCs emitted by receptive male figs in spring differed significantly

| Varieties | Total estimated quantity | | | | | Relative proportion | | | | |
|-----------|--------------------------|----------|----------------|-----------|----------------|---------------------|----------|----------------|-----------|----------------|
| | Df | All VOCs | | Four VOCs | | Df | All VOCs | | Four VOCs | |
| | | F | <i>p</i> value | F | <i>p</i> value | | F | <i>p</i> value | F | <i>p</i> value |
| Qouti | 2, 14 | 9.11 | <0.01 | 5.60 | 0.02 | 2, 17 | 2.99 | 0.01 | 4.29 | 0.02 |
| Messari | 2, 13 | 10.28 | <0.01 | 11.35 | <0.01 | 2, 16 | 3.79 | <0.001 | 6.75 | <0.001 |
| Lemdar | 2, 14 | 33.25 | <0.001 | 148.36 | <0.001 | 2, 17 | 13.58 | <0.001 | 9.84 | <0.001 |
| Harchi | 2, 14 | 5.33 | 0.02 | 17.82 | <0.01 | 2, 17 | 4.7 | <0.001 | 10.72 | <0.001 |
| Aassal | 2, 14 | 1.46 | 0.27 | 2.72 | 0.1 | 2, 17 | 2.29 | 0.03 | 2.26 | 0.1 |
| Qellal | 2, 14 | 1.76 | 0.21 | 9.43 | <0.01 | 2, 17 | 5.48 | <0.001 | 11.78 | <0.001 |
| Hamri | 2, 14 | 1.30 | 0.3 | 42.73 | <0.001 | 2, 17 | 4.17 | <0.001 | 3.06 | 0.04 |
| Aicha | 2, 13 | 2.99 | 0.09 | 10.36 | <0.01 | 2, 16 | 3.52 | 0.01 | 1.13 | 0.35 |

Table 3. Global results of the comparison among pollination periods of the total estimated quantity and on the relative proportions, of all volatile organic compounds (VOCs) and of the four pollinator-attractive VOCs emitted by figs of each variety. For the estimated quantity, analysis of deviance was performed on a linear regression model of VOCs (log-transformation). For the relative proportions, permutational multivariate analysis of variance (PERMANOVA) performed on proportions of VOCs (standardization prior to the analysis). Degrees of freedom (Df) and results of the test (F) are indicated. Significant *p* values ($p < 0.05$) are indicated in bold.

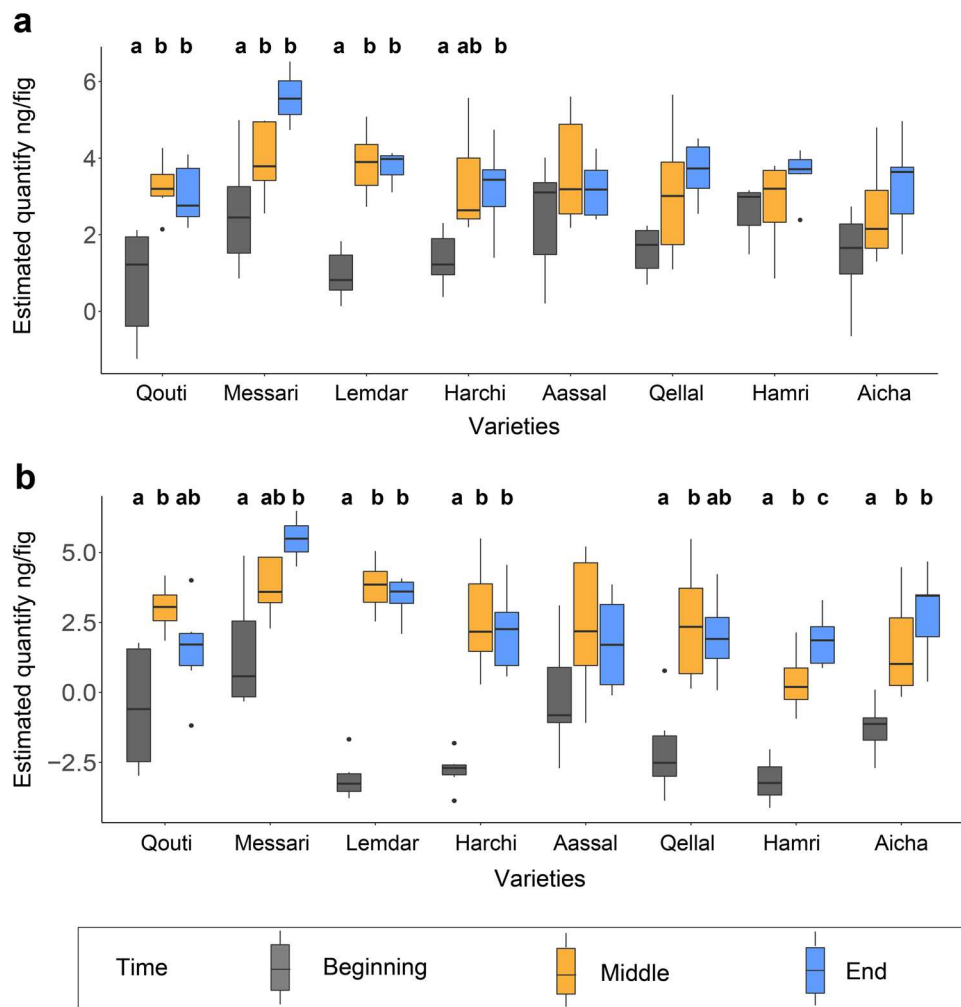


Figure 4. Comparison of the estimated quantities of all volatile organic compounds (VOCs) (a) and of the four VOCs responsible for pollinator attraction (b) at the three different times within the manual pollination period for each of the eight varieties. Data were log-transformed. Results of pairwise comparisons of estimated total quantities within each variety using least-squares means comparisons are presented, with different letters indicating statistically significant differences.

from those of receptive male and female figs produced in summer, with higher relative proportions of several sesquiterpenes in summer in both male and female figs compared to spring males³⁰. The authors of these studies hypothesized that this seasonal difference in VOC emission could be related to abiotic factors such as hydric stress and temperature. Indeed, it is known that while emission by plants of monoterpenes, such as linalool, can decrease under drought conditions, emission of sesquiterpenes, such as β -caryophyllene, can increase under high temperature³¹. Also, high temperature has generally been found to increase the rates of total emissions of VOCs in plants^{32,33}. Interestingly, in *F. carica* studied in southern France, the relative proportions of the four VOCs emitted at receptivity to attract the specific pollinator were not significantly different between spring male figs and summer male and female figs²³. However, the absolute quantity of these four VOCs was significantly higher for male figs in spring than for male and female figs in summer²³. It has been suggested that this quantitative inter-seasonal difference in VOC emission by receptive figs - which is in the opposite direction to the difference generally driven by temperature- could be the result of pollinator-mediated selection: because the density of pollinators is much lower in spring than in summer^{22,29}, spring male figs should be selected to emit a greater quantity of VOCs to attract pollinators²³.

In the present study, the chemical profiles of the receptive figs of the early (Qouti) and three of the middle-pollinated varieties (Messari, Lemdar and Harchi) (Fig. 1) were closer to that already described²³ for wild spring male figs than to that of wild females (greater relative proportions of monoterpenes than sesquiterpenes, Table 1 and Supplementary Fig. S1). On the contrary, Aassal and the three late-pollinated varieties (Fig. 1) presented a “classical” volatile profile of wild female figs (greater relative proportions of sesquiterpenes compared to monoterpenes, Table 1 and Supplementary Fig. S1). These inter-varietal differences in the emission of all VOCs, for both total estimated quantity and relative proportions, could be driven by abiotic factors, human driven-selection, or both combined. However, in this study, we can reject the hypothesis that abiotic factors affected emission of

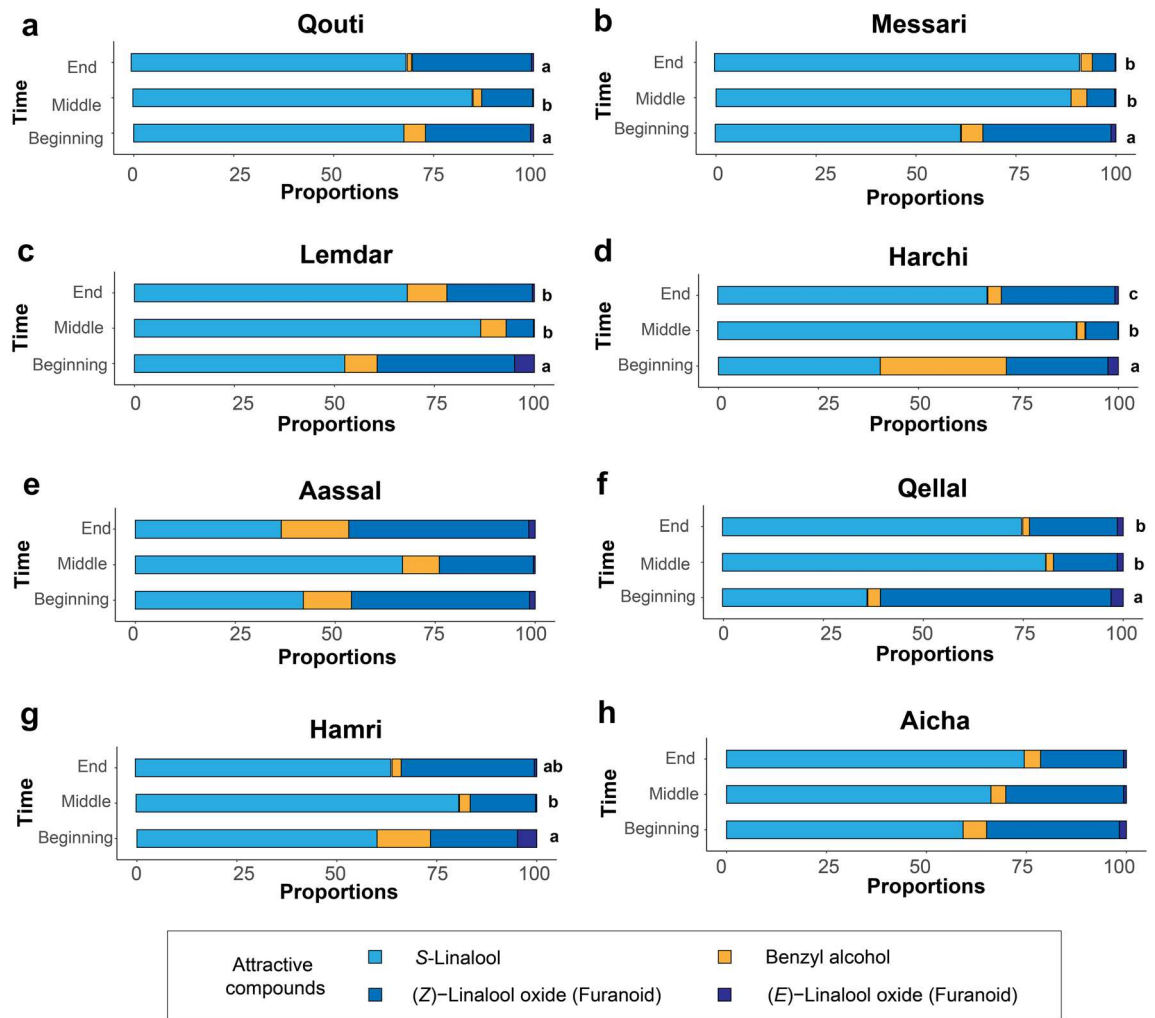


Figure 5. Relative proportions of the four pollinator-attractive volatile organic compounds (VOCs) at the three different time within the manual pollination period of the eight varieties (a, b, c, d, e, f, g and h). Results of pairwise comparisons between times were performed with correction of p -values using the “fdr” procedure. Different letters indicate statistically significant differences. When no letter is indicated, the global test was not statistically significantly different.

VOCs, as all individuals studied were cultivated on the same site, thus exposed to similar environmental conditions. In addition, although slight variations in micro-climatic conditions may have occurred, we found that (1) ambient temperature during the collection phases was very homogeneous (Fig. 1) and that the temperature registered inside the sample bag had no significant effect on the total quantity emitted on the relative proportions of all VOCs, or on the relative proportions of the four attractive VOCs, for all varieties. Therefore, the inter-varietal differences found in this study were most likely related to whether they were pollinated early or late in the season, and therefore variation results mainly from human-driven diversification. We hypothesize that the varieties emitting the highest quantities of the VOCs responsible for pollinator attraction (Messari, Qouti and Lemdar) are more effective at attracting pollinators early in the flowering season when pollinator density is low. These female varieties with very different chemical profiles from wild females were probably selected during the domestication process because their pollination early in the season was facilitated by their high VOC emissions. Indeed, efficient pollination of figs increases the yield and quality of the fruits³⁴.

Compared to the other VOCs emitted by receptive figs, in these eight varieties, the relative proportions of the four VOCs that attract the pollinator are relatively homogeneous, except for Aassal and Qellal (Table 1, Figs. 2, S2). Despite this small variation, the proportions of the four VOCs are still within the range that ensures pollinator attraction²³. Indeed, for all the varieties studied here, fruit production requires attracting female fig wasps. Thus, our results seem to indicate that in *F. carica*, stabilizing selection during human-driven varietal diversification has maintained the chemical signal responsible for pollinator attraction. This hypothesis could be tested by comparing the chemical profiles of varieties that do not require the presence of pollinators (*i.e.* parthenocarpic varieties) and those that depend exclusively on pollinators for fruit production.

In the genetic analyses, the difference we found between expected and observed heterozygosity is typical for clonally propagated entities. Indeed, clonal propagation in domesticated plants can result in an excess of

heterozygosity for several reasons³⁵. First, highly heterozygous genotypes may have been selected by humans for clonal propagation. Also, clonal lineages accumulate mutations, and since it is unlikely that the same mutation occurs at the same locus on both chromosomes, the clonal lineage becomes more heterozygous over time. For instance, it has been shown in manioc that domestication by clonal propagation has resulted in the fixation of deleterious mutation³⁶. As *F. carica* is an emblematic plant of the Mediterranean region with a large number of varieties and a long history of domestication²¹, it would be interesting to study the consequences of human driven-selection on the genetics of these different varieties in order to shed light on the evolutionary trajectories of this species under domestication.

The lack of correlation between chemical and genetic distances is probably not surprising, as neutral markers were used in the present study, but correlation could occur at the level of biosynthetic pathways³⁷, or when specific loci are selected. For instance, in *Cannabis sativa* and *C. indica*, differences between cultivars are driven by terpene synthase genes on different chromosomes, which control the concentrations of terpenes in this species³⁸. Terpene synthase genes involved in the synthesis of some monoterpenes (including linalool) and sesquiterpenes in *F. carica* figs have been identified³⁹. In the present study, since significant differences in the emission of mono- and sesquiterpenes have been shown between some varieties (Table 1, Supplementary Fig. S1), comparison of the expression of these genes in receptive figs of these varieties would be interesting in exploring the links between chemical and genetic differences.

Our results revealed that in the eight varieties studied, the total estimated amount of all VOCs and the four attractive VOCs changed at different times during the manual pollination period. As different figs were collected for each sampling time, we cannot know whether there are quantitative or qualitative variations in VOC emission (1) for a single fig along the whole receptive stage or (2) among different figs of the same individual tree. Nevertheless, in wild male and female fig trees, receptivity lasts 2–3 weeks if the figs are not pollinated⁴⁰ and variation in the VOC emission along the receptive period has been described²⁹. In other plants, floral VOC emission is known to be developmentally regulated and displays variable patterns: emission increasing before the flowers are ready for pollination and either decreasing or remaining relatively constant during flower receptivity^{41,42}. In the future, it would be interesting to compare the VOC emission patterns of individual figs along the receptive stage between varieties and wild individuals of *F. carica* to examine whether human-driven selection may have affected these patterns.

For most of the varieties studied here, during the manual pollination times, the total estimated quantity of the four attractive VOCs was generally higher at the middle and end compared to the beginning. Therefore, for most varieties, the best time for pollination would be the middle of the manual pollination period and this is when most manual pollination takes place (Fig. 1). Nevertheless, farmers start manual pollination very early for some varieties, possibly because all varieties need to be pollinated during a short period. For practical reasons of availability of male figs, this pollination must be spread out over time during this period. Thus, manual pollination is controlled²⁸ and repeated two to three times for each tree during this period to enhance the pollination of figs until these are considered fully pollinated (Fig. 1).

Three varieties (Aassal, Qouti and Messari) show a pattern of temporal variation different from the others. For these three varieties, the quantity of VOCs emitted at the beginning of the manual pollination period is already high, which can explain the fact that farmers start pollination with Qouti. Usually, Messari is manually pollinated at the beginning of the season too, but the year we conducted our sampling, farmers decided to start pollinating this variety in the middle of the season (Fig. 1). For these three varieties, as the period of manual pollination does not correspond to the peak of VOC emission, reasons other than the intensity of the chemical signal must also contribute to explaining the farmers' pollination strategy.

Materials and methods

Study site and plant materials. The sample collection took place in the village of Talandaoued (34.83124 North, 5.0757267 West), part of the rural commune of Bni Ahmed Charqia (Chefchaouen, Morocco), a mountainous region of the western Rif belonging to the Jbala country (Mountain Arabic-speaking socio-cultural group). The region is characterized by hills, mountains and plains. The climate is of the Mediterranean type, with a wet winter and a dry summer. The average annual temperature is 18.2 °C and the average annual precipitation is 512 mm. Choice of the study site is justified by the significant agro-diversity of fig trees and the persistence of the practice of manual pollination^{27,28}. Thus, it is characterized, within an area of eight hectares, by the presence of 15 locally-identified varieties of fig tree (*Ficus carica* L.) of which only eight are non-parthenocarpic and require manual pollination (caprification) at the receptive stage to ensure fruit production. In this study, we only considered these eight varieties, no wild plants, and relied on farmers' knowledge for receptive periods and timing of the manual pollination, as this may vary from year to year according to climate. The manual pollination lasted around five weeks in total and was spread out during this period between the varieties that bear most of their receptive figs early (Qouti, Messari), at the middle (Harchi, Aassal and Lemdar) or late (Aïcha, Hamri and Qellal) in the season (Fig. 1). During this season, after the first manual pollination, the trees of each variety were pollinated two to three times at an interval of about five days. In 2018, manual pollination of Messari was only conducted at the middle of the manual pollination period. It should be noted that this study complies with the relevant institutional, national and international guidelines and legislation. Thus, the farmers contacted for access to the cultivated plant material gave their agreement to obtain the plant samples as well as the data on manual pollination periods. The Moroccan team involved in this project also obtained the required authorizations from the local village administration.

Genetic analysis. DNA was extracted from 70 mg of young leaves of the eight varieties (ten trees of each variety, including the trees that were used for odor collection in 2018) using the Dneasy Plant Mini Kit (Qia-

gen). Among the SSR markers developed^{43–45}, we selected 15 loci²⁶ (Supplementary Table S1). The microsatellite amplifications were carried out according to the standard Multiplex PCR protocol and using five multiplexes [(LMFC30, LMFC32 and FSYCO4), (MFC2, FSYCO1 and MFC11), (MFC3, MFC1 and LMFC28), (LMFC24, MFC8 and LMFC26) and (MFC9, MFC4 and LMFC19)]. SSR genotyping was performed in an Abi 3500XL automated capillary sequencer and analyses were performed using Genemapper software (v. 5.0).

Collection of VOCs. Collection of volatile organic compounds (VOCs) of receptive figs (*i.e.*, ready to be pollinated) was conducted during two successive years on different trees between the two years, in situ using dynamic headspace^{46,47}. In July 2017, VOCs were collected from three individuals per variety from eight different fig varieties. In 2018, samples from six individuals per variety were collected to analyze the VOCs of the varieties at three different time during the manual pollination period; (i) at the beginning (when the first manual pollination of the early variety was conducted), (ii) at the middle (when the first manual pollination of the middle varieties was conducted for the first time, corresponding to the same development stage as in the previous year), and (iii) at the end (when the last manual pollination of the late varieties was conducted) (Fig. 1). For each collection, 20 receptive figs per individual were enclosed in a polyethylene terephthalate (Nalophane[®], Kalle Nalo GmbH, Würsthüllen, Germany) bag of volume around 8 L. Microtraps, ChromatoProbe[®] quartz microvials (Varian Inc., length: 15 mm; inner diameter: 2 mm), containing 3 mg of a 1:1 mixture of Tenax-TA and Carbotrap (60–80 and 20–40 mesh respectively, Sigma-Aldrich, Munich, Germany) were used as adsorbent traps. One microlitre of a solution of internal standards (*n*-nonane and *n*-dodecane, 110 ng µl⁻¹ of each) was added to each trap before scent collection. Bags containing figs were left in the shade for 30 min and during 10 min, air (flow rate 200 ml min⁻¹) was drawn out of the bag and through the adsorbent trap for 5 min. In parallel, for each control collection, extractions were performed using empty bags, to control for ambient contaminant compounds. We collected one blank sample per site per collection day. Collections were done under natural light and ambient temperature, between 10:30 and 17:00 h. Data on additional parameters were collected during harvesting, including the temperature at the time of harvesting, inside and outside the collection bags.

Chemical analysis of VOCs. Traps were stored in the dark in a freezer (–20 °C) until analysis. Samples were analyzed by gas chromatography-mass spectrometry (GC–MS) using a similar method as Souto-Vilarós et al.⁴⁷. These samples were analyzed by a gas chromatograph (GC, Trace 1310, Thermo Scientific, Milan, Italy) coupled with a mass spectrometer (ISQ QD Single Quadrupole, Thermo Scientific, Milan, Italy) with an Optima 5-MS capillary column (30 m, 0.25 mm ID, 0.25 µm film thickness, Machery-Nagel, Düren, Germany). The injection system composed of a Thermal Desorption Unit (TDU) and a Cold Injection System (CIS) (Gerstell, Mülheim, Germany), adsorbent traps were handled with a Multi-Purpose Sampler (Gerstell, Mülheim, Germany). The program for the analysis was as follows: oven held at 40 °C for 3 min, then increased from 40 °C to 220 °C at 5 °C min⁻¹, and finally from 220 to 250 °C at 10 °C min⁻¹. Temperature was then maintained at 250 °C for 2 min. The transfer line was 250 °C. The ion source of the mass spectrometer was 200 °C. The acquisition was from 38 m/z to 350 m/z at 70 eV ionisation energy. Xcalibur TM 266 software (Thermo Scientific TM, Milan, Italy) was used for data processing. Retention index (RI) was calculated by a series of *n*-alkanes (alkanes standard solution, 04070, Sigma Aldrich, Munich, Germany). Compounds were identified based on computer matching of the mass spectra with a library (NIST 2017 MS), and confirmation by comparison of retention index reported in literature⁴⁸. When possible, mass spectra and retention index were compared to those of reference compounds run in the same GC–MS with the same method. Putative contaminant compounds were subtracted from our chromatograms by comparing spectra of each sample with the respective control sample. Trace compounds, which always occurred in mean proportions per variety of less than 0.1%, were excluded from the analyses. In addition, the stereochemistry of linalool of one individual per variety was determined using the same analytical method as indicated above in the same GC–MS equipped with a β-cyclodextrin chiral capillary column (Cyclosil-B, 30 m–0.25 mm i.d., 0.25 µm film thickness, Agilent J&W columns, USA).

Chemical dataset. In order to estimate the quantity of VOCs emitted by a receptive fig of each sample, the calculation was performed using the two internal standards as reference and the following Eq. (1):

$$Q_x = \left(\left(\frac{A_x}{A_{C9}} \right) + \left(\frac{A_x}{A_{C12}} \right) * 110 \right) / 2 * 20 \quad (1)$$

where Q_x : estimated quantity of the VOCx (ng fig⁻¹); A_x : peak area of the VOCx, A_{C9} : peak area of the *n*-nonane, A_{C12} : peak area of the *n*-dodecane. In each sample 110 ng of each internal standard were injected. Twenty figs were used during odor collection.

For the comparison of volatile profiles among different varieties and between years, two estimated-quantity datasets (total of all VOCs and total of the four pollinator-attractive VOCs) were created combining data from 2017 and the middle of manual pollination of 2018. To test the effect of the time within the manual pollination period for each variety separately, two estimated-quantity datasets per variety (all VOCs and four attractive VOCs) were built using all data from 2018.

In addition, for each sample, the percentage relative to the total peak area (*i.e.* relative proportion), for all VOCs on the one hand and only for the four pollinator-attractive VOCs on the other, of each VOC was calculated. As with the estimated quantity, we generated four datasets for the relative proportions. For the effect of varieties and year, we combined data from 2017 and the middle of the 2018 manual pollination for all VOCs and the four attractive VOCs. To test the effect of time within the manual pollination period on each variety separately, we used all 2018 data. Moreover, we generated a dataset of relative proportions of the VOCs belonging to the

different chemical classes (monoterpenes, sesquiterpenes, fatty acid derivatives, and benzenoids) from the eight varieties using the samples from 2018 for all VOCs.

In order to compare chemical and genetic distance, a dataset using allele frequencies among all replicates of each variety was calculated, thus with only one point per variety. Mirroring this, for each variety, the available replicates on the relative proportions of all VOCs (2017 data and the middle of the 2018 manual pollination) were averaged by variety.

Statistical analyses. All the data on chemical analyses were performed in R (v. 4.2.1;⁴⁹) with the packages *emmeans*⁵⁰, *vegan*⁵¹, *RVAideMemorie*⁵², *ade4*⁵³ and *hierfstat*⁵⁴. Estimation of the various parameters of genetic diversity was performed using GENETIX software (v. 4.03) and NTSYSpC software (v. 1.05).

The significant effect of varieties and time of the manual pollination period on the total estimated quantity of VOCs emitted by one fig (for all VOCs and only the four attractive VOCs), was tested using linear regression models including temperature in the bag and year as co-factors, and followed by a deviance analysis. The data were log-transformed prior to the analyses to obtain normality of the residuals. Then pairwise comparisons between varieties were performed computing estimated marginal means for the factor varieties (least-squares means).

For the comparison of the relative proportions of VOCs between varieties, a distance matrix comparing the dissimilarity between all samples in pairs using a Bray–Curtis index was built, on the dataset including all VOCs on the one hand, and on the dataset including only the four attractive VOCs on the other. Data were standardized prior to the analysis. Then, for each dataset the distances between all pairs of samples in the distance matrix were plotted using non-metric multidimensional scaling analysis (NMDS). Effects of varieties were then tested on the distance matrix, including temperature and year in the bag as co-factors, using permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations. If significant effects were found, pairwise comparisons between varieties were performed with correction of *p*-values using the false discovery rate (fdr) method⁵⁵. Similar analysis was used on the datasets of relative proportions of all samples from 2018, for all VOCs, for only the four attractive VOCs, and for the relative proportions of four different chemical classes, to test the effect of time within the manual pollination period with temperature as co-factor.

For genetic data, polymorphism was estimated by the average number of alleles per locus (*NA*), allelic richness (*AP*), number of genotypes (*NGP*), polymorphism information content (*P*)⁵⁶, observed heterozygosity (*HO*), expected heterozygosity (*HE*), and Wright's fixation index (*Fis*)⁵⁷. Hierarchical clustering using Nei's distance⁵⁸ and the UPGMA clustering method was performed. In addition, to compare genetic and chemical distances, we created independent matrices coding the standard Nei genetic distance between varieties, based on allele frequency data, and the Bray–Curtis distance for the average chemical distance of all VOCs. Then, for these two matrices we created dendrograms using the neighbor-joining method⁵⁸. Finally, we tested the correlation between chemical and genetic distances using the Mantel test.

Data availability

All data generated or analyzed during this study are included in this published article. The raw data supporting these analyses will be made available by the authors, without undue reservation.

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Author contributions

M.P., Y.H., Y.A.T. and M.H.M. designed the study. Y.H. and S.E. collected the data. L.C., M.P., S.E., M.P.D., M.L.M. and B.B. analysed and interpreted the data. Y.A.T. and M.P. obtained the funding. L.C., M.P. and A.G.B. wrote the manuscript with the assistance of all authors.

Competing interests

The authors declare no competing interests.

Additional information

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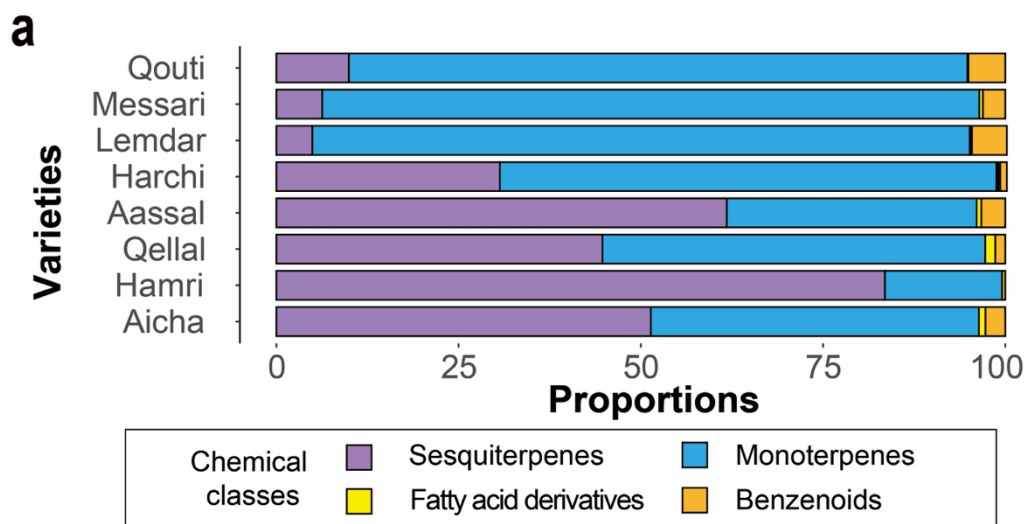
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Supplementary information

Floral scent of the Mediterranean fig tree: significant inter-varietal difference but strong conservation of the signal responsible for pollinator attraction

Li Cao, Younes Hmimsa, Salama El fatehi, Bruno Buatois, Marie-Pierre Dubois, Maily Le Moigne, Martine Hossaert-McKey, Yildiz Aumeeruddy-Thomas, Anne-Geneviève Bagnères, Magali Proffit

Figure S1. Mean relative proportions of all volatile organic compounds (VOCs) from the different chemical classes emitted by the eight varieties of *Ficus carica* (a) and pairwise comparisons (b). Pairwise comparisons of the relative proportions were conducted using permutational multivariate analysis of variance (PERMANOVA). Significant codes: P < 0.01 “***”, P < 0.05 “*”, P > 0.05 “NS”.



b

| | Qouti | Messari | Lemdar | Harchi | Aassal | Qellal | Hamri |
|---------|-------|---------|--------|--------|--------|--------|-------|
| Messari | NS | | | | | | |
| Lemdar | NS | NS | | | | | |
| Harchi | * | * | ** | | | | |
| Aassal | ** | ** | ** | * | | | |
| Qellal | ** | * | ** | NS | NS | | |
| Hamri | ** | ** | ** | ** | NS | ** | |
| Aicha | * | ** | ** | NS | NS | NS | * |

Figure S2. Mean relative proportions of the four attractive volatile organic compounds emitted by the eight varieties of *Ficus carica*.

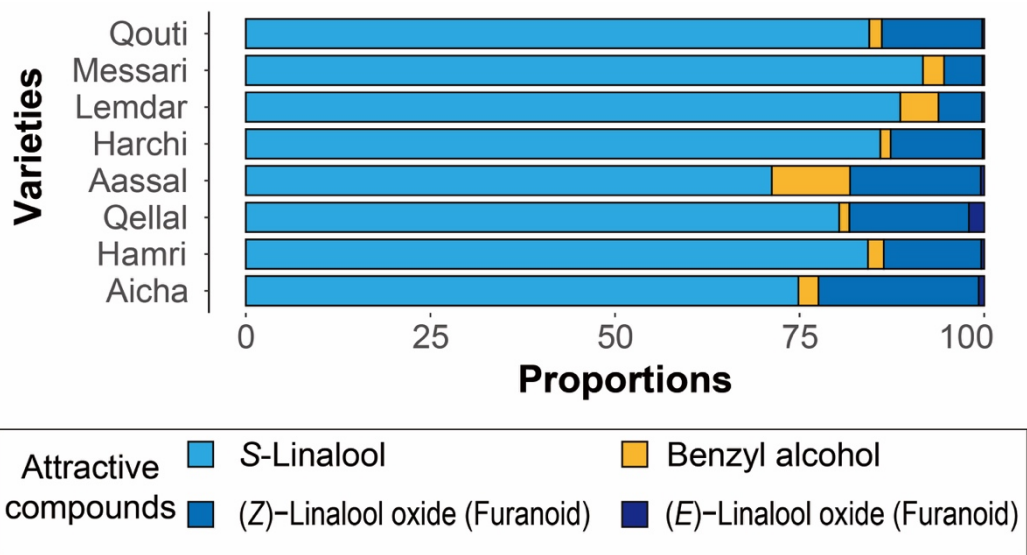


Table S1. Information on the 15 microsatellite markers used for the genetic analysis. Locus name, fluorescent dye-labeled (HEX, FAM and NED fluorochromes), size range of amplified fragments, hybridization temperature, number of individuals analyzed (N_I). Genetic parameters include number of alleles (A) and the genotype number (N_G) per locus.

| Locus | Fluorescent dye | Range Size (pb) | Hybridization temperature | N_I | A | N_G |
|--------|-----------------|-----------------|---------------------------|-------|-----|-------|
| LMFC30 | HEX | 231-261 | 50 | 80 | 6 | 8 |
| LMFC32 | FAM | 197-223 | 55 | 80 | 5 | 6 |
| FSYC04 | HEX | 177-193 | 55 | 80 | 3 | 3 |
| MFC2 | HEX | 152-190 | 50 | 80 | 4 | 3 |
| FSYC01 | FAM | 117-160 | 50 | 80 | 7 | 7 |
| MFC11 | NED | 156-203 | 55 | 80 | 2 | 3 |
| MFC3 | FAM | 96-144 | 55 | 80 | 4 | 5 |
| MFC1 | HEX | 161-199 | 58 | 80 | 3 | 5 |
| LMFC28 | NED | 192-202 | 55 | 80 | 4 | 5 |
| LMFC24 | FAM | 272-276 | 55 | 80 | 3 | 4 |
| MFC8 | HEX | 143-193 | 50 | 80 | 2 | 2 |
| LMFC26 | NED | 224-236 | 55 | 80 | 2 | 2 |
| MFC9 | FAM | 184-225 | 58 | 80 | 4 | 4 |
| MFC4 | NED | 210-226 | 50 | 80 | 2 | 3 |
| LMFC19 | HEX | 296-332 | 50 | 80 | 2 | 2 |

Figure S3. UPGMA tree showing the relationships among the ten individual trees of each of the eight studied varieties of *Ficus carica*. Nei distance was calculated on 15 SSR markers.

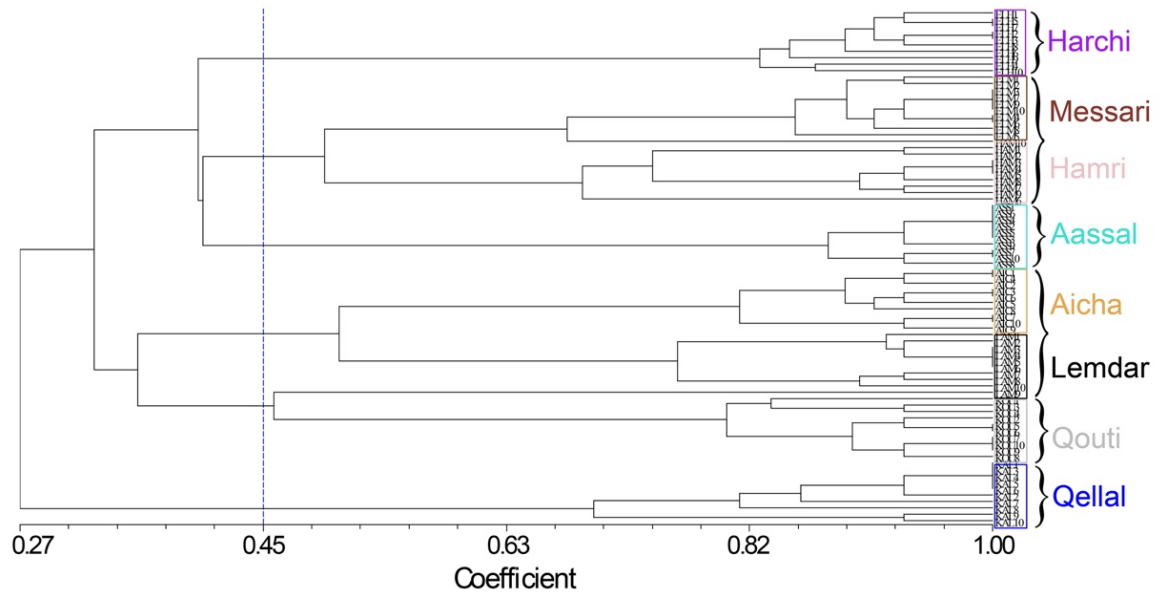
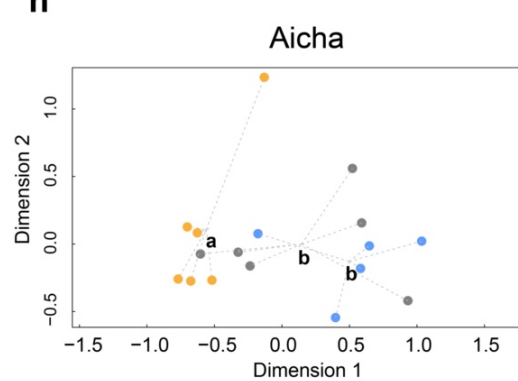
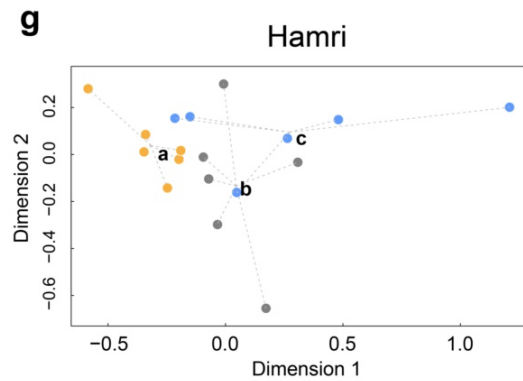
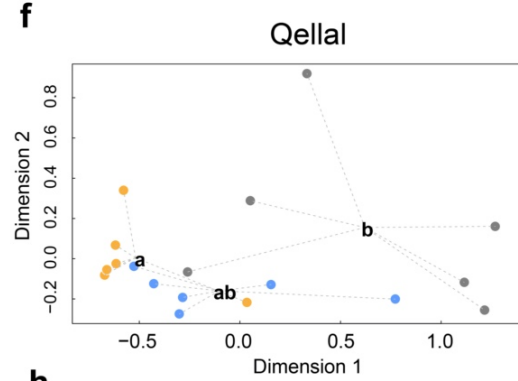
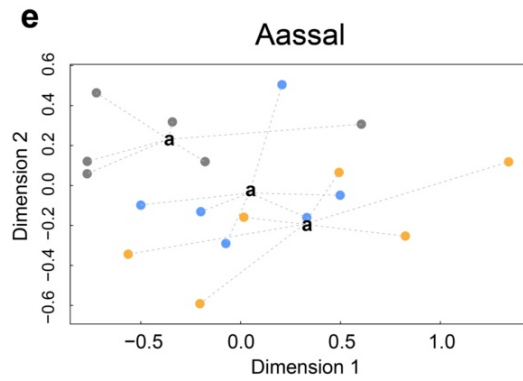
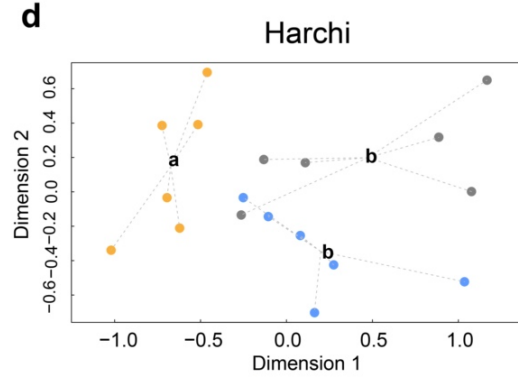
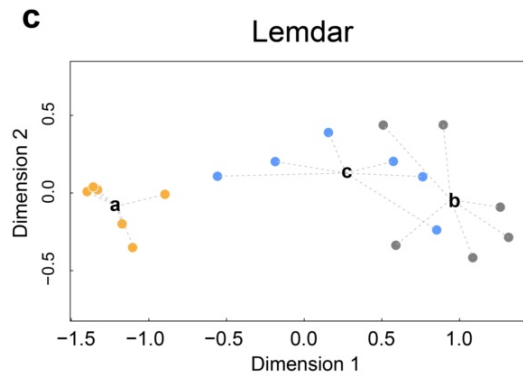
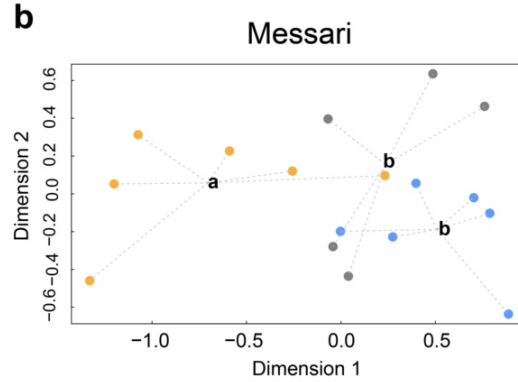
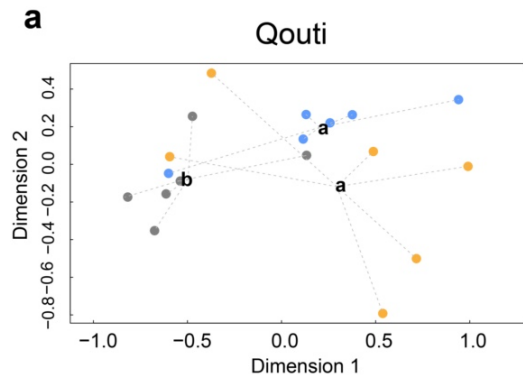


Figure S4. Non-metric multidimensional scaling ordination (NMDS) of the relative proportions of all volatile organic compounds (VOCs) at the three times within the manual pollination period for each of the eight varieties of *Ficus carica* (a, b, c, d, e, f, g and h), based on Bray-Curtis distances. Stress: A = 0.097, B = 0.066, C = 0.105, D = 0.089, E = 0.039, F = 0.085, G = 0.057, H = 0.079. Results of pairwise comparisons between times were performed using permutational multivariate analysis of variance (PERMANOVA) with correction of *p*-values using the fdr (false discovery rate), different letters indicating statistically significant differences.

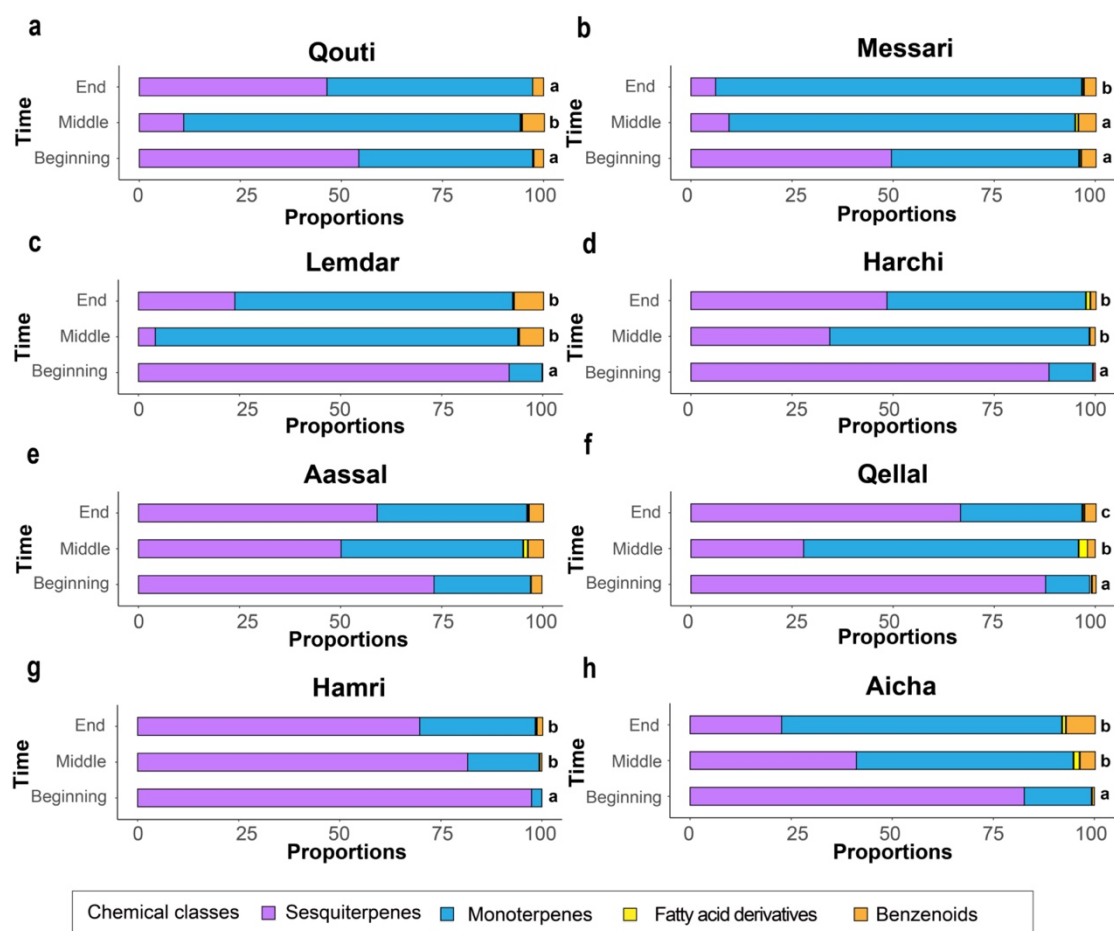


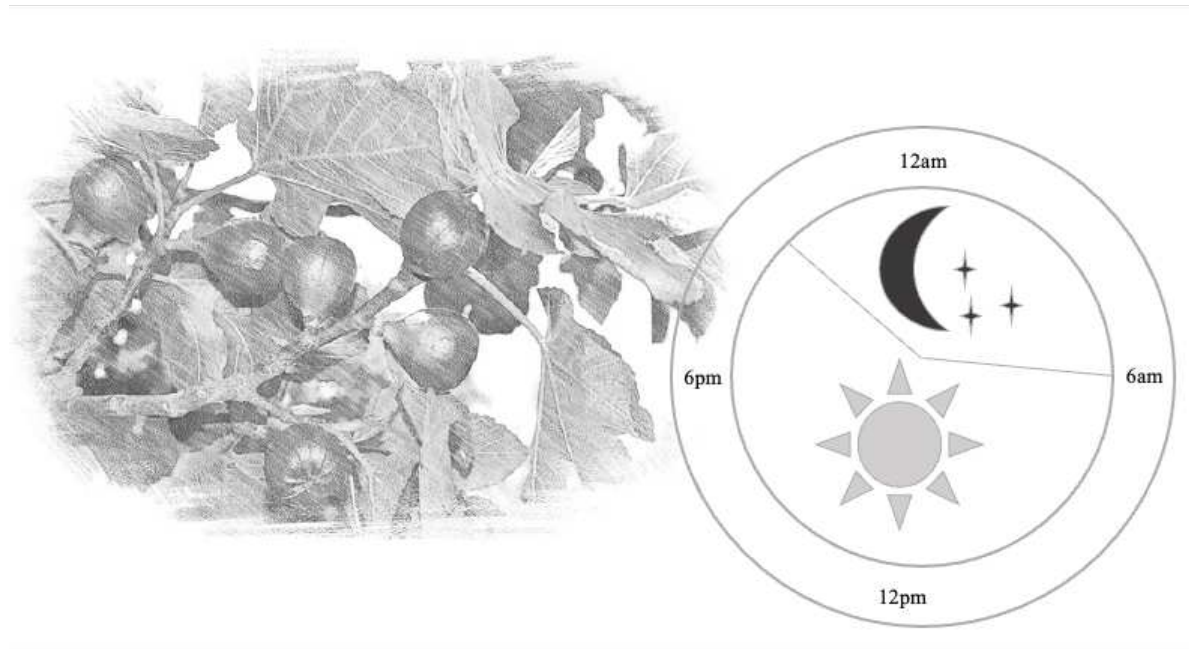
Time ● Beginning ● Middle ● End

Table S2. Results on comparison among times within the manual pollination period of relative proportions of the four chemical classes of volatile organic compounds (VOCs) for each variety of *Ficus carica* during the manual pollination period. Permutational multivariate analysis of variance (PERMANOVA) was performed on relative proportion of the four chemical classes (standardization before the analysis). Degrees of freedom (Df) and results of the test (F) are indicated. Significant *p*-values ($p < 0.05$) are indicated in bold.

| Varieties | Df | all VOCs | |
|-----------|-------|----------|-------------------|
| | | F | <i>p</i> |
| Qouti | 2, 17 | 3.43 | 0.04 |
| Messari | 2, 16 | 4.73 | 0.01 |
| Lemdar | 2, 17 | 29.28 | < 0.001 |
| Harchi | 2, 17 | 5.71 | 0.01 |
| Aassale | 2, 17 | 0.98 | 0.40 |
| Qellal | 2, 17 | 8.17 | < 0.001 |
| Hamri | 2, 17 | 6.27 | < 0.001 |
| Aicha | 2, 16 | 8.57 | < 0.001 |

Figure S5. Mean relative proportions of all volatile organic compounds (VOCs) from the different chemical classes at the three different times within the manual pollination period of the eight varieties of *Ficus carica* (a, b, c, d, e, f, g and h). Results of pairwise comparisons between times were performed using permutational multivariate analysis of variance (PERMANOVA) with correction of *p*-values using the *fdr* (false discovery rate) procedure. Different letters indicate statistically significant differences.





*Chapter 3. The temporal dynamic of floral odour emission of *Ficus carica* is influenced by pollinator activity and abiotic factors*

La dynamique temporelle de l'émission d'odeurs florales de *Ficus carica* est influencée par l'activité des pollinisateurs et par des facteurs abiotiques

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Résumé

Les parfums floraux, formant un mélange complexe de composés organiques volatils (COVs), jouent un rôle majeur dans la rencontre entre partenaires dans les interactions plantes-pollinisateurs, leurs patterns temporels d'émission pouvant être régulés pour répondre à la fois à l'activité des pollinisateurs et à l'état de pollinisation. Dans le mutualisme de pollinisation -ou nursery pollination- entre le figuier dioïque, *Ficus carica*, et son pollinisateur exclusif, la guêpe du figuier, *Blastophaga psenes*, il a été vu auparavant que les COVs émis par les fleurs des figes réceptives médiaient l'interaction spécifique et qu'une combinaison de quatre COVs (l'alcool benzylique, le *S*-linalool, le (*Z*)-linalool-oxide et le (*E*)-linalool-oxide (furanoids)) sont responsables de l'attraction des pollinisateurs, mais le schéma temporel de l'émission de ces COVs pendant la journée et après la pollinisation n'a jamais été étudié. Dans la présente étude, nous avons testé l'hypothèse selon laquelle les figes réceptives de *F. carica* présentent un schéma diurne d'émission d'odeurs correspondant à la période d'activité des pollinisateurs, et que le signal chimique attirant les pollinisateurs change après la pollinisation pour réduire la visite d'un trop grand nombre de pollinisateurs. Nous avons

étudié en conditions contrôlées la cinétique d'émission des COVs floraux des figes réceptives et pollinisées de *F. carica* pendant trois jours consécutifs, ainsi que l'influence de plusieurs facteurs abiotiques, tels que la luminosité, la température ou l'humidité, en effectuant des mesures en temps réel à l'aide d'un PTR-ToF-MS (Proton-transfer-reaction time-of-flight mass spectrometer) et des identifications fines à l'aide d'un GC-MS (Gas chromatography - mass spectrometry). En outre, nous avons testé à l'aide d'olfactomètres si les changements induits par la pollinisation modifiaient l'attraction des pollinisateurs. Les COVs émis par les figes réceptives ont suivi un schéma d'émission diurne, avec un pic à la mi-journée correspondant à la période où le pollinisateur est le plus actif. La température et l'humidité ont affecté l'émission de ces COVs de manière non linéaire. Après la pollinisation, l'émission de COV attirant les pollinisateurs a diminué très rapidement (en moins de 5 heures) et le changement induit a stoppé l'attraction des pollinisateurs. En étudiant la variation à petite échelle des COVs floraux, cette étude montre que le schéma d'émission des parfums floraux de *F. carica* peut être optimisé pour attirer ses pollinisateurs exclusifs, mais aussi que les facteurs abiotiques peuvent influencer l'émission de ces COV.

Mots clefs : Figes, Composés Organiques Volatiles, Attraction du pollinisateur, Emission rythmique, Facteurs abiotiques

The temporal dynamic of floral odour emission of *Ficus carica* is influenced by pollinator activity and abiotic factors

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Abstract

Since floral scents, a complex mixture of Volatile Organic Compounds (VOCs), play major roles in mediating encounter with partners in plant-pollinator interactions, their temporal patterns of emission may be regulated to respond to both pollinator's activity and pollination status. In the nursery pollination mutualism between the dioecious fig tree, *Ficus carica*, and its exclusive pollinator the fig wasp, *Blastophaga psenes*, it has been reported that floral VOCs emitted by receptive figs mediated species-specific interaction and that a combination of four VOCs (Benzyl alcohol, *S*-linalool, (*Z*)-linalool oxide (furanoids) and (*E*)-linalool oxide (furanoids)) are responsible for pollinator attraction, however the temporal pattern of emission of these VOCs during the day and after pollination has never been studied. In the present study, we tested the hypothesis that receptive figs of *F. carica* exhibit a diurnal pattern of odor emission corresponding to the period of pollinator activity, and that the chemical signal attractive to the pollinator changes after pollination to reduce pollinator visitation. We studied under controlled conditions the emission kinetics of floral VOCs from receptive and pollinated figs of *F. carica* during three consecutive days as well as the influence of several abiotic factors, such as luminosity, temperature or humidity by performing real time measurements using PTR-ToF-MS (Proton-transfer-reaction time-of-flight mass spectrometry) and fine identifications using GC-MS (Gas chromatography - mass

spectrometry). In addition, we tested using behavioral olfactometer tests whether pollination-induced changes altered pollinator attraction. VOCs emitted by receptive figs followed a diurnal pattern of emission, with a peak at mid-day corresponding to the period when the pollinator is most active. Temperature and humidity affected the emission of these VOCs in a non-linear way. Following pollination, the emission of pollinator-attractive VOCs decreased very rapidly (within 5 h) and the induced change impaired pollinator attraction. Through the study of the fine-scale variation of floral VOCs, this study shows that the floral fragrance emission pattern in *F. carica* can be optimized to attract its exclusive pollinators, but also that abiotic factors can influence the emission of these VOCs.

Keywords : Figs, volatile organic compounds, pollinator attraction, rhythmic emission, abiotic factors.

Introduction

Floral Volatile Organic Compounds (VOCs) play major roles in the interactions between plants and their biotic environment. For example, they play a major role into attracting pollinators, and other beneficial animals such as parasitoids, also to defend plants against herbivores, pathogens and microorganisms (Agrawal, 2011; Beccera et al., 2009; Raguso, 2008; Junker and Parachnowitsch, 2015). The production and emission of floral VOCs could be very variable in time. Floral VOCs emission is often developmentally and rhythmically regulated (Picazo-Aragonés et al., 2020) and influenced by the endogenous biological clock, i. e. circadian clock (Zeng et al., 2017). For instance, in *Hoya carnosa*, a circadian rhythm of floral VOCs emission has been reported as this rhythm was maintained even during exposure to continuous dark (Matile and Altenburger, 1988). Also, temporal variation between day and night in floral VOCs emissions has been demonstrated in snapdragons and petunias (Kolosova et al., 2001; Fenske et al., 2015). Nevertheless, only a very limited recent studies (Powers et al., 2020) have examined the temporal variation in VOC emissions on a very

fine time scale and the factors that may influence these emission patterns.

As floral odours are crucial for plant reproduction, they often mediate partner encounters in plant-pollinator interactions (Raguso, 2008; Hossaert-McKey et al., 2010), the timing of pollinator activities and attraction are major factors affecting the temporal pattern of floral VOC emission. Indeed, it has been reported that nocturnally pollinated plant species emit strong scents in the night and a diurnal pollinated species during the day (Dötterl et al., 2006). Flowers of *Petunia axillaris* (Oyama-Okubo et al., 2005), the wild tobacco, *Nicotiana attenuata* (Kessler et al., 2008; Yon et al., 2016) and *Myrtaceae* species (Cordeiro et al., 2019) emit VOCs at night to attract a night-active pollinator (Fenske et al., 2015; Yon et al., 2016). The floral scent emission is increased when their pollinators are the most active (Friberg et al., 2014; Jürgens et al., 2014). Also, it is expected that after successful pollination the emission of floral VOCs should be reduced to limit pollinator attraction but also the physiological costs of VOC biosynthesis (Schiestl and Ayasse, 2001; Chen et al., 2009; Proffit et al., 2018). Indeed, it has been reported that the time for a significant reduction of floral VOCs can vary from 24 hours (Dötterl et al., 2005; Proffit et al., 2018) to several days after pollination (Schiestl and Ayasse, 2001; Chen et al., 2009). However, if there is significant pollinator limitation, changes in the attractive signal should be observed more quickly after pollination to limit the attraction of pollinators to already pollinated flowers and transfer pollinators to unpollinated flowers of the same plant. Unfortunately, to our knowledge, there are no studies on changes in VOCs attracting pollinators on scale smaller than 24 hours after pollination.

In addition to biotic factors, the emission of floral VOCs could be affected by abiotic factors such as temperature, humidity or light. Some of these factors, can affect VOCs emissions in difference ways: (1) through their direct effects on the biosynthesis of VOCs; (2) by affecting various other plant physiological trait such as photosynthesis and therefore carbon availability for VOCs production (Loreto et al., 1996; Niinemets et al., 2002; Rasulov et al., 2009); (3) through their effects on the physicochemical properties of VOCs such as volatility (Niinemets et al., 2004). Compared to foliar

VOCs, only a few studies have addressed the effects of abiotic factors on the emission of floral VOCs. For instance, it has been reported for various Mediterranean plants, that floral emission increased with temperature to an optimum that depended to the maximum temperatures that plant species can experience during flowering and thereafter decreased (Farré-Armengol et al., 2014). Drought decreased floral visual traits and pollinator visitation rates but increased volatile organic compound (VOC) emissions in forb species *Potentilla recta* L., sulphur cinquefoil and *Campanula rotundifolia* L., harebell. (Glenny et al., 2018). Light intensity and temperature increase the emission of floral scent in *Lilium* especially terpenoid compounds (Hu et al., 2013). Thus, effects of abiotic factors on VOC emission are dependent of the plant species, the chemical class of the VOC and are not necessarily linear.

The Mediterranean fig tree, *Ficus carica* L. (Moraceae), represents a perfect model to investigate the timing of emission of floral VOCs and the factors affecting this dynamic. *Ficus* trees possess figs being sycones, which are characterized by an enclosed wall with a unique entrance (i.e., the ostiole) with the hundreds of tiny florets lining the inside of a central cavity, inflorescences are exclusively pollinated the agaonid wasps, *Blastophaga psenes* L. (Agaonidae), that develop exclusively in the figs of their host species. Once emerging from its natal fig, pollinators leave less than 24h. Pollinators are active exclusively during the day, with a peak of activity. It has been reported that receptive figs of *F. carica* emitted 36 VOCs (mainly terpenoid compounds) at the receptive stage (i.e. in the developmental stage where they are ready to be pollinated) (Soler et al., 2012) but that this wasp use four of them (Benzyl alcohol, *S*-linalool, (*Z*)-linalool oxide (furanoids) and (*E*)-linalool oxide (furanoids)) within a particular proportion to locate their host (Proffit et al., 2020). Previous studies have shown that that receptive period of *Ficus carica* can last for more than two weeks, and that after the introduction of three wasps into a receptive fig, receptivity stops in less than 72h (Hossaert-McKey and Gibernau, 1994; Khadari et al., 1995). We hypothesize that the VOCs emitted by receptive figs of *F. carica* and in particular the four pollinator-attractive VOCs are influenced by pollinator attraction an activity. Thus, we predict that

these VOCs emission follow a diurnal emission rhythm, with a peak emission at mid-day as the pollinators most activity. We also predict that after pollination, the emission of these VOCs decreases within less than three days and that these pollinated figs no longer attract pollinators. In addition, as temperature, humidity and light are the three factors than can affect the emission of VOCs, particularly in the Mediterranean region, we hypothesis that these factors affect the emission of VOCs of figs. For temperature, we predict, as for other Mediterranean plants (Farré-Armengol et al., 2014), VOCs emission increase up to a temperature and then decrease.

To test our hypothesis in control conditions, we analyse in real-time the VOCs emitted by fig trees bearing inflorescence of *Ficus carica* that were either maintained receptive or that were pollinated during 72h using PTR-ToF-MS combined with GC-MS analysis. During VOCs analyses, we measured the relevant environment parameter (light intensity, temperature and humidity) to test their possible effects on VOCs emission. In addition, behavioural olfactometer tests were carried out to test the attraction of pollinators to VOCs emitted by figs either receptive or 72 hours after pollination. With these experiments we aimed to characterize: (1) The emission patterns of VOCs in *Ficus carica* during the receptivity phase and following pollination; (2) The effects of the major abiotic factors on the emission of fig VOCs; (3) The changes in VOCs composition and proportions following pollination; (4) Whether quantitative and qualitative changes in the chemical mixture after pollination affect pollinator attraction behaviour.

Materials and methods

Study site and materials

Ficus carica is a dioecious fig species with male tree bearing figs where its specific pollinator, fig-wasp, oviposit and develop, and female tree bearing figs that produce seeds where pollinators cannot oviposit and thus develop (Kjellberg et al., 1987). Male fig tree bear figs twice a year (in spring and summer) and female only once

(in summer) (for more details about the reproductive biology, see Kjellberg and Lesne, 2020; Proffit et al., 2020).

The experiment took place in spring (April-May 2022), where males of the fig tree bear receptive figs for the first time in the year, in the Terrain d'Expérience (TE) technical platform of the Laboratoire d'Excellence Centre Méditerranéen Environnement et Biodiversité (LabEx CeMEB) in the Centre d'Ecologie Fonctionnelle et Evolutive (CEFE, CNRS Montpellier, France). The temperature was controlled and fixed at 25 °C in the day and 15 °C during the night under natural light conditions (15 h/9 h day/night periods) in TE. For VOCs analysis we used 6-year-old trees obtained by cuttings male wild fig trees provided by nurserymen bearing 1-3 figs (Pépinières Baud©, <http://www.fig-baud.com>). The receptive and pollinated figs for behavioural tests collect from fig trees in field of CEFE. Two branches for each tree were covered by a nylon bag until the end of the experiment. When figs reached receptivity one branch figs was pollinated by three fig-wasps, after pollination 3 days, pick up the pollinated and receptive figs for behavioural tests. In order to pollinated fig trees and for behavioural tests, we collected figs from trees with emerging insects from natural populations around the Montpellier area. Only female fig-wasps were used because they are the only ones to disperse from one fig to another.

Effects of abiotic factors and pollination on VOCs emission

Experimental setup

The fig trees bearing figs were covered by a nylon bag to prevent pollinator entrance. In order to measure the VOCs emitted by small fig trees, plants were individually enclosed in approximately 25 L inert and odourless PTFE (polytetrafluoroethylene) frame closed by a 50 µm thick PTFE film (Figure 1A). Air was pushed using a PTFE pump (KNF, N816.1.2K.18®, Germany) through a charcoal filter into the chambers at 3.5 L/min. A PTFE fan ensured air mixing in the chambers. Air flow rates were controlled by flowmeters a flow rate of 100 mL/min and all tubing lines were made of PTFE. For each series of measurement, we used 5 chambers: one with a fig tree with

only leaves and branches, as blank, two with a fig tree bearing receptive fig that were not pollinated, as receptive group; two with a fig tree bearing receptive figs that were just pollinated with 3 pollinators per fig, as pollinated group (See Figure 1A). The system was set up over 72 hours, repeated five times (five series), so there are 10 replications of pollinated and receptive group.

During the 72 hours of the experiment, light intensity (PAR, Photosynthetically Active Radiation), humidity and temperature were recorded continuously every 10 minutes. Light intensity was measured on top of the chambers using a PAR sensor (LI-1000 light meter, LI-COR®, Biosciences). Temperature and humidity inside each chamber were measured using iButtons® data loggers (DS1923 iButton Temperature/Humidity Logger, 1-Wire).

After each series with the 5 chambers (Blank, Receptive and Pollinated), the number of figs and leaves from each tree were recorded. The numbered figs were dried in oven at 40°C for at least 72 hours and dry weight was measured using an analytical balance (LS-120A SCS, Precisa, Swiss). The length of the film was also recorded in each chamber to determine the volume of air arrive at the chamber.

VOCs analyses in real-time by PTR-ToF-MS

To monitor the emission kinetics of VOCs from receptive and pollinated figs on a large scale, we took advantage of a proton transfer reaction time-of-flight mass spectrometer (PTR-ToF-MS) (Vocu, ToFwerk, Switzerland) to generate continuous measurements of volatile emissions over several days. As soon as fig trees were enclosed into the Teflon chambers, PTR-ToF-MS analysis was launched. In the PTR-ToF-MS, the reaction chamber pressure was set at 0.99 mbar, drift tube voltage at 300 V and drift tube temperature at 60°C corresponding to an electric field strength applied to the drift tube (E) to buffer gas density (N) ratio of ≈ 120 Td. Mass spectra were recorded up to m/z 502 at 30 seconds integration time, for one chamber, the VOCs were collected by PTR-ToF-MS 12 acquisitions in 6 minutes.

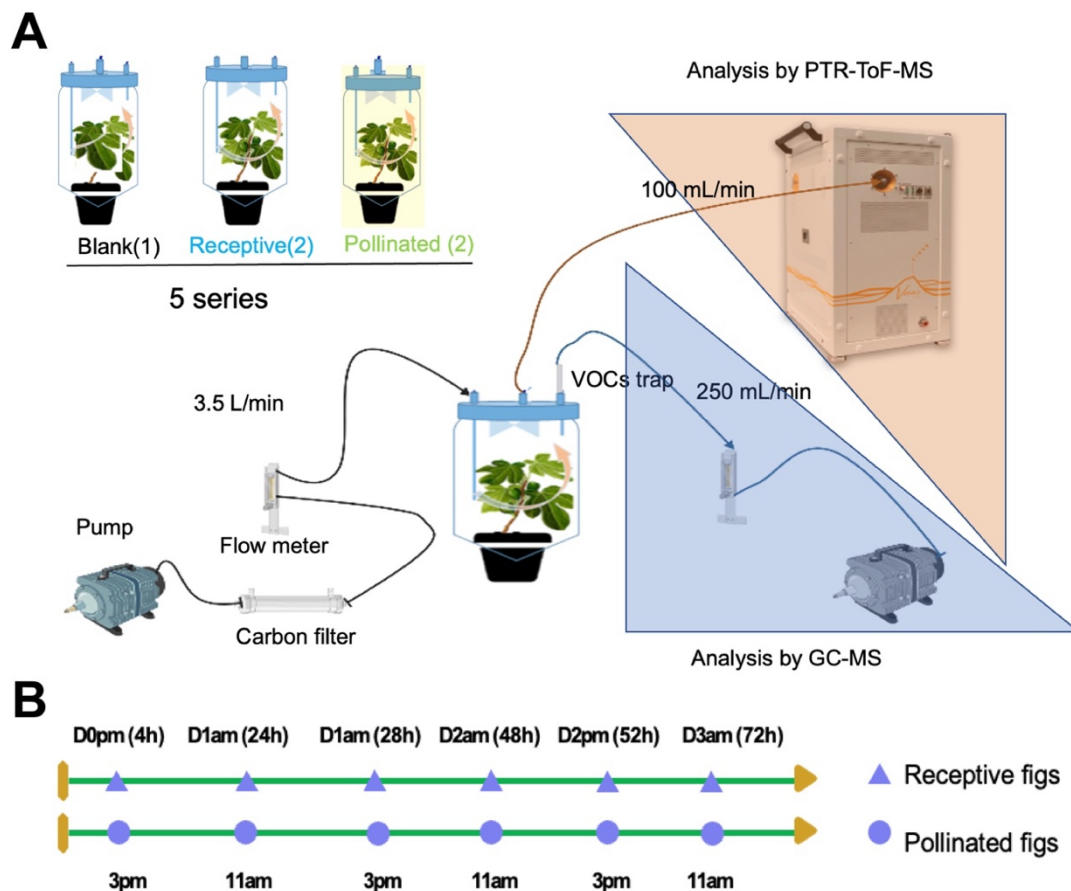
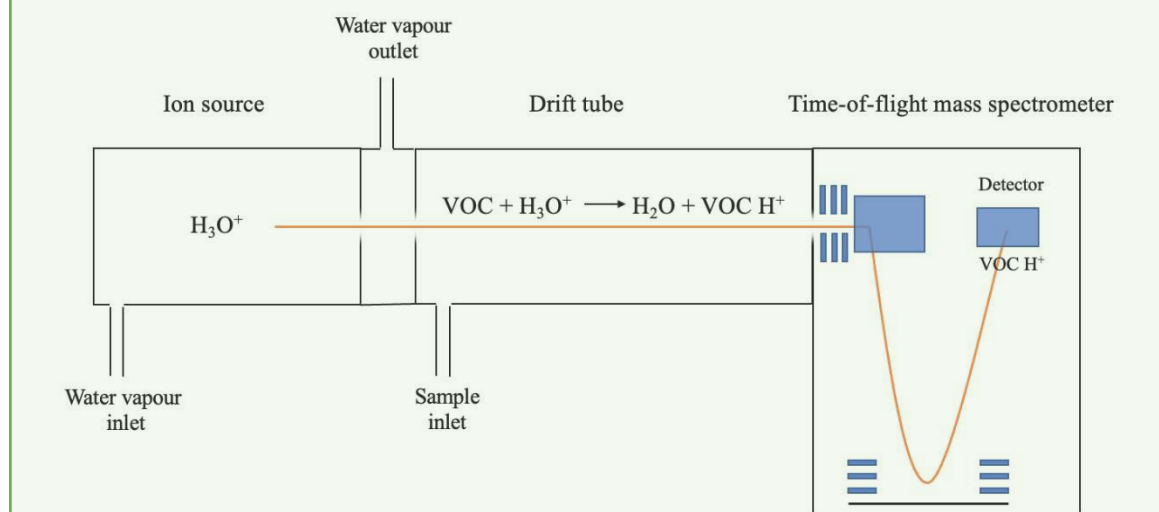


Figure 1. A. Experimental setup for the study of VOC emission rates from *F. carica*. The numbers in brackets illustrate the number of fig trees tested on this modality for one serie. For each series, five plants were individually enclosed in an inert and odourless polytetrafluoroethylene (PTFE) chamber. Air was pushed using a PTFE pump through a carbon filter into the chambers at 3.5 L/min. A PTFE fan ensured air mixing in the chambers. Air flow rates were controlled by flowmeters and all tubing lines were made of PTFE. Each chamber was connected to PTR-ToF-MS and VOCs trap of GC-MS analysis with PTFE tubes and measured the VOCs composition in the chambers at a flow rate of 100 and 250 mL/min, respectively. **B. Timeline of VOCs collection for GC-MS analysis. Collected VOCs of each plant for 5 minutes twice per day (11 am and 3 pm).** Triangles represent receptive figs, circles represent pollinated figs.

Box 1. Method of Proton-transfer-reaction time of flight mass spectrometry (PTR-ToF-MS).

This is a real-time monitoring of trace VOC analysis. It provides dynamic measurements, increasing the understanding of volatile compounds' generation dynamics. PTR-ToF-MS (Proton-transfer-reaction mass spectrometer) consists of an ion source that is directly connected to a drift tube and an analyzing system (time-of-flight mass spectrometer). Hydronium ions generated from water vapor in an ionizer are reacting with analytes in a drift chamber. Ions are separated in an analyzer based on the mass-to-charge ratio and are subsequently transferred to a detector where identification takes place (as shown in the picture). PTR-ToF-MS is a soft ionization mass spectrometry technique, with VOCs producing less fragments than with other ionization methods. The ions produced by such fragments will have an additional mass unit. For example, the volatile organic compound linalool emitted by the fig tree has a molecular weight of 154.249 g/mol, but will be detected as 155.256 g/mol.



VOC analyses by GC-MS

In addition to PTR-ToF-MS measurements, VOCs were collected by pulling the air out of the chambers at 250 mL/min through an adsorbent steel cartridge acting as VOCs trap filled with 40 mg of Carbotrap and 80 mg of Tenax phases (Sigma-Aldrich, Munich, Germany). We collected VOCs of each plant for 5 minutes twice per day (11 am and 3 pm) (See Figure 1B). These traps were stored at -20°C until analysis by GC-MS.

Samples were analysed by gas chromatography-mass spectrometry (GC-MS) using similar protocol as Proffit et al, 2020. These samples were analysed by a gas

chromatograph (GC, Trace 1310, Thermo Scientific, Milan, Italy) coupled with a mass spectrometer (MS ISQ QD Single Quadrupole, Thermo Scientific, Milan, Italy) equipped with an Optima 5-MS capillary column (30 m, 0.25 mm ID, 0.25 μ m film thickness, Machery-Nagel, Düren, Germany). The injection system was composed of a Thermal Desorption Unit (TDU) and a Cold Injection System (CIS) (Gerstell, Mülheim, Germany). Absorbent traps were handled with a Multi-Purpose Sampler (Gerstell, Mülheim, Germany). The GC temperature program for the analysis was as follow: oven held at 40 °C for 3 minutes, then increased from 40 °C to 220 °C at 5 °C /min, and finally from 220 to 250 °C at 10 °C/min, temperature was maintained at 250 °C for 2 minutes. The GC-MS transfer line was set-up at 250 °C. The ion source of the mass spectrometer was at 200 °C. The MS acquisition was from 38 m/z to 350 m/z at 70 eV ionisation energy. The Xcalibur TM 266 software (Thermo Scientific TM, Milan, Italy) was used for data processing. Retention index (RI) was calculated by a series of *n*-alkanes (Alkanes standard solution, 04070, Sigma Aldrich, Munich, Germany). Compounds were identified based on computer matching of the mass spectra with library (NIST 2017 MS), and confirmation by comparison of retention index reported in literature (Adams, 2007). When possible, mass spectra and retention index were compared to those of reference compounds run in the same GC-MS with the same method. Putative contaminant compounds were subtracted from our chromatograms by comparing spectra of each sample with the respective control sample. Trace compounds, which always had a mean per variety of less than 0.1%, were excluded from the analyses.

Statistical analysis

All the data on chemical analyses were performed in R (v. 4.2.1; R Development Core Team, 2013) with the different packages *provoc* (Huguenin, 2022), *vegan* (Oksanen et al. 2022), *RVAideMemoire* (Hervé, 2022), *lme4* (Douglas et al., 2015) and *gamm4* (Wood and Scheipl, 2020).

The processing of the raw data from PTR-ToF-MS was performed with the R package *provoc*. Four attractive compounds were identified by their major fragment

weight (MW) plus 1 in the PTR-ToF-MS. The intensities of ion peaks of the four attractive compounds for pollinators, respectively, 137 (*S*-linalool MW 136+1), 109 (benzyl alcohol MW 108+1) and 159 ((*Z*)-linalool oxide (furanoids) and (*E*)-linalool oxide (furanoids) MW 158+1), were extracted. For comparison with the four attractive VOCs, the 205 peak (a mixture of sesquiterpenoids MW 204+1) is known to be not involved in attracting pollinators (Proffit et al., 2020). For each measurement, the intensity of each ion peak was estimated by the average of the last six acquisitions (from 12 acquisitions). For each measurement point, the peak intensity of the corresponding empty control chamber (blank) was then subtracted to eliminate background odour. The peak intensities were converted by dividing by the dry weight of the fig in the chamber and then by the time required for the total renewal of the air. The time for total air change in the chamber was calculated by dividing the volume of each chamber by the airflow entering the chamber (in minutes).

Statistical analyses for the effect of abiotic factors on the emission of the VOCs from receptive figs in *F. carica*, statistics were based on the construction of Generalized Additive Models (GAMs) with the peak intensities of VOCs (137 (linalool MW 136+1), 109 (benzyl alcohol MW 108+1), 159 ((*Z*)-linalool oxide (furanoids) and (*E*)-linalool oxide (furanoids) MW 158+1) and 205 peak (a mixture of sesquiterpenoids MW204+1)). The GAMs allow to determine statistically whether the variability of the intensity of the peaks were significantly related to parametric variables and to smooth the fit of the model in case the relationship was non-linear. GAMs were built for data located between 6 am and 7 pm, VOCs emission between 8 pm and 5 am being almost zero for the 72 hours measurement. For each VOC, a model was constructed with the three abiotic parameters (humidity, light intensity, temperature) as fixed effects and individual plant and series as random effects. Significance was measured by analysis of deviance (ANOVAs). Pearson correlation tests were also performed on between abiotic factors to determine confounding effects between them.

For the effect of pollination, GAMs were also built on the peak intensities of VOCs (137 (*S*-linalool MW 136+1), 109 (benzyl alcohol MW 108+1) and 159 ((*Z*)-linalool

oxide (furanoids) and (*E*)-linalool oxide (furanoids) MW 158+1) and 205 peak (a mixture of sesquiterpenoids MW 204+1)) with individual plant and series as random effects, followed by analyses of deviance (ANOVAs). It was also tested whether the smoothness was significant and whether there was a linear relationship. As we want to know when the pollinator-attractive VOCs begin to decrease after pollination. Linear Mixed Models (LMMs) (fixed effects: time, treatment and their interaction; and random effect: individual and series) were built only of the data comprising the 13 hours after pollination (i.e., between 11am and 12 pm on average). ANOVAs were carried out on the LMEs and then comparison of the VOCs between the two treatments (receptive and pollinated) at each hour within this interval.

The proportions of the four attractive VOCs were estimated by the intensity of the total peak area of four attractive VOCs from GC-MS for receptive and pollinated group at 4, 24, 28, 48, 52 and 72 hours (D0pm, D1am, D1pm, D2am, D2pm, D3am) (See Figure 1B). In order to compare of the relative proportions of the four attractive between pollinated and receptive group a distance matrix calculating the dissimilarity between all samples in pairs using a Bray-Curtis index was built. Then the distance matrix was plotted using non-metric multidimensional scaling analysis (NMDS). Effects of treatments (receptive and pollinated) and time were tested on the distance matrix, using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations. If significant effects were found, pairwise comparisons between samples were performed with correction of *p*-values using the “false discovery rate” (fdr) method (Benjamini and Hochberg, 1995).

Attractions tests with pollinators

To test whether females of *B. psenes* were attracted by VOCs emitted by receptive and 72 hours pollinated figs of *F. carica*, we used the same olfactometer system as the one employed in a previous study (Proffit et al, 2020). Y-tubes were in glass with a diameter of 40 mm, with each lateral arm of 200 mm and the central arm of 150 millimetres (mm) long. Odour sources were placed in 500 ml glass containers and connected to one of the lateral arms of the Y-tube, the other with only air (blank). Air

was purified with activated charcoal, then humidified with distilled water and blown into the glass containers (200mL/min per arm). Y-tubes and glass containers were cleaned with acetone after each test to remove any chemical traces left by the insects. The positions of odour sources were inverted between the two arms in each successive trial in order to avoid a directional bias. Fifty fig wasps were tested per treatment for the bioassays. Air was blown for one min before insects were introduced into the olfactometer. The choices made by each individual were recorded over a ten-minute period. We considered that wasps did not choose when they stayed motionless in the departure section and/or the central arm before the olfactometer bifurcation after these ten minutes. These wasps were not considered in the statistical analyses.

Adult female wasps were collected from mature figs taken randomly from different individual male trees. Due to their very short lifespan outside the fig (less than 24 h), individuals of *B. psenes* were tested shortly after their exit. Each day a maximum of 20 individuals were tested per treatment. The tests were conducted between 1pm and 5pm to limit deviations in insect behaviour over time.

For each odour tested, a non-parametric binomial test was performed to test if selection made by the wasps differed from chance (i.e. 50% on each side).

Results

1. Daily kinetic of emission of VOCs of tree with receptive figs

The intensity of the four masses monitored during the 72h for the receptive fig trees maintained consistent rhythm throughout the monitoring in *F. carica*. with a daily higher concentration peak between 1 and 3 pm, followed by a linear decline of the emission until the middle of the night (around 1 am). This non-linear trend following a daily rhythmicity was further confirmed by the adaptive smoothing of GAMs with linalool (137) (GAM, $\chi^2=3537$, edf (the effective degrees of freedom) =25, $p<0.001$), linalool oxides (159) (GAM, $\chi^2=546$, edf=18, $p<0.001$), benzyl alcohol (109) (GAM, $\chi^2=5590$, edf=28, $p<0.001$) and sesquiterpenes (205) (GAM, $\chi^2=692$, edf=18, $p<0.001$) (Figure 2).

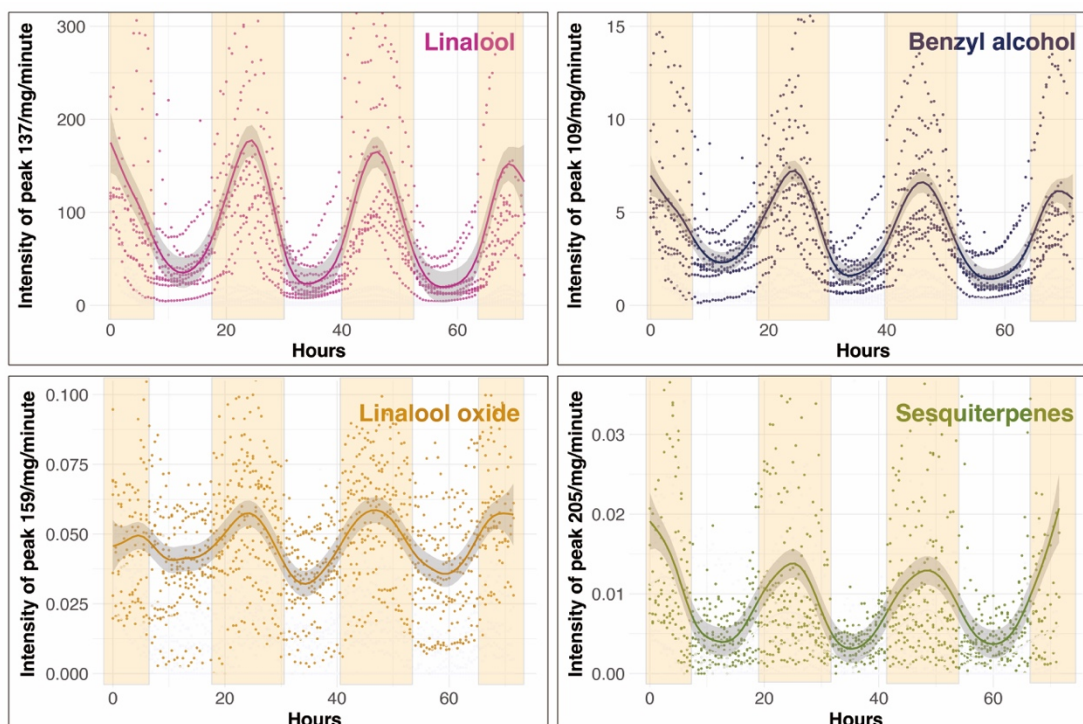


Figure 2. Dynamic emission of pollinator attractive VOCs (137, 159, 109) and sesquiterpenes (205) over 72 hours in fig trees bearing receptive figs. Each dot represents the peak intensity of VOCs of one individual. The line is the regression of GAM modelling of the peak intensity of VOCs over 72 hours. The grey bands represent the standard error of line. Yellow areas represent the day (7am-6pm) and white areas represent the night (6pm-7am). The vertical coordinate of dots represents the peak intensity of VOCs in receptive figs (mg/min), the horizontal coordinate of dots represents the time.

2. Effects of abiotic factors on emission of VOCs from receptive figs in *F. carica*

The abiotic parameters recorded (temperature, humidity and light) had contrasting effects on the intensity of the different Mass of the trees bearing receptive figs. Significant positive correlation was observed between temperature and light ($R^2= 0.70$, $t=45$, $df=2112$, $p<0.001$), significant negative correlations were observed between light and humidity ($R^2 =0.38$, $t=-18$, $df=2112$, $p<0.001$) and between temperature and humidity ($R^2=0.52$, $t= -29$, $df=2256$, $p<0.001$).

Temperature had a significant and non-linear effect on linalool (137) (GAM, temperature: $\chi^2 =12202$, $df=1$, $p<0.001$; $\chi^2 =1408$, $edf=6$, $p<0.001$), benzyl alcohol (109) (GAM, temperature: $\chi^2 =3976$, $df=1$, $p<0.001$; $\chi^2 =509$, $edf=6$, $p<0.001$) and linalool oxides (159) (GAM, temperature: $\chi^2 =5888$, $df=1$, $p<0.001$; $\chi^2 =1659$, $edf=6$, $p<0.001$). The effect was also significant and non-linear for sesquiterpenes (205) (GAM, temperature: $\chi^2 =3426$, $df=1$, $p<0.001$; $\chi^2 =127$, $edf=4$, $p<0.001$) although graphically

the trend seems linear (Figure 3). The emission of linalool, linalool oxides and benzyl alcohol increase with temperature until a peak is reached at 22°C, after which it gradually decreases. This contrasts with the emission dynamics of the sesquiterpenes, which appear to be non-linear and positive over the range of temperature recorded.

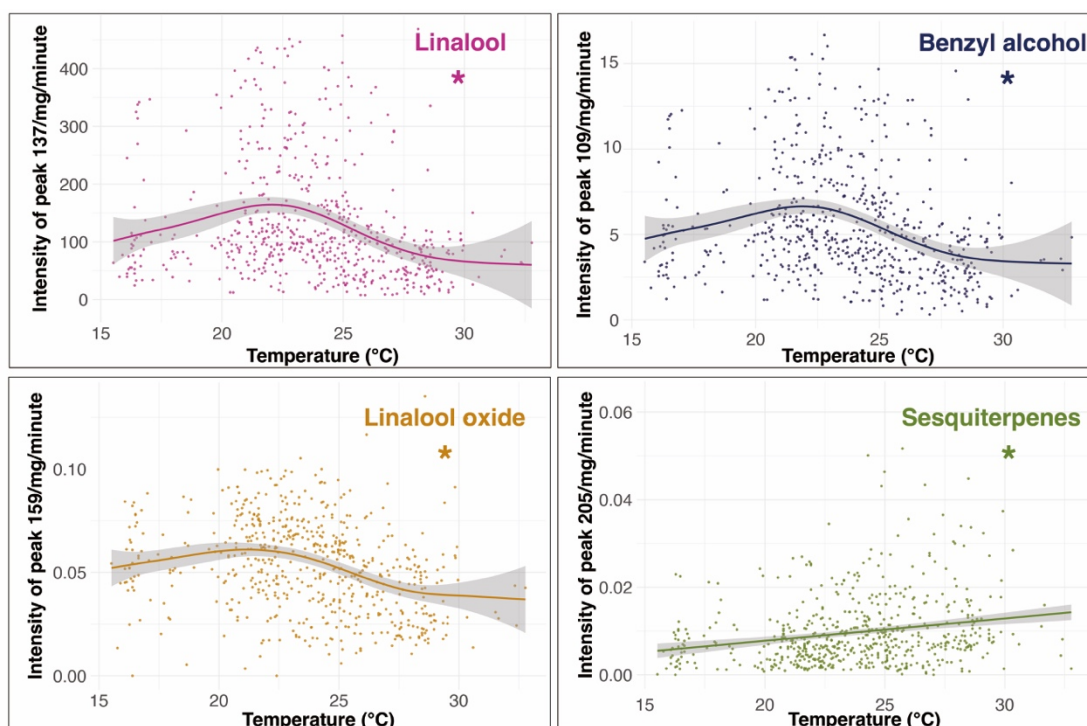


Figure 3. Effect of temperature on the emission of VOCs attracting pollinators (137, 159, 109) and sesquiterpenes (205) in fig trees bearing receptive figs. Each dot represents the peak intensity of VOCs of one individual. The line is the regression of GAM modelling of the peak intensity of VOCs with temperature over 72 hours. The grey bands represent the standard error of line. The vertical coordinate of dots represents the peak intensity of VOCs in receptive figs (mg/min), the horizontal coordinate of dots represents the temperature. The presence of stars (*) means that the effect of temperature is significant ($p < 0.001$).

The relative humidity in the chamber showed a significant effect on the emission of linalool (GAM, humidity: $\chi^2 = 4766$, $df=1$, $p < 0.001$; $\chi^2 = 421$, $edf=8$, $p < 0.001$), benzyl alcohol (GAM, humidity: $\chi^2 = 2549$, $df=1$, $p < 0.001$; $\chi^2 = 200$, $edf=8$, $p < 0.001$), linalool oxides (GAM, humidity: $\chi^2 = 10444$, $df=1$, $p < 0.001$; $\chi^2 = 762$, $edf=7$, $p < 0.001$) and sesquiterpenes (GAM, humidity: $\chi^2 = 1540$, $df=1$, $p < 0.001$; $\chi^2 = 296$, $edf=6$, $p < 0.001$). The peak intensity of linalool, benzyl alcohol and linalool oxides emissions did not differ until the relative humidity (RH) reached 60%, then, after this threshold, the

emission appeared to increase strongly until saturation (100% RH), the peak intensity of linalool oxide start at 60% RH increase with humidity until a peak is reached at 90% RH and decreased after (Figure 4).

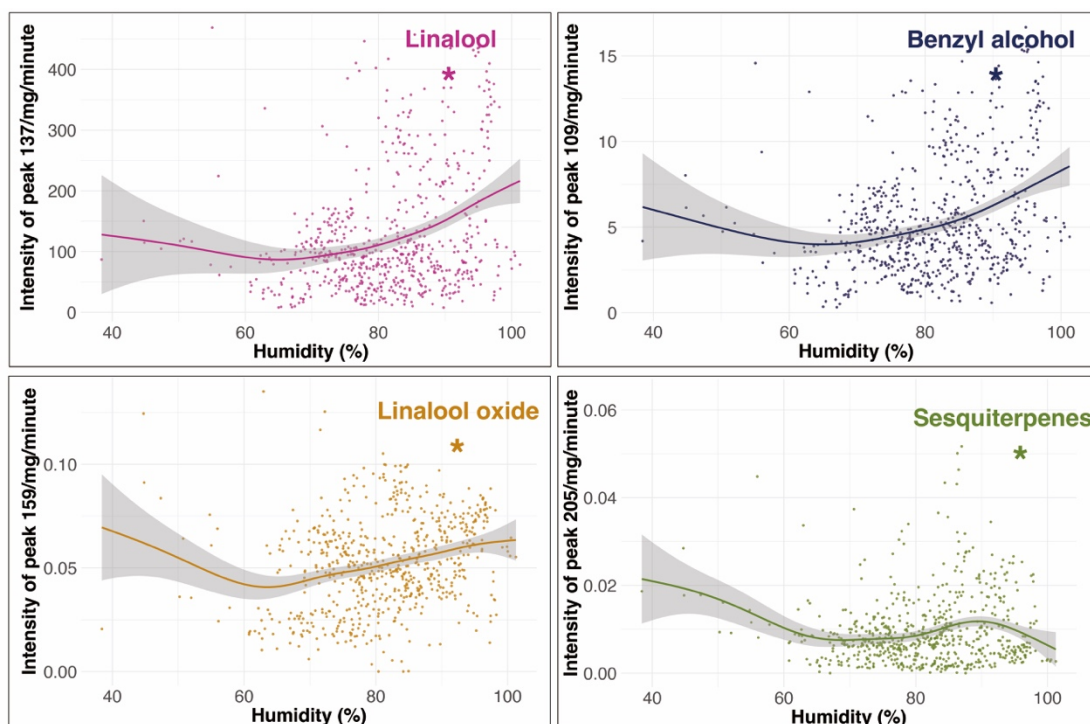


Figure 4. Effect of humidity on the emission of VOCs attracting pollinators (137, 159, 109) and sesquiterpenes (205) in fig trees bearing receptive figs. Each dot represents the peak intensity of VOCs of one individual. The line is the regression of GAM modelling of the peak intensity of VOCs with humidity over 72 hours. The grey bands represent the standard error of line. The vertical coordinate of dots represents the peak intensity of VOCs in receptive figs (mg/min), the horizontal coordinate of dots represents the humidity. The presence of stars (*) means that the effect of humidity is significant ($p < 0.001$).

No significant effect on the emission of linalool (GAM, light: $\chi^2 = 0.1$, $df=1$, $p=0.8$), benzyl alcohol (GAM, light: $\chi^2=0.7$, $df=1$, $p=0.4$) and linalool oxides were detected (GAM, light: $\chi^2 = 0.57$, $df=1$, $p=0.45$). In contrast, light intensity had a significant but non-linear effect on sesquiterpene emission (GAM, light: $\chi^2 = 13$, $df=1$, $p < 0.001$; $\chi^2=52$, $edf=5$, $p < 0.001$) (Figure 5).

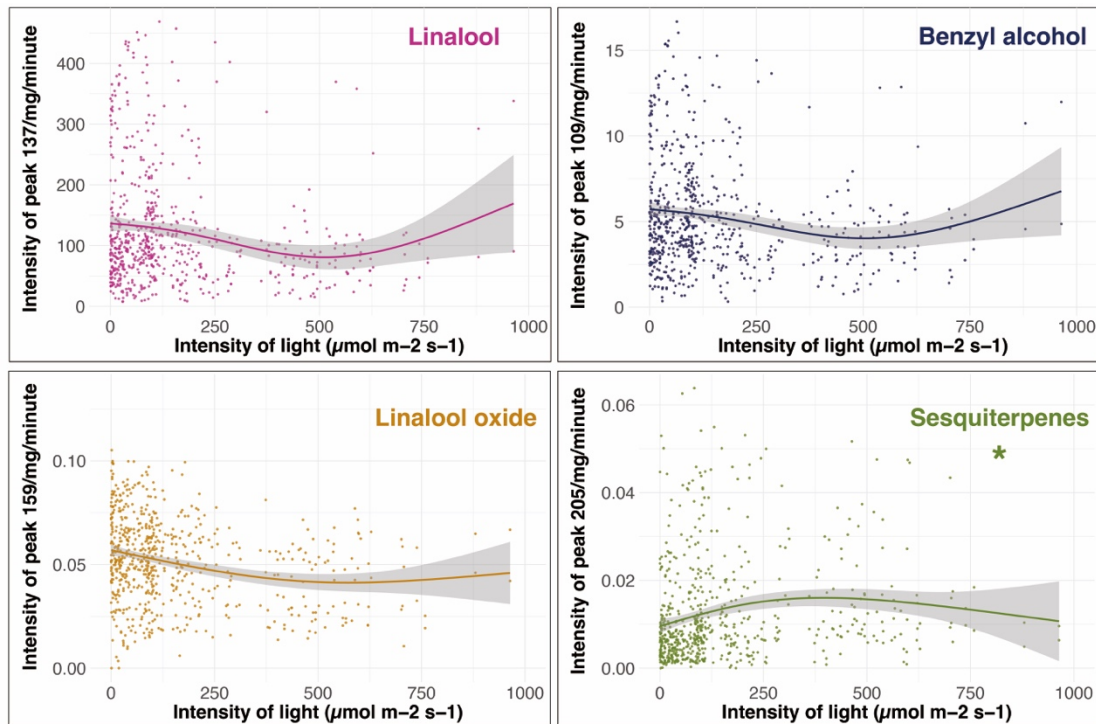


Figure 5. Effect of light on the emission of VOCs attracting pollinators (137, 159, 109) and sesquiterpenes (205) in fig trees bearing receptive figs. Each dot represents the peak intensity of VOCs of one individual. The line is the regression of GAM modelling of the peak intensity of VOCs with light intensity over 72 hours. The grey bands represent the standard error of line. The vertical coordinate of dots represents the peak intensity of VOCs in receptive figs (mg/min), the horizontal coordinate of dots represents the light intensity. The presence of stars (*) means that the effect of light is significant ($p < 0.001$).

Effects of pollination on emission of VOCs in *F. carica*

For the 72 hours of monitoring, the effect of pollination was significant on the emission of linalool (GAM, pollination: $\chi^2 = 6906$, $df=1$, $p < 0.001$), linalool oxides (GAM, pollination: $\chi^2 = 3359$, $df=1$, $p < 0.001$), benzyl alcohol (GAM, pollination: $\chi^2 = 9420$, $df=1$, $p < 0.001$) and sesquiterpenes (GAM, pollination: $\chi^2 = 134$, $df=1$, $p < 0.001$) (Figure 6).

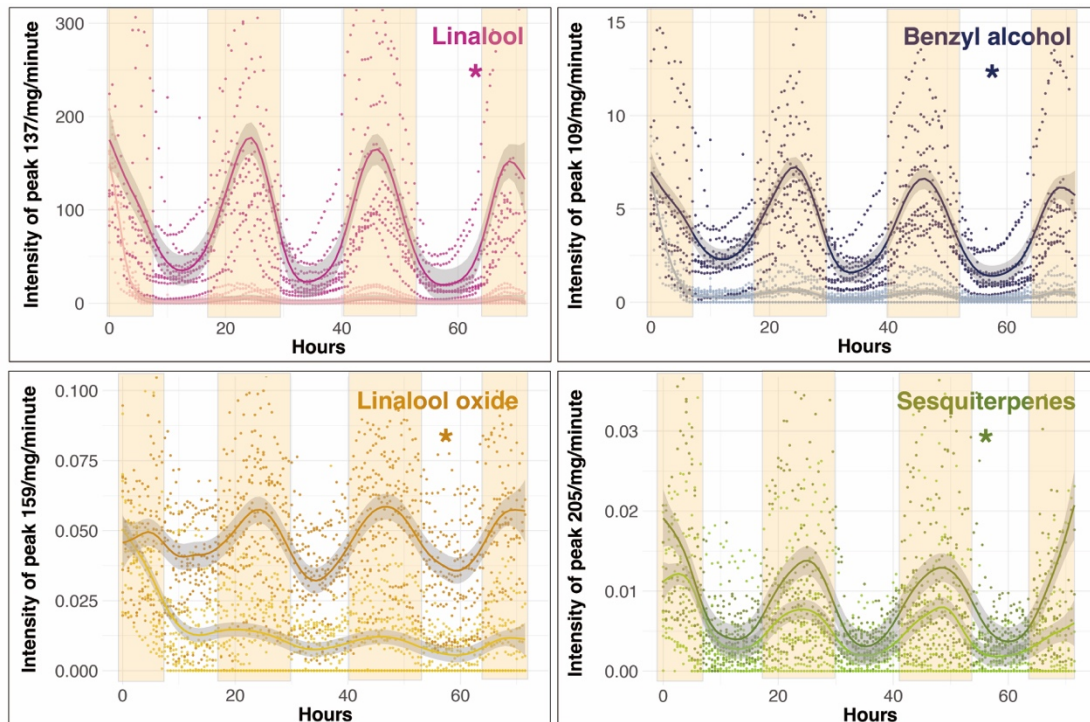


Figure 6. Dynamic emission of VOCs attracting pollinators (137, 159, 109) and sesquiterpenes (205) over 72 hours in fig trees bearing receptive figs (dark colour) and pollinated figs (light colour). Each dot represents the peak intensity of VOCs of one individual. The line is the regression of GAM modelling of the peak intensity of VOCs over 72 hours. The grey bands represent the standard error of line. The dark and light dots(line) represent the peak intensity of VOCs in the receptive and pollinated fig plant trees, respectively. The vertical coordinate of dots represents the peak intensity of VOCs in the receptive and pollinated fig plant trees, the horizontal coordinate of dots represents the time after pollination. Yellow areas represent the day (7am-6pm) and white areas represent the night (6pm-7am). The presence of stars (*) means that the effect of pollination is significant ($p < 0.001$).

For the 13 hours following pollination a significant effect of time, pollination and their interaction on linalool (LMM, time: $\chi^2 = 2652$, $df = 26$, $p < 0.001$; pollination: $\chi^2 = 56$, $df = 1$, $p < 0.001$; interaction: $\chi^2 = 525$, $df = 26$, $p < 0.001$), benzyl alcohol (LMM, time: $\chi^2 = 2631$, $df = 26$, $p < 0.001$; pollination: $\chi^2 = 25$, $df = 1$, $p < 0.001$; interaction: $\chi^2 = 304$, $df = 26$, $p < 0.001$) and linalool oxides (LMM, time: $\chi^2 = 415$, $df = 26$, $p < 0.001$; pollination: $\chi^2 = 5.4$, $df = 1$, $p = 0.01$; interaction: $\chi^2 = 150$, $df = 26$, $p < 0.001$). For sesquiterpenes, the time had a significant effect (LMM, time: $\chi^2 = 836$, $df = 26$, $p < 0.001$), but neither the pollination nor the interaction of the two factors (LMM, pollination: $\chi^2 = 0.55$, $df = 1$, $p = 0.45$; interaction: $\chi^2 = 12.1$, $df = 26$, $p = 0.99$) (Figure 6). The pairwise comparisons showed a significant divergence between fig trees bearing receptive and pollinated fig from 5 hours until 13 hours after pollination for linalool ($t = 2.28$, $df = 17$, $p = 0.036$) and

benzyl alcohol ($t=2.3$, $df=17$, $p=0.034$), as well as a significant difference from 12 hours until 13h after pollination for the linalool oxides ($t=2.16$, $df=17$, $p=0.046$).

Fig trees bearing pollinated figs showed a reduction in emissions of VOCs that attracted pollinators compared to fig trees bearing receptive figs from the first hour after pollination, followed by a cessation the following day. In contrast, the sesquiterpenes maintained an emission rhythm close to the original one even after pollination.

Time (PERMANOVA, $F_{5,113} = 6.0$, $df=5$, $p=0.001$), pollination ($F_{1,113} = 210.2$, $df=1$, $p=0.001$) and their interaction ($F_{5,113} = 7.8$, $df=5$, $p=0.001$) had a significant effect on the relative proportion of the four pollinator-attractive VOCs. More precisely, pairwise analysis showed a significant divergence in the floral scent between pollinated and receptive fig trees, as early as the first VOCs collection and analysis by GC-MS, on the day of launch (D0pm, about 4h after pollination). The difference between pollinated and receptive trees persists throughout the follow-up. The first significant change in relative proportion from receptive figs occur from 5 hours after pollination (D0pm), a second significant change from receptive figs occur from 5 hours after pollination till 28 hours after pollination (D1am, D1pm), a third significant change in proportions occurred from 28 till 72 hours (D2am, D2pm, D3am) (Figure 7).

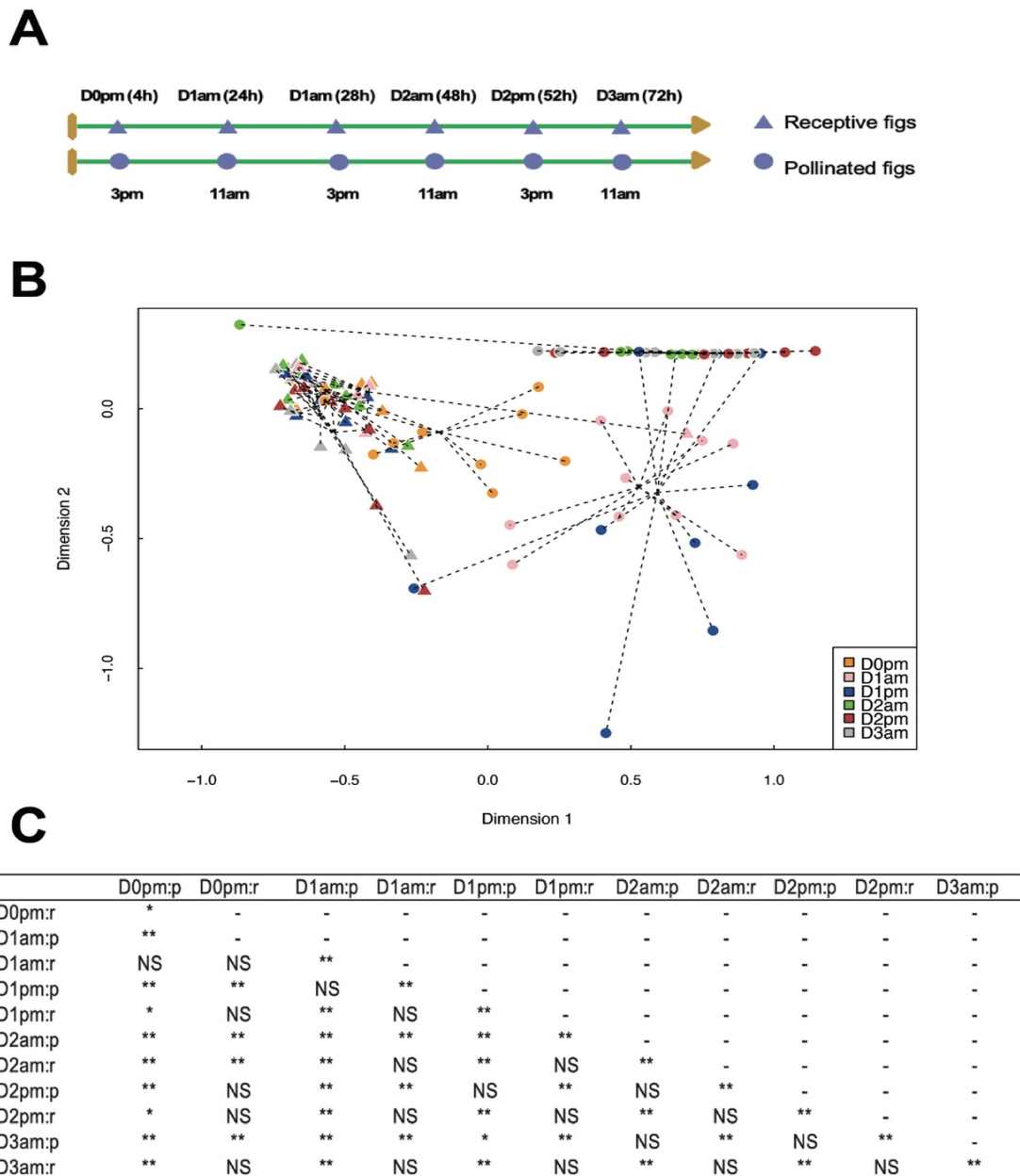


Figure 7. A. Timeline of VOCs collection for GC-MS analysis. Collected VOCs of each plant for 5 minutes twice per day (11 am and 3 pm). Triangles represent receptive figs, circles represent pollinated figs. B. Non-metric multidimensional scaling ordination (NMDS) of the relative proportions of the four pollinator-attractive VOCs of fig trees bearing receptive and pollinated figs based on Bray-Curtis distance (Stress = 0.042). The circles illustrate pollinated fig trees while the triangles illustrate receptive fig trees. C. Results of pairwise comparisons of the relative proportions using PERMANOVA are presented for the four VOCs responsible for pollinator attraction. $p < 0.01$: **, $p < 0.05$: *, $p > 0.05$: NS. p represents pollinated figs. r represents receptive figs.

Behavioural response of the pollinator *Blastophaga psenes*

In our olfactometer bioassays, females of *B. psenes* were significantly more attracted by VOCs released by receptive figs than by the control (empty) (binomial test, $N = 47$, $P < 0.001$). On the contrary, no significant difference was noted between the 72 hours figs after pollination (binomial test, $N = 48$, $P = 0.19$) and the control (empty) (Figure 8).

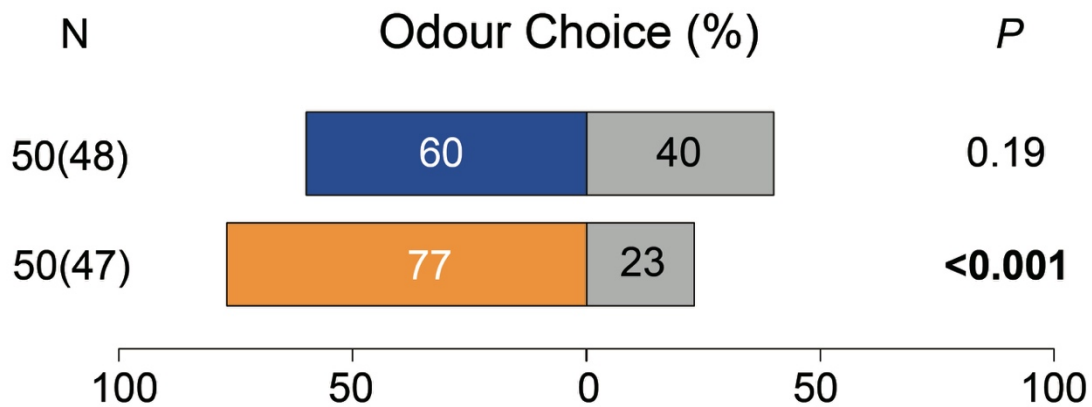


Figure 8. Attraction responses of *Blastophaga psenes* females towards VOCs 72 hours after pollination and receptive figs. Tests were conducted in Y-tube olfactometers where females were allowed to choose between VOCs of either 72 hours after pollination (blue) or receptive (i.e. non pollinated) figs (orange) in one branch, and control odour (blank) (grey) in the other. Number of wasps tested (N), number of individuals that making a choice (in parentheses), and P -values (exact binomial test) are indicated for each comparison.

Discussion

This study provides new insights into the emission dynamics of floral VOCs from fig trees. We had an original approach with a new kind of apparatus, a PTR-ToF-MS, used for the first time to monitor changes in VOCs emitted from the figs of the Mediterranean fig tree (*Ficus carica*) on a fine time scale. To our knowledge, only one study performed real-time measurements of flower VOCs by directly injecting volatiles into a PTR-ToF-MS for floral scent composition and fine-scale timing in two moth-pollinated Hawaiian *Schiedea*. (Powers et al., 2020). This technique allowed to characterise precisely the emission dynamics of fig trees at the receptive phase, and to describe the effect of major abiotic factors. In addition, we tested experimentation the effect of pollination on the emission of floral scents.

Daily kinetic of emission of VOCs of fig tree with receptive figs

We showed, as hypothesized, that fig tree bearing receptive figs presented an emission rhythm of VOCs through the day. The larger emission peak was from 11 am to 3 pm which correspond to the main activity time of *B. psenes*. In several other systems, it has been reported that the rhythmicity of floral scent emission is coherent with pollinator activity (Hoballah et al, 2005; Steen et al, 2019). The timing of VOCs emissions by *Petunia axillaris* is in tune with nocturnal hawk moth activity (Hoballah et al, 2005). Furthermore, the rate of emission of the VOCs involved in the attraction of pollinators seems to be controlled by an internal clock of the plant, that regulate diurnal rhythms of VOCs emission in *Pentunia* (Fenske et al., 2015). This certainly happens in *Ficus* trees too. Several studies have shown that gene expression of key enzymes, final enzymes in biosynthetic pathways and their enzyme activity play a role in control of the circadian rhythmic emission of plant VOCs (Zeng et al, 2017). For instance, in snapdragon flowers, the rhythmicity of terpenoids emission were controlled by the endogenous clock through the methylerythritol phosphate (MEP) pathway (key enzyme) (Dudareva et al., 2005). Also, transcript levels of QH6, a β -pinene synthase gene (final enzyme) continue to fluctuate with circadian rhythms, resulting in diurnal patterns of β -pinene content and emissions, when *Artemisia annua* was cultivated under constant light or constant dark conditions (Lu et al., 2002). Another example shown that salicylic acid carboxyl methyltransferase activity (enzyme activity) oscillations were coincided with nocturnal methyl salicylate emission in *Stephanotis* flowers (Pott et al., 2003). Additionally, the efficiency of VOC emission may depend on substrate availability (Benzoic acid's substrate regulated the rhythmic production and emission of VPBs such as methyl benzoate in petunia flowers), but also on transcription factors (PhLHY regulates the production of floral volatiles in petunia) (Fenske et al., 2015). Therefore, it would be interesting in the future to conduct experiments keeping the plants in darkness to determine whether the diurnal rhythm of floral VOC emission in fig trees is due to an internal clock (i.e., a circadian rhythm). In addition, monitoring of VOC emissions and the expression of certain genes in fig trees under these conditions

(in the dark) would allow us to deepen our knowledge of the factors regulating this rhythm whether through gene expression, transcription factors and or substrate availability.

Interestingly, the rhythmicity of floral emissions from receptive figs was very homogeneous during the 72 hours of measurements. Moreover, multivariate analyses of relative proportions also revealed a homogeneity of relative proportions of the four compounds attracting pollinators during the day in the receptive figs during these 72 hours. However, in *Ficus carica*, only a particular proportion of the four VOCs can attract pollinators (Proffit et al., 2020). Thus, our results suggest that pollinator attraction is probably the main factor affecting the floral VOC emission as has been suggested for other systems (Schiestl and Johnson, 2013).

Effects of abiotic factors on emission of VOCs in *F. carica*

The hypothesis that abiotic factors influence VOC emissions has also been confirmed and the results observed for temperature and humidity (climatic factors) on receptive fig trees are consistent with our hypothesis, but not in terms of light.

For the temperature factor, VOC emission of the four attractive-VOCs increases with increasing temperature to a threshold value around 22°C and then decreases. This pattern of emission is coherent with what has been reported in other Mediterranean plants (Farré-Armengol et al., 2014). The increase in floral emission with temperature may be in part due to the temperature dependence of the physicochemical properties of VOCs and to the enzymatic activities of terpene synthases (Holopainen and Gershenzon, 2010). For instance, the activities of key pathway enzymes and expressions of several scent-related genes under varying temperatures exhibited similar trends with the scent emission patterns in *Jasminum auriculatum Vahl* (Barman and Mitra, 2021). However, as the optimal temperatures for VOC emission vary between several Mediterranean plants growing in the same area, it has been pointed out that the observed pattern cannot be due solely to differences in the physicochemical properties of specific compounds, but that specific characteristics of floral physiology probably play an important role in determining the responses of floral emissions to temperature (Farré-Armengol et al.,

2014). On the contrary, for sesquiterpenes, which have a lower volatility than monoterpenes, the continuous linear increase with temperature could be mainly due to the physico-chemical properties of VOCs. In addition, sesquiterpenes may have some heat-tolerant enzymes that can increase even at high plant temperatures (Holopainen and Gershenzon, 2010).

For the humidity factor, there is a significant and positive effect after 60% on the emission of the pollinator-attractive VOCs, but negative on the concentration of sesquiterpenes. Stomatal conductance increases with increasing humidity and therefore VOCs emissions from fig trees are increasing (Lange et al., 1971). The decrease in sesquiterpenes with increasing humidity may occur since they are mainly involved in aerosol formation (Ormeño et al., 2007).

For the light factor, non-linear effect was expected on sesquiterpenes and no effect for four attractive-VOCs. Different biosynthetic pathways, localize in different parts of the plant cell (i.e., cytosol or plastid), are responsible for the production of these VOCs (Pichersky et al., 2006, Maffei, 2010, Picazo-Aragonés et al., 2020). Since the monoterpene biosynthetic pathway, the methylerythritol phosphate (MEP) pathway, is partially located in chloroplasts and requires carbon substrate and co-factors from photosynthesis (Loreto et al., 1996; Niinemets et al., 2002; Rasulov et al., 2009), sesquiterpene biosynthesis, which occurs mainly in the cytoplasm, is independent of light (Lynch et al. 2020). Light dependency is more uncertain for sesquiterpene emissions, whose biosynthesis are thought to proceed in the cytosol. The light responses of sesquiterpene emissions are not exactly known although evidence for light dependency has been reported in some studies (Duhl et al., 2008; Geron and Arnts, 2010).

Effects of pollination on the emission of VOCs in *F. carica*

Floral scent decreases significantly soon after pollination. The effect of pollination on VOC emission variation in *F. carica* is faster than expected. Pollination-induced decrease in the pollinator-attractive VOCs emissions between 5 and 12 hours. Pollination-induced decrease in methyl benzoate emission takes between 35 and 40 h

in snapdragon and 32 h in Petunia, in order to ensure successful fertilisation; they begin only after the pollen tubes reach the ovary (Negre et al., 2003). Unlike other plants, a fig is a hollow and fleshy structure called syconium, with hundreds of tiny flowers surrounded by a thick wall with a unique entrance (i.e. the ostiole) with a central cavity. Only the pollinator can access through the ostiole to be in contact to the flowers. Therefore, an experiment to investigate the post-pollination decrease in scent emission in *F. carica* whether triggered by pollinator or and by pollen deposition on the flower stigma could be an interesting prospect.

The result shows here that the proportion of VOCs responsible for pollinator attraction varies from the five first hours after pollination. This reveals that the selection pressures that kept the proportions stable disappear rapidly after pollination. This was also found in *Ficus racemosa* (Proffit et al., 2018), *Ficus hispida* (Proffit et al., 2008) and *Ficus semicordata* (Chen et al., 2009). Also, behavioural tests showed that receptive figs were significantly attractive, while figs 72 hours after pollination were no more attractive. These can be explained by emissions of VOCs by the figs, which were very low 72 hours after pollination and the proportion of the four attractive-VOCs was different from that of receptive figs. The continued release of sesquiterpenes after pollination could be explained by the fact that they are not attractive to pollinators and are therefore unaffected by these selection pressure. Sesquiterpenes have the other function such as playing a role against herbivores by attracting natural enemies of the herbivores. At the contrary, in *Cirsium repandum*, quantities of aromatic and sesquiterpenoid volatiles declined after pollination, whereas fatty acid derivatives and monoterpenoids, continued to be emitted (Theis and Raguso, 2005). These behavioural test results confirm that the attractiveness of figs after pollination lasts into a 72-hour time. This cessation of attractiveness could even occur within a very few hours after pollination, according to the results of the PTR-ToF-MS monitoring. This rapid change after pollination would limit the attraction of pollinators to figs already visited and redirect pollinators to other figs of the same individual, thus maximising their reproductive success. Indeed, in spring, access to pollinators is a limiting resource

for receptive figs. In addition, these changes would reduce the costs associated with VOC biosynthesis.

Conclusion

This work reveals that pollinator selection pressures can shape the temporal dynamics of floral VOC emissions. Although this is an obligate mutualism based exclusively on an olfactory signal, the effect of these selective pressures on floral odour emission must be considered to better understand VOCs mediated plant-insect interactions. This research also highlights for the first time the rapidity of the VOC emission response to pollination: a significant drop after a few hours, when it was thought to be over a few days at least. This rapid change raises the question of the signal that drives the regulation of floral VOC emissions. Finding the answer to the questions would bring us a more comprehensive and broader view of the regulatory networks for daily scent emission.

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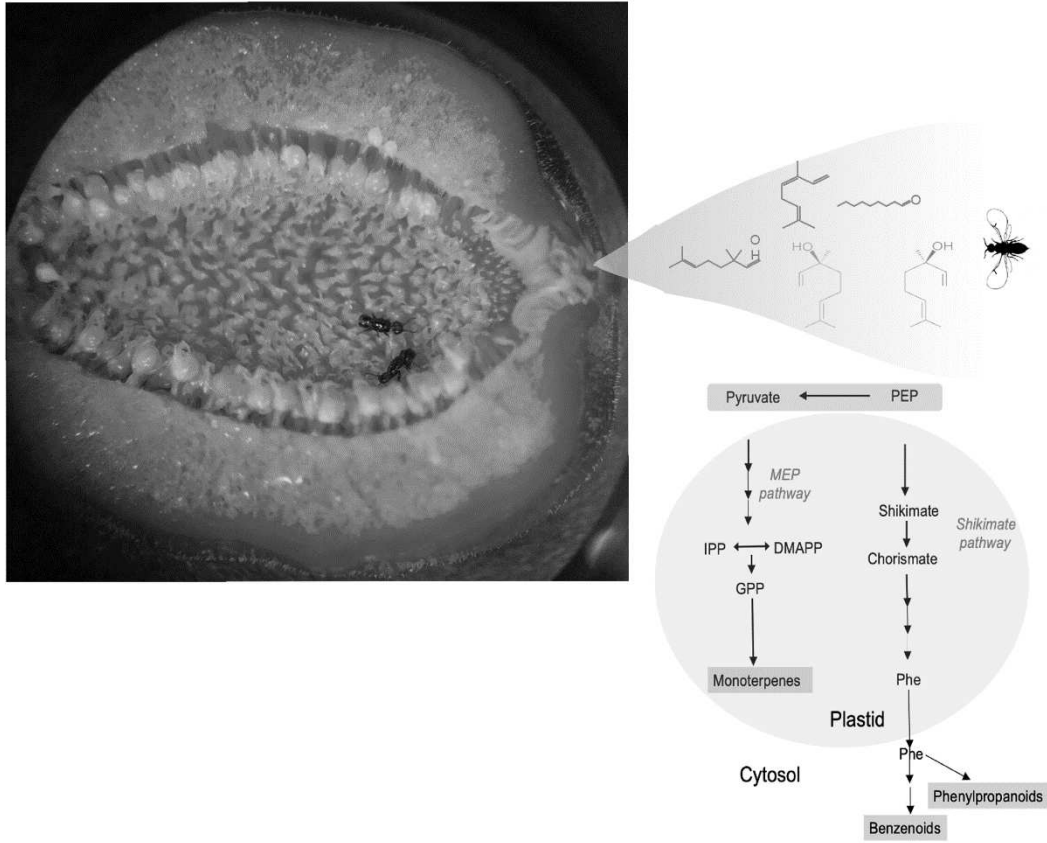
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Chapter 4. Localization of the production of pollinator-attractive volatile organic compounds in Ficus carica

Localisation de la production des composés organiques volatils attractifs pour les pollinisateurs de *Ficus carica*

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Résumé

Les parfums floraux forment des mélange complexe de composés organiques volatils (COVs), jouant un rôle majeur dans la médiation entre partenaires dans les interactions entre plantes et pollinisateurs. Ces COVs sont le résultat de voies de biosynthèse impliquant un grand nombre d'enzymes et sont émis par différentes structures végétales. Jusqu'à présent, un nombre limité d'études ont caractérisé les produits formés par les enzymes impliqués dans les voies de biosynthèse des COVs attirant les pollinisateurs, ainsi que celles sur la localisation de la production de ces produits. Dans la présente étude, la localisation de la production de COVs attirant les pollinisateurs a été étudiée chez le figuier méditerranéen, *Ficus carica*. Dans cette interaction, le pollinisateur est attiré par un mélange de 4 COVs émis en quantité significative par les figes réceptives, c'est-à-dire par leurs inflorescences, mais dont l'émission diminue après la pollinisation (voir chapitre 3). En utilisant la transcriptomique comparative entre figes réceptives et figes pollinisées et leur expression hétérologue, certains gènes liés à la production des COVs responsables de l'attraction des pollinisateurs ont été étudiés. L'expression protéique dans *Escherichia coli* des terpènes synthases (TPS) a révélé qu'une monoterpène synthase catalyse la formation du *S*-linalool (FcTPS1), et qu'une sesquiterpène synthase catalyse la formation du germacrène D (FcTPS3). La localisation de la partie de la fige produisant des COV attractifs pour les pollinisateurs a été étudiée par l'expression des gènes trouvés liés à ces COVs et par des analyses

cytologiques de différents tissus de la figue. La libération des COVs attractifs pour les pollinisateurs à partir de l'ostiole, c'est-à-dire l'entrée de la figue, chez *F. carica* a été directement démontrée pour la première fois. En intégrant des méthodologies de métabolomique, de bioinformatique, de biochimie et de biologie moléculaire, cette étude est l'une des rares à avoir identifié certains gènes liés à la communication chimique entre plantes et pollinisateurs. Les résultats de cette étude ouvrent ainsi la voie à de futures études visant à mieux comprendre la régulation de l'émission de COVs chez *Ficus carica* et les mécanismes moléculaires impliqués dans la diversité chimique d'autres espèces de *Ficus*.

Mots clefs : Figes, Ostiole, COVs attractifs dans la pollinisation, expression des gènes

Localization of the production of pollinator-attractive volatile organic compounds in *Ficus carica*

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Abstract

Floral scents as a complex blend of Volatile Organic Compounds (VOCs), play major roles in mediating encounter between partners in plant-pollinator interactions. These VOCs are the result of biosynthetic pathways involving a large number of enzymes and are emitted from different plant structures. So far, a limited number of studies have characterized the products formed by the enzymes coded by genes involved in biosynthesis of pollinator-attractive VOCs, as well as the location of the production of these products. In the present study, the localization of pollinator-attractive VOC production were investigated in the Mediterranean fig, *Ficus carica*. In this interaction, the pollinator is attracted to a blend of 4 VOCs emitted in significant quantity by the receptive figs, i.e. close inflorescence, but whose emission decreases after pollination. Using comparative transcriptomics between receptive and pollinated figs and heterologous expression, some genes related to VOC production responsible for pollinator attraction were studied. The protein expression in *Escherichia coli* of terpene synthases (TPSs) revealed that one monoterpene synthase catalyzes the formation of *S*-linalool (FcTPS1), and one sesquiterpene synthase catalyzes the formation of germacrene D (FcTPS3). The location of the part of the fig producing VOCs attractive to pollinators was studied using VOCs, transcripts of VOC-related genes and cytological analyses of different tissues. The release of pollinator-attractive VOCs from the ostiole, i.e. the entrance of the fig, of *F. carica* was directly demonstrated for the

first time. By integrating metabolomics, bioinformatics, biochemistry, and molecular biology methodologies, this study is one of the few that has identified certain genes related to plant-pollinator chemical communication. The results of this study pave the way for further studies to better understand the regulation of VOCs emission in *Ficus carica* and the molecular mechanisms involved in the chemical diversity of other *Ficus* species.

Keywords : Fig, ostiole, pollinator-attractive VOCs, gene expression.

Introduction

Pollination by insects is considered to be one of the key events that promote the diversification and evolution of flowering plants. The conservation of plant-insect interactions depends on the success of partner encounter. This encounter is usually mediated by the emission of volatile organic compounds (VOCs) by plants and their reception by pollinators (Raguso, 2008; Hossaert-McKey et al., 2010). So far, more than 1700 VOCs are mainly found in floral scents (Knudsen et al., 2006). This diversity offers infinite possibilities in the composition of olfactory signals. Among the VOCs emitted by plants only a small portion is detected by insect antennae (Bruce et al., 2005, Raguso, 2008). These VOCs belong to four main chemical families according to their metabolic origin: terpenoids, phenylpropanoids/benzenoids, derivatives of fatty acid and amino acid (Knudsen et al., 2006, Maffei, 2010). The VOCs emitted by plants are the result of biosynthetic pathways involving a large number of enzymes (*e. g.* terpene synthases) (Dudareva et al., 2013; Magnard et al., 2015). Even though part of terpenes' biosynthetic pathway and the role of synthase genes for VOC formation have been reported in some species (Nawade et al., 2020), there is a lack of information on genes related to VOCs attractive to pollinators, as well as on the location of VOC production. Therefore, improving our understanding on the VOCs involved in plant-pollinator

encounters and the spatial patterns of these VOCs, is crucial to better apprehend VOCs' mediated plant-pollinator interactions.

Flowers are one of the major biotic sources of emitted scent VOCs. In general, petals are the primary tissue where floral VOCs are emitted. For example, in *Rosa rugosa* flowers, the floral scent is produced by the corolla (Dobson et al., 1990) and in petunia VOC production is primarily localized in the corolla and more specifically the petal limb (Verdonk et al., 2003). In the flowers of *Polianthes tuberosa*, it was clearly found that the petaloid tepals emitted most of the VOCs compared to other floral parts. (Maiti and Mitra, 2017). But other flower parts such as the stamen, pistil and sepals of the calyx and nectaries sometimes can also play an important role in VOCs emission. For instance, the main sources of volatiles were the petals and stamens in *Ranunculus acris* flowers (Bergström et al., 1995) and in *Dianthus inoxianus* flowers, the petals or the calyx emitted almost eightfold relative to the whole flower headspace (Balao et al., 2011). In *Penstemon digitalis*, tissue-specific scents for the stigma and stamens were found (Burdon et al., 2015). In the particular closed inflorescences of the genus *Ficus*, i. e. figs, in the unique entrance of the fig, i.e. the ostiole, composed of imbricated bracts, terpene-secreting cells (Souza et al., 2015) and two types of ostiolar osmophores (Castro-Cárdenas et al., 2022) were found. However, in all these studies, there is no direct evidence that the source of VOC production attracts pollinators from these flowers.

Floral VOCs attract beneficial insects, leading to pollination of the flower. Several studies document changes in floral VOC emission after pollination (Chen et al., 2009; Delle-Vedove et al., 2017; Gu et al., 2012; Muhlemann et al., 2006; Negre et al., 2003; Proffit et al., 2018; Proffit et al., 2008; Rodriguez-Saona et al., 2011; Theis and Raguso, 2005; Tollsten, 1993). The pollinator attractive VOCs emission and related genes expression has been reported to change after pollination. In petunia, the floral VOC methylbenzoate (MeBA) decreases after pollination (Negre et al., 2003). Genes characterized in vitro to catalyze MeBA synthesis, S-adenosyl-L-methionine: benzoic acid/salicylic acid carboxyl methyltransferases (PhBSMTs), also show decreased

expression after pollination in whole flowers in petunia (Underwood et al., 2003). However, there are no more studies on post-pollination changes in the expression genes for pollinator attractive VOCs.

The Mediterranean fig tree, *Ficus carica* L. (Moraceae), represents a perfect model to investigate the genes involved in the biosynthetic pathways of pollinator attracting VOCs and their emission site. *Ficus* trees possess figs being sycones, which are characterized by an enclosed wall with a unique entrance (i.e. the ostiole), with hundreds of tiny florets lining the inside of a central cavity. For *Ficus carica*, those inflorescences are exclusively pollinated by the agaonid wasp *Blastophaga psenes* L. (Agaonidae). This fig-wasp uses VOCs emitted by receptive figs (i.e. at the developmental stage where they are ready to be pollinated) to locate their plant host (Hossaert-McKey et al., 2010). It has been reported that figs of *F. carica* emitted 36 VOCs at the receptive stage (Soler et al., 2012) but this wasp uses only four of them (i.e. Benzyl alcohol, *S*-linalool, (*Z*)-Linalool oxides (furanoids) and (*E*)-Linalool oxide (furanoids)) within a particular proportion to locate their host (Proffit et al., 2020). The pollinating wasps can only enter the syconium through the ostiole, which is covered by superficial bracts (Castro-Cárdenas et al., 2022). Today, there is still no direct evidence showing that the ostiole is a source of the VOCs that attract the pollinating wasps. However, Souza et al. (2015) found terpene-secreting cells in the ostiolar bracts and Castro-Cárdenas et al. (2022) found two types of ostiolar osmophores (mesophyll and diffuse), that could be a source of volatile compounds attracting fig wasps. Therefore, it is necessary to localize the tissue of the figs producing the VOCs in *F. carica*.

Based on these previous results, we hypothesize in figs of *F. carica* that the four pollinator-attractive VOCs and their related genes are expressed in high quantity at the receptive stage and decrease after pollination. We also hypothesize that these VOCs are produced in the ostiole in *F. carica*. To test our hypothesis, we performed a transcriptome-based identification of potential genes involved in the emission of the four pollinator-attractive VOCs by comparing receptive and pollinated figs. In addition, we conducted heterologous expression of some of these genes in *Escherichia coli*. We

then analyzed the VOCs and the expression of related genes from different tissues of receptive figs using GC-MS and q-RT-PCR. In addition, a cytological analysis of the different tissues of receptive figs was performed. With these different experiments we want to characterize: (1) The chemical communication plant/pollinator related genes in *F. carica*, looking at the function of several genes involved in the emission of VOCs responsible for the attraction of pollinators.; (2) The localization of the tissue(s) of the figs producing the VOCs in *F. carica*.

Materials and methods

Study site and plant materials

Ficus carica is a dioecious fig species with the male tree bearing figs where its specific pollinator, the fig-wasp *B. psenes*, oviposits and develops, and the female tree bearing figs that produce seeds where pollinators cannot oviposit and thus cannot develop (Kjellberg et al., 1987). A male fig tree bears figs twice a year (in spring and summer) and the female tree only once (in summer) (for more details about the reproductive biology, see Kjellberg and Lesne, 2020; Proffit et al., 2020). The experiments took place at the Terrain d'Experience (TE) of the Centre d'Écologie Fonctionnelle et Évolutive (CEFE, Montpellier, France, 43°38'19"N, 3°51'49"E) a plateforme of the Labex CeMEB.

1/ The collect for the experiment of the transcriptomic and the genes functional study took place in spring (April-May, 2019) and summer (July-August, 2019). Two branches (for each tree were covered by a nylon bag until figs reached receptivity. In order to pollinate fig trees, we collected figs with emerging insects from trees from natural populations around the Montpellier area. Only female fig-wasps were used because they are the only ones to disperse from one fig to another.

2/ The collect for the experiment for VOCs location study took place in spring (April-May, 2022). Two branches (each branch having more than 3 figs) for each tree were covered by a nylon bag until figs reached receptivity.

VOCs collections and fig sampling

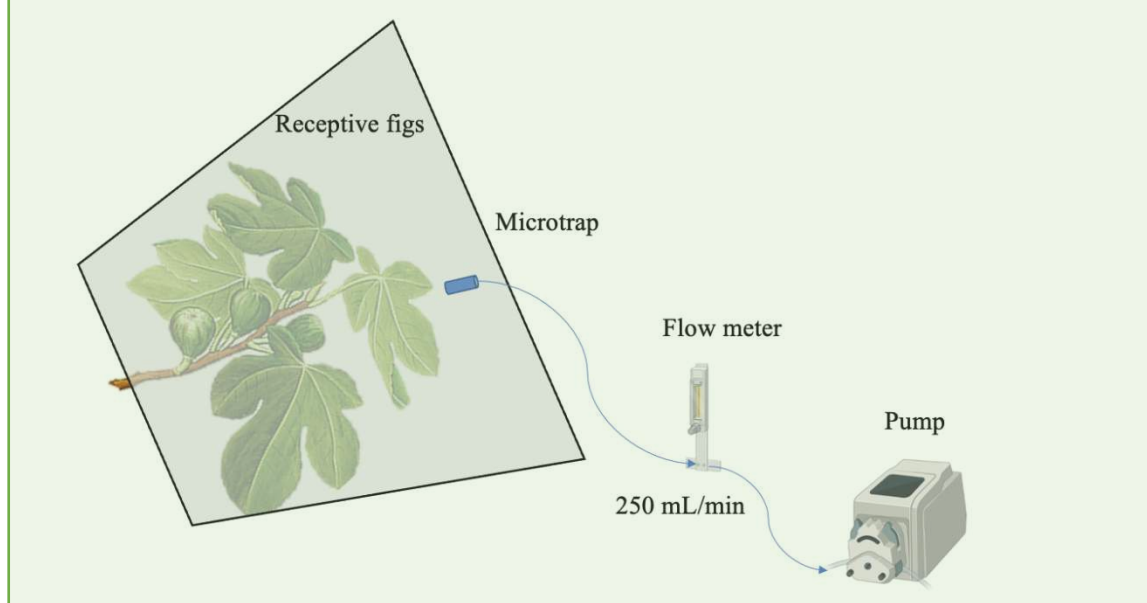
1/ VOCs collections for transcriptomic and genes functional study were conducted on spring male (N = 3) and female fig trees in summer (N = 3). Two branches (each branch having more than three figs) for each *F. carica* tree were covered by a nylon bag until figs reached receptivity. Four VOCs collections were performed for each tree, from which one branch was used just before the experimental pollination ('receptive' group), and then at five days after introducing pollinators ('pollinated' group); the other branch was used just before the experimental pollination ('receptive' group), and after five days without pollination ('nonpollinated' group). For these collections, at least 3 receptive figs per individual tree were enclosed in a polyethylene terephthalate (Nalophane®, Kalle Nalo GmbH, Wursthüllen, Germany) bag, ChromatoProbe® quartz microvials (Varian Inc., length: 15 mm; inner diameter: 2 mm), containing 3 mg of a 1:1 mixture of Tenax-TA and Carbotrap (60–80 and 20–40 mesh respectively, Sigma-Aldrich, Munich, Germany) were used as adsorbent traps. One microliter of a solution of internal standards (*n*-nonane and *n*-dodecane, 110 ng/μl of each) was added to each trap before scent collection. Bags containing figs were left in the shade for 30 min and air (flow rate 300 ml/min) was drawn out of the bag and through the adsorbent trap for 5 minutes in spring and 30 minutes in summer (Proffit et al., 2020). In parallel, for each control collection, extractions were performed using empty bags, to control for ambient contaminant compounds. We collected one control per site per collection day. Collections were done under natural light and ambient temperature, between 11 am and 4 pm with correspond to the maximum dial emission of VOCs (Li et al., unpublished data). These microtraps were stored at -20°C until analysis by GC-MS. After VOCs collection, all the figs were collected and immediately transferred to liquid nitrogen container and stored at -80 °C for RNA isolation.

2/ VOCs' extraction for VOCs location study was performed with spring male figs (N = 3). When figs reached receptivity, we collected one fig from one branch, then extracted VOCs from different tissues of the three figs: flowers, ostiole and wall. Each tissue was extracted by immersion in 1mL hexane for 1 minute. These liquid samples

were stored at -20°C until analysis by GC-MS. The different tissues were dried in an oven at 40°C for at least 72 hours to get the dry weight of each tissue. We also cut one fig fruit from the same branch with clean knife, separating flowers, ostiole and wall from the fig, each tissue was then immediately transferred to liquid nitrogen and stored at -80°C for RNA isolation.

Box 2. Method of VOCs collection.

For each collection, at least 2 receptive figs per individual were enclosed in a polyethylene terephthalate (Nalophane®, Kalle Nalo GmbH, Würsthüllen, Germany) bag of volume around 5 L. Microtraps, ChromatoProbe® quartz microvials (Varian Inc., length: 15 mm; inner diameter: 2 mm), are containing 3 mg of a 1:1 mixture of Tenax-TA and Carbotrap (60–80 and 20–40 mesh respectively, Sigma-Aldrich, Munich, Germany) were used as adsorbent traps. One microliter of a solution of internal standards (*n*-nonane and *n*-dodecane, 110 ng/ul of each) was added to each trap before scent collection. Bags containing figs were left in the shade for 30 min, and during 10 min air (flow rate 250 ml/min) was drawn out of the bag and then through the adsorbent trap for 5 min. In parallel, for each control collection, extractions were performed using fig trees without figs, to control for compounds from leaves and ambient compounds.



Chemical analysis of VOCs

Samples were analysed by gas chromatograph (GC, Trace 1310, Thermo Scientific, Milan, Italy) coupled with a mass spectrometer (MS, ISQ QD Single Quadrupole, Thermo Scientific, Milan, Italy), with an Optima 5-MS capillary column (30 m, 0.25

mm ID, 0.25 μm film thickness, Machery-Nagel, Düren, Germany). The stereochemistry of linalool was determined in the same GC-MS apparatus equipped with a β -cyclodextrin chiral capillary column (Cyclosil-B, 30 m–0.25 mm i.d., 0.25 μm film thickness, Agilent J&W columns, USA). The injection system (for microtraps) is composed of a Thermal Desorption Unit (TDU) and a Cold Injection System (CIS) (Gerstell, Mülheim, Germany), absorbent traps were handled with a Multi-Purpose Sampler (Gerstell, Mülheim, Germany). Liquid samples were injected manually. The temperature program for absorbent traps and liquid samples analyses was as follow: oven held at 40 °C for 3 minutes, then increased from 40 °C to 220 °C at 5 °C min^{-1} , and from 220 to 250 °C at 10 °C min^{-1} , temperature was maintained at 250 °C for 2 minutes. The transfer line was 250 °C. The ion source of the MS was 200 °C. The mass spectra acquisition was from 38 m/z to 350 m/z at 70 eV ionisation energy. The Xcalibur TM 266 software (Thermo Scientific TM, Milan, Italy) was used for data processing. Retention index (RI) was calculated by a series of *n*-alkanes (Alkanes standard solution, 04070, Sigma Aldrich, Munich, Germany). Compounds were identified based on computer matching of the mass spectra with library (NIST 2017 MS), and confirmation by comparison of retention index reported in literature (Adams, 2007). When possible, mass spectra and retention index were compared to those of reference compounds run in the same GC-MS with the same method. Putative contaminant compounds were subtracted from the chromatograms by comparing spectra of each sample with the respective control sample. Trace compounds, which always had a mean per individual of less than 0.1%, were excluded from the analyses.

In order to estimate the quantity of VOCs emitted by receptive and pollinated fig (2019 experiment (1)) of each sample, the calculation was performed using the two internal standards as reference and the following equation:

$$Q_x = \left(\left(\frac{A_x}{A_{C9}} \right) + \left(\frac{A_x}{A_{C12}} \right) \right) * 110/2 * 20$$

Where Q_x : estimated quantity of the VOCx (ng fig^{-1}); A_x : peak area of the VOCx, A_{C9} : peak area of the *n*-nonane, A_{C12} : peak area of the *n*-dodecane. In each sample 110

ng of each internal standard were injected.

In order to evaluate the concentrations of the different VOCs emitted from different tissues (2022 experiment (2)), peak areas of VOCs were integrated using the Total Ion Chromatogram (TIC) and calibration factors were calculated with the same spectral method. To do so, we prepared a stock solution in hexane (Pestnorm, >99.8%) of four pollinator-attractive VOCs, each of them at a mean concentration of 10 $\mu\text{g}/\mu\text{L}$: benzyl alcohol (Fluka, CAS Number: 100-51-6; purity: >99.5), (*Z*) and (*E*)-linalool oxide furanoid (Fluka, CAS Number: 68780-91-6; purity: >97%) and *S*-linalool (Fluka, CAS Number: 78-70-6; purity: >99%). In order to have calibration in the concentration range of the VOCs detected in fig samples, dilutions of 1:100,000, 1:10,000, and 1:1,000 of this stock solution were prepared in hexane. For each of the solution, 1 μL was injected in the adsorbent trap, with three replicates per solution, and analyzed by GC-MS using the same method as for the fig samples. Calibration factors were obtained for each VOC using the slope of the regression between their peak area (integrated from TIC) and their concentration in the three replicates of the four dilutions, setting the intercept to zero.

RNA isolation and transcriptome sequencing

The following experienceq wer conducted at the Laboratory LBVpam (Laboratoire de biologies végétales appliquées aux plantes aromatique et médicinales) in Saint Etienne. Figs were ground in liquid nitrogen and total RNA extraction was performed according to manufacturer's instructions using the kit NucleoSpin RNA Plus. RNAs were quantified using a Nanodrop 2000C (Thermo Scientific, Wilmington, USA). The samples from 2019 (exp 1: gene functional study) are analyzed with TapeStation and send to BGI (The Beijing Genomics Institute) for transcriptome resequencing. The samples from 2022 (exp 2: VOCs location study) were reverse transcribed at 50 °C for one hour with an oligo-dT primer using the SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, USA), then stored at -80 °C for q-RT-PCR.

Total RNA was sequenced on the illumina NovaSeq platform (BGI, HongKong). Approximately 70 m 150 bp long paired-end reads were obtained for each sample.

Reads were cleaned with trimmomatic v0.39 (Bolger et al., 2014), removing the first 13 bases of each read, low quality bases (< 35) and short reads (< 50 bases). *F. carica* genome (Usai et al., 2020) was corrected using the cleaned RNA-Seq reads with pilon v1.23 (Walker et al., 2014) and annotated using UniProt database and a custom perl script. Reads were mapped onto the genome with Star v2.7.5a (Dobin et al., 2013) and genome feature counts were obtained using Stringtie v2.1.4 (Pertea et al., 2015). Differential gene expression analysis was conducted within R using the packages tximport (Soneson et al., 2016) and DESeq2 (Love et al., 2014).

Heterologous expression of recombinant TPS in *Escherichia coli*

A total of three terpene genes were identified from these transcriptome databases and gene-specific primer pairs were designed online (<http://www.ncbi.nlm.nih.gov/>). The *F. carica* fig cDNA was amplified with gene-specific primers using a Platinum Taq DNA polymerase (Invitrogen, USA), yielding a targeted product size for three FcTPS genes. The PCR products were first cloned in the pENTR/D-TOPO entry vector (Invitrogen, USA), and subsequently transferred into the pHMGWA (FcTPS1, FcTPS2 and FcTPS3), pHNGWA (FcTPS1) or pdest17 (FcTPS3) vectors of the pHGWA-based series (Busso et al., 2005) using the Gateway LR cloning reaction. *Escherichia coli* Rosetta (DE3) pLysS cells (Novagen, Darmstadt, Germany) were transformed by heat shock with the recombinant expression vector. After induction with 0.5 mmol/L isopropyl-1-thio- β -D-galactopyranoside (IPTG) in terrific broth (TB, a bacterial growth medium) supplemented with 0.5% glycerol, 250 mmol/L D-sorbitol and 2.5 mmol/L betaine, heterologous protein production overnight incubation at 17 °C and 140 rpm. The cells were recovered by centrifugation and immersed in native lysis/binding buffer (50 mmol/L NaH₂PO₄ (pH 8), 0.5 M NaCl, 20 mmol/L imidazole, 5% glycerol, 0.5 mg/mL lysozyme) for 30minutes, followed by sonication. After centrifugation to clarify the lysate, the recombinant proteins were purified by binding to the Talon® metal affinity resin (BD Biosciences Clontech, Palo Alto, USA) according to the manufacturer's instructions. The 5' primer used to clone genes in the pENTR/D-TOPO entry vector includes an additional thrombin recognition sequence for hydrolysis of the

His-tag/TPS (pHMGWA), His-tag/TPS (pHNGWA) or His-tag/TPS (pdest17) recombinant protein by thrombin. Resin-bound proteins were incubated overnight at 4 °C in 200 µl of 25 mmol/L Tris-HCl (pH 7.5), 5% glycerol, 10 mmol/L MgCl₂, 1 mmol/L MnCl₂ supplemented with 10 units of thrombin (GE Healthcare). The next day, the released TPS was recovered from the mixture by filtration. Proteins concentrations were measured using the Bio-Rad protein assay dye reagent (Bio-Rad, München, Germany) with bovine serum albumin as standard.

Enzymatic assays of recombinant protein activity

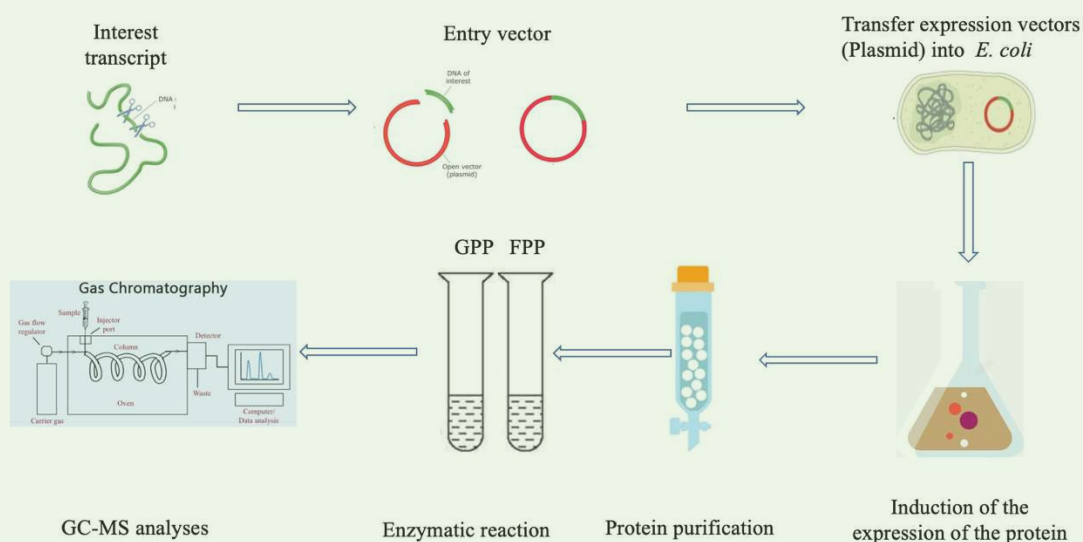
Twenty to 50 µg of purified recombinant protein were suspended in 490 µl of 25 mM Tris-HCl, (pH 7.5), 5% glycerol, 1 mmol/L DTT, 1 mg/mL BSA supplemented with 10 mmol/L MgCl₂ and 1 mmol/L MnCl₂ cofactors. At the beginning of the reaction, 10 µl geranyl or farnesyl diphosphate (Sigma) (final concentration 60 µM) was added and the mixture was covered with 500 µl of hexane. After incubation at 30 °C for three hours, the mixture was mixed vigorously and the upper hexane phase was collected, concentrated under a nitrogen stream and analyzed by GC-MS.

Transcript analysis

The quantitative PCR (qRT-PCR) was performed with CFX96™ real-time detection system (Bio-Rad, Hercules, USA) using the SsoAdvanced™ SYBR Green Supermix (Bio-Rad, Hercules, USA). All reactions were carried out according to the manufacturer's protocol in a total volume of 10 µl, 5 ng reverse-transcribed total RNA, and 100 ng of each primer. The PCR reactions were performed according to the same thermal profile: 95 °C for 30 s, followed by 40 quantification cycles (95 °C for 5 s, 60 °C for 30 s). At the end of the amplification, a melting-curve analysis (65 °C–95 °C, one fluorescence read every 0.5 °C) allowed to check the specificity of the amplification. Normalized expression values ($2^{-\Delta\Delta C_q}$ method, Livak and Schmittgen, 2001) were calculated by the CFX96™ data manager (BioRad, Hercules, USA) using Actin, Cap and Perixon4 as reference genes. All analyses were performed using three biological replicates.

Box 3. Gene functional study in *Escherichia coli*.

PCR was performed on cDNA using primers designed on the transcripts, cloning the gene in the entry vector, transferring it into expression vectors. It was followed by induction of the expression of the protein in *E.coli*, purification of the proteins from the bacterial culture, making an in vitro enzymatic reaction on GPP and FPP substrates, finalizing with GC-MS analysis to detect the compounds produced.



Cytological study in different tissues

Experience was conducted at the LBVpam in St Etienne after fig collection at the CEFE in Montpellier. To study the chemical nature of substances produced by the different collected tissues (flowers, ostiole, wall) in *F. carica* the Nadi reagent (David and Carde, 1964) was used to detect terpenes. Observations were made with a Leitz DMRB microscope. Fresh sections of fig tissue were placed for 30 min in a freshly made mixture of 0.001% 1-naphtol, 0.001% N,Ndimethyl-p-phenylenediamine dihydrochloride and 0.4% ethanol in 100 mM sodium cacodylate-HCl buffer (pH 7.2) and then directly observed. Droplets are stained purple when they contain terpenes.

Statistical analysis

All the data on chemical analyses were performed in R (v. 4.2.1; R Development Core Team, 2013).

One-way ANOVA (analysis of variance) was used to analyse transcripts between receptive and pollinated figs in *F. carica* and to compare the four pollinator-attractive VOCs and transcripts among different tissues of fig in *F. carica*. If significant difference were found, post hoc comparisons were performed Tukey HSD test (The Tukey Highly Significant Difference test).

Results

1. Characterization of the potential genes involved in the plant/pollinator chemical communication in *Ficus carica*

1.1 Comparative analyses of VOCs between receptive and pollinated figs

Pollinated figs showed a huge reduction in emissions of the four pollinator-attractive VOCs compared to receptive figs both in spring male (A) and summer female (B) figs (Figure 1).

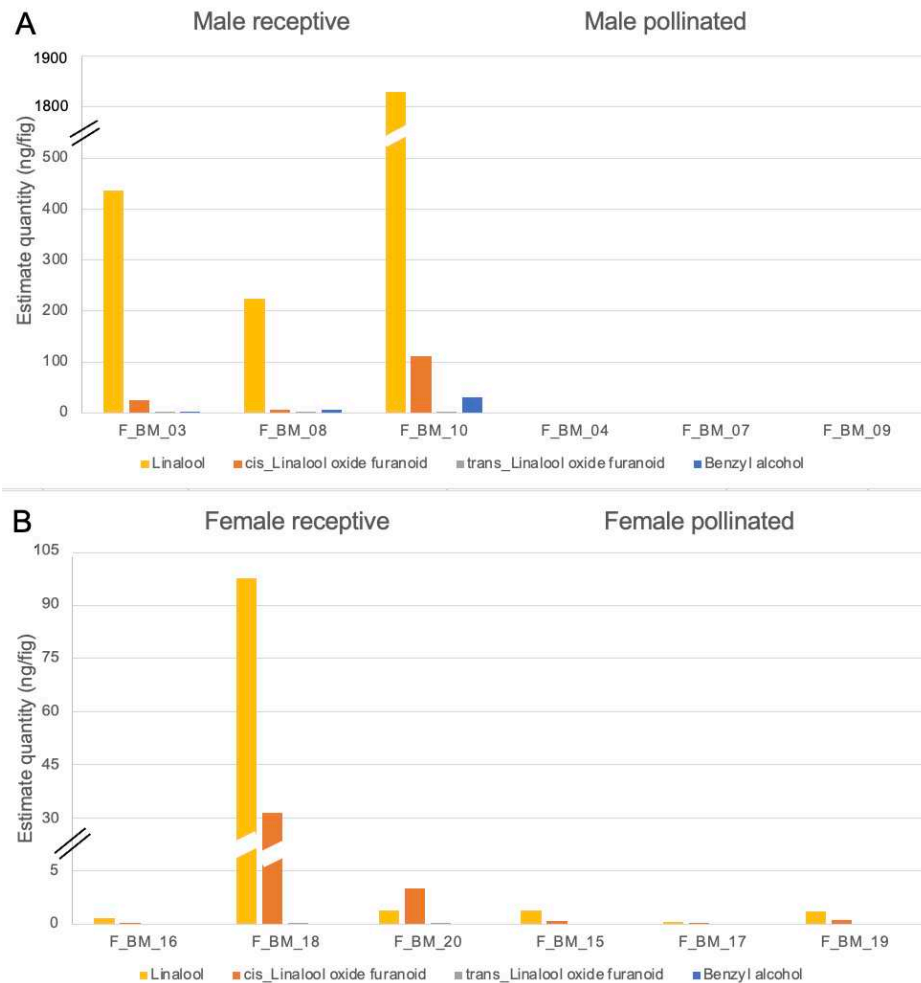


Figure 1. Estimate quantity by GC-MS of the four pollinator-attractive VOCs in receptive and pollinated spring male (A) and summer female (B) figs in *F. carica* (2019 exp 1). GC-MS analysis of compounds present in the different samples was performed to quantify the VOCs with internal standards (ng/fig). F_BM_* represent individual of samples.

1.2 Transcriptome analyses

A total of 60 genes were DE (differential gene expression) between male receptive and pollinated figs (Table S1), 5 genes were DE between female receptive and pollinated figs (p -value < 0.05) (Table S2). Among the DE genes, 39 were downregulated and 21 were upregulated in male pollinated figs relative to receptive figs, 4 were downregulated and 1 were upregulated in female pollinated figs relative to receptive figs. When visualizing the rlog (regularized log) transformed read counts of the significantly DE genes, 4 female samples had a high expression in stress induced proteins (Figure 2A). We combined the results of comparative analyses of the four pollinator-attractive VOCs between receptive and pollinated figs with the ones of

differential expression analyses to look at on the genes with homology to synthases. Then we selected the 3 genes (2 monoterpene synthases and 1 cytochrome P450 synthase) that were highly expressed in receptive figs compared to pollinated figs and 1 sesquiterpene synthase which was not DE gene as follow (Figure 2B and 2C):

- The FcTPS1- Transcripts FCD_00002972 (and FCD_00002971 which is similar) corresponds to the linalool synthase characterized by Nawade et al., 2020.
- The FcTPS2-Transcripts FCD_00023747 presents no strong homology to previously characterized terpene synthase. The best homology (60%) following a Blast P analysis is with a *Quercus myrcene* synthase and with the *F. carica* cineole synthase (Nawade et al 2020). At this point the only realistic assumption that can be made is that this sequence corresponds to an unknown monoterpene synthase.
- The FcTPS3-Transcripts FCD_00013313 corresponds to the cadinene synthase characterized by Nawade et al., 2020.
- The FcCYP1- Transcripts FCD_00006785 is a candidate for a linalool oxide synthase.

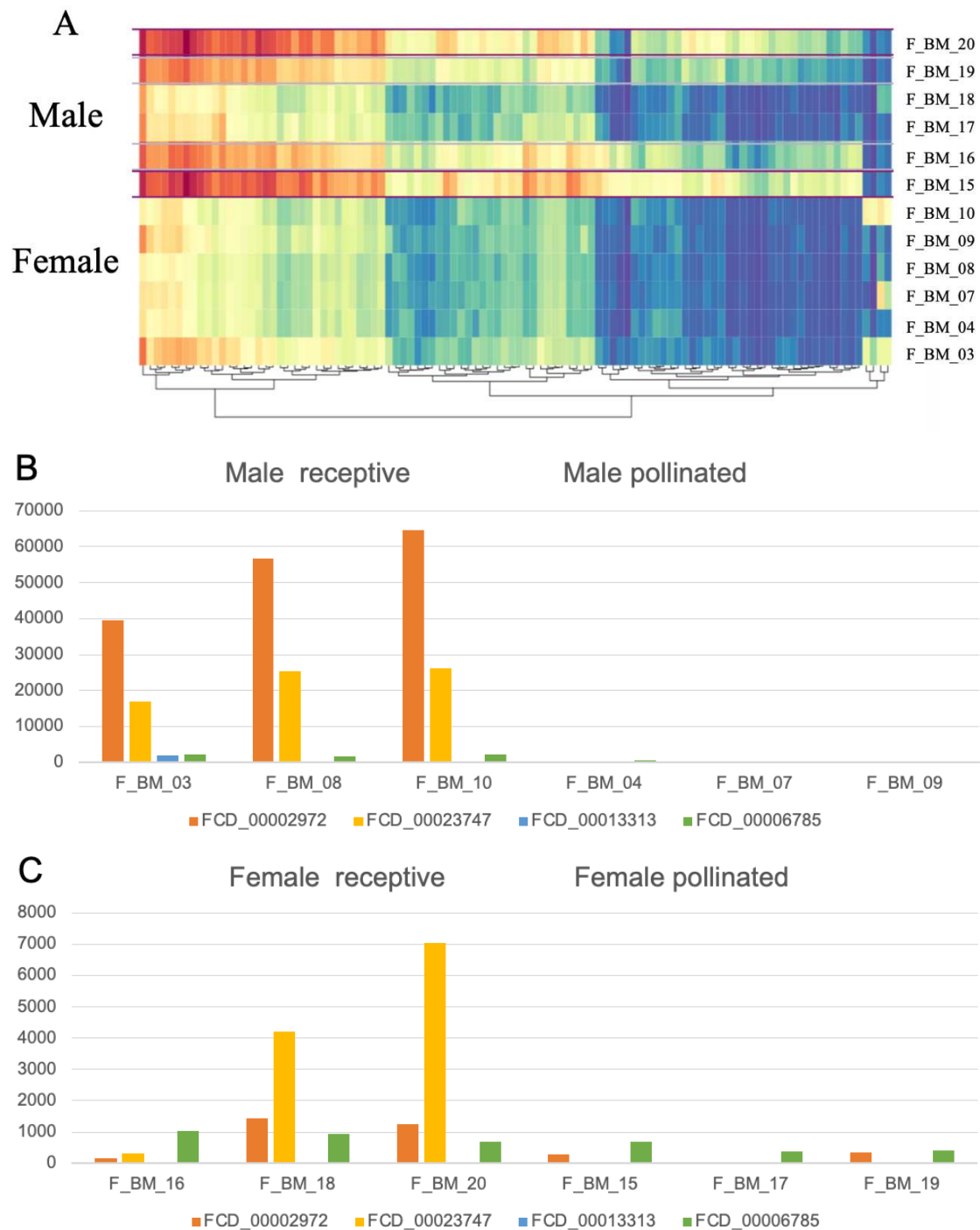


Figure 2. Gene expression levels (A) between receptive and pollinated spring male and summer female figs. Transcripts gene level normalized counts between receptive and pollinated spring male (B) and summer female (C) figs of *F. carica* (2019 exp 1). Heatmap of regularized log (rlog) normalized values of expression levels and hierarchical clustering for these genes DE genes. Rlog values are coded on the from red to blue gradient scale (high expression: red; intermediate expression: yellow; low expression: blue). F_BM_03, F_BM_08 and F_BM_10 represent receptive male figs; F_BM_16, F_BM_18 and F_BM_20 represent receptive female figs; F_BM_04, F_BM_07 and F_BM_09 represent pollinated male figs; F_BM_15, F_BM_17 and F_BM_19 represent pollinated female figs.

2. Functional study of the genes involved in the emission of VOCs responsible for pollinator attraction.

2.1 Enzymatic activity of recombinant fig terpene synthases

Our bioinformatic analysis led to amplification from cDNA using gene-specific primers resulted in the isolation of three full-length FcTPS (*F. carica* Terpene synthase) coding regions. We designated these gene models as FcTPS1 to FcTPS3.

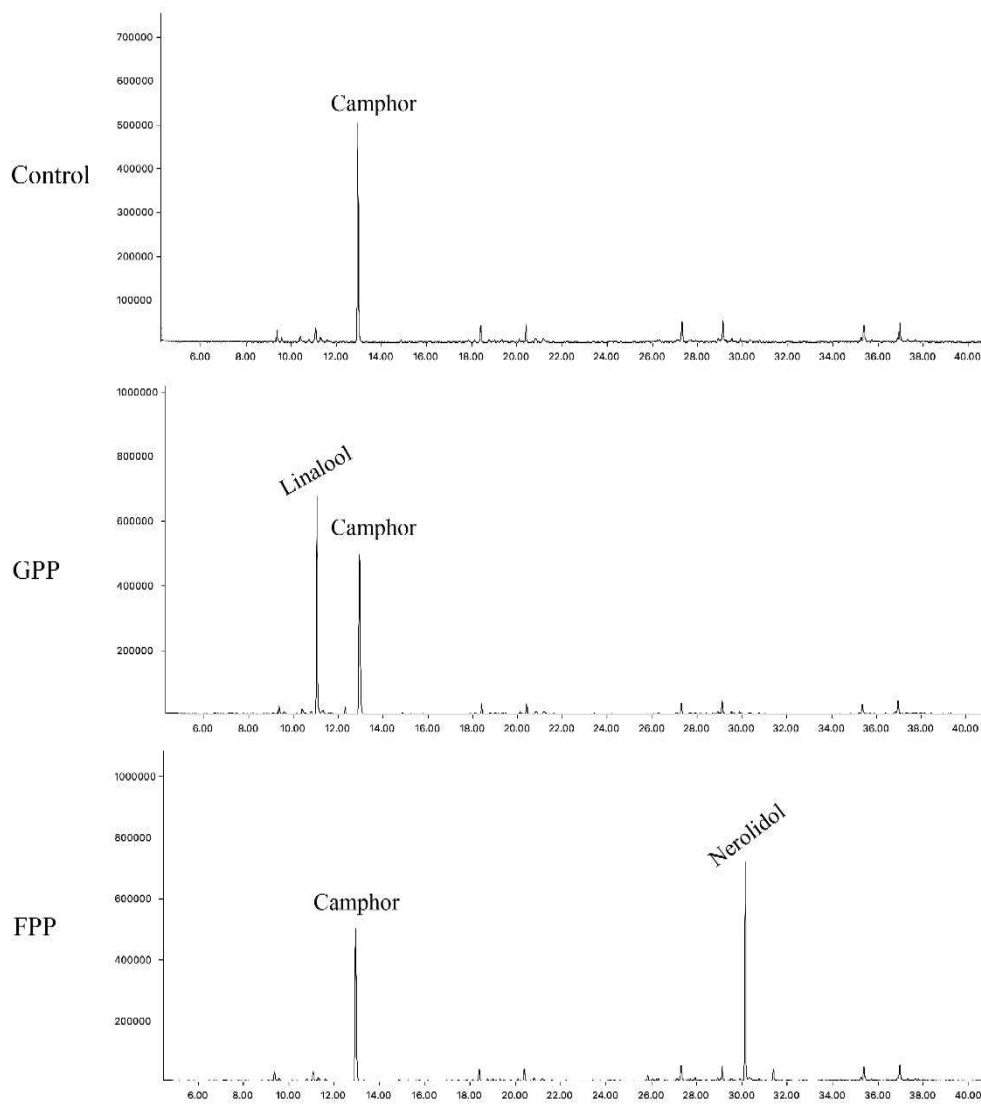
The three FcTPS coding sequences were used for production of recombinant proteins in the *E. coli* Rosetta strain. Activities of the purified recombinant proteins were assessed on the GPP and FPP substrates, and the products analyzed by GC-MS (Figure 3).

Incubation of FcTPS1 with GPP catalyzed the formation of monoterpene *S*-linalool (Figure 3A1, A2). FcTPS1 was designated as *S*-Linalool synthase. FcTPS1 reactions with FPP resulted in production of the sesquiterpene nerolidol.

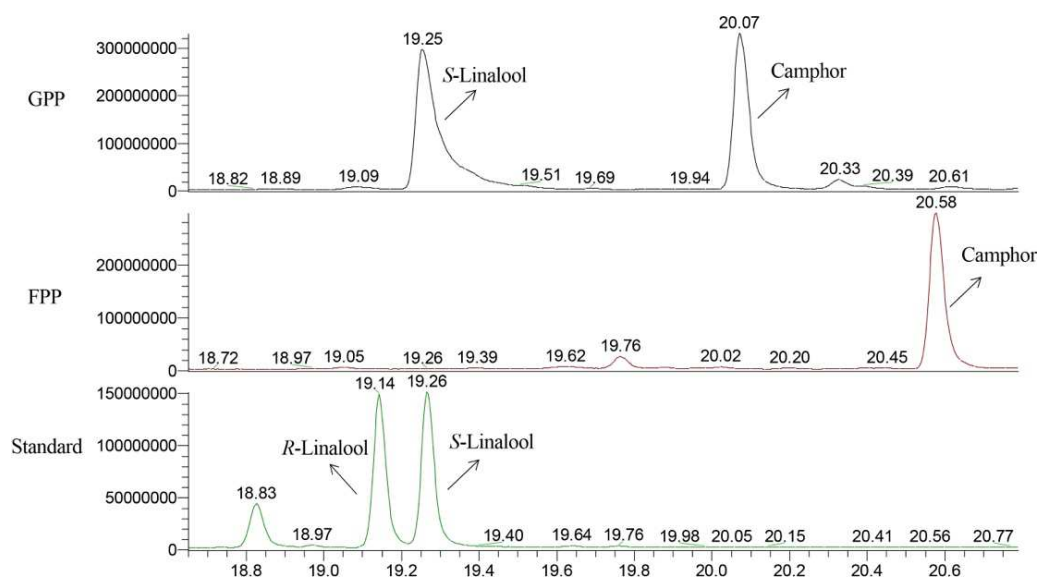
The FcTPS2 recombinant enzyme showed a low activity when GPP and FPP was used as a substrate (Figure 3B).

Upon incubation with GPP, FcTPS3 led to the production of six monoterpenes, including myrcene, nerol, geraniol, linalool (Figure 3C), while the FcTPS3 reactions with FPP resulted in production of several sesquiterpenes, i.e. germacrene D, beta-copaene, elemene (Figure 3C). Since germacrene D was the major product of the reaction, FcTPS3 was designated as a germacrene D synthase.

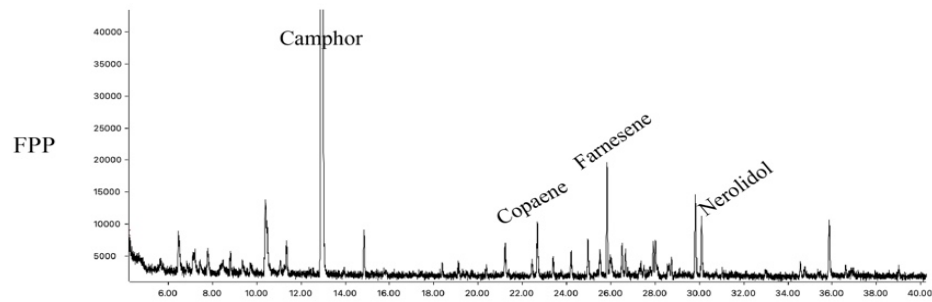
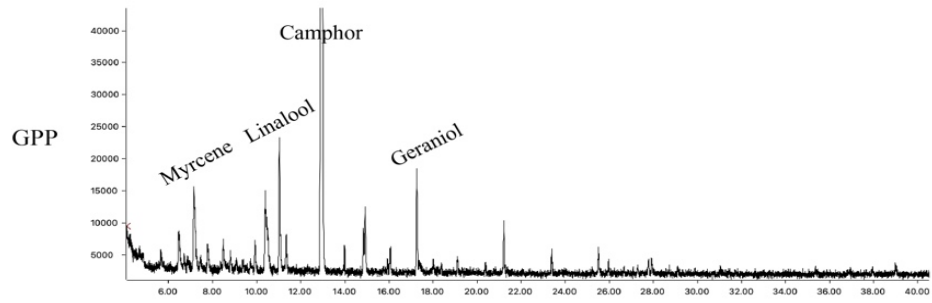
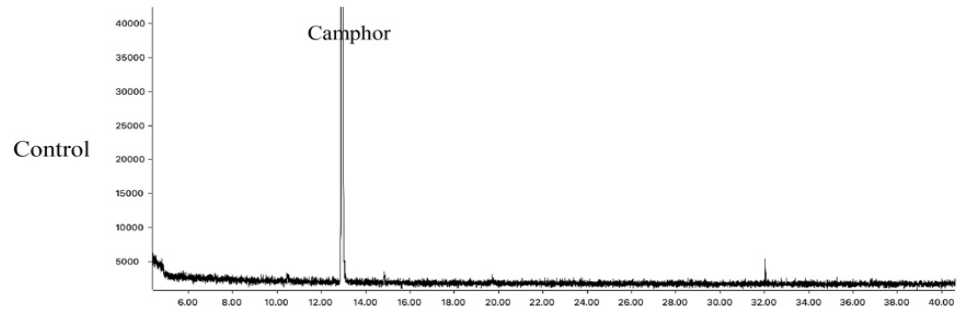
(A1)



(A2)



(B)



(C)

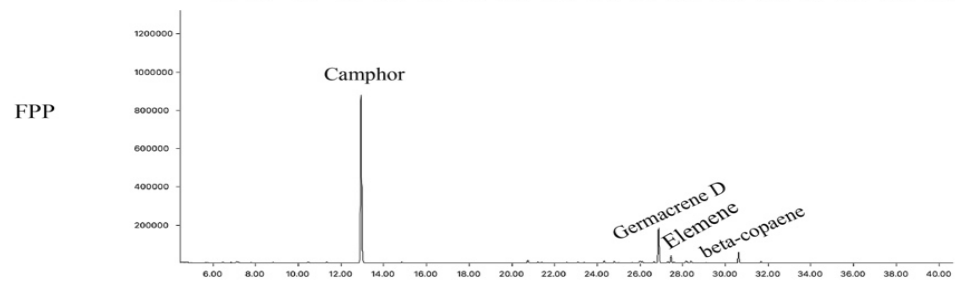
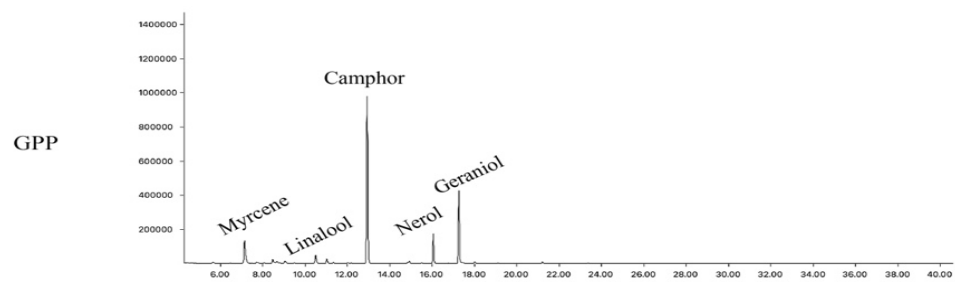
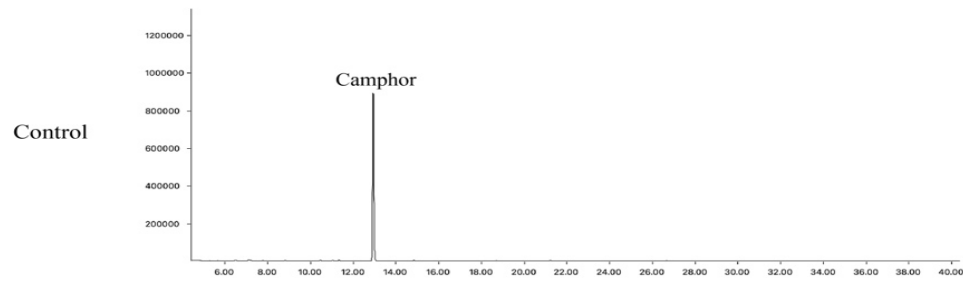


Figure 3. GC-MS analyses of the volatile products synthesized by recombinant FcTPS1 (A1, A2), FcTPS2 (B) and FcTPS3 (C). After expression in *Escherichia coli* and purification, the enzymes were incubated with the GPP and FPP substrates. A1: GC-MS analysis (Optima 5-MS column) of the resulting products by FcTPS1 enzyme upon incubation with GPP and FPP. A2: Enantioselective analysis (Cyclosil-B column) of linalool produced by FcTPS1 enzyme upon incubation with GPP and FPP. A mixture of the *S*-linalool and *R*-linalool stereoisomers was used as standard. Retention times and mass spectra were compared to those of a linalool standard. B: GC-MS analysis (Optima 5-MS column) of the resulting products by FcTPS2 enzyme upon incubation with GPP and FPP. C: GC-MS analysis (Optima 5-MS column) of the resulting products by FcTPS3 enzyme upon incubation with GPP and FPP. Camphor was the internal standard in all samples.

2.2 Transcript expression analysis between receptive and pollinated figs

Our results show that *S*-linalool and linalool oxide accumulation is generally higher in receptive figs. The expression of *S*-linalool (FcTPS1) and FcCYP1 synthases was consequently evaluated in receptive figs using quantitative RT-PCR (Figure 4). The expression of FcTPS1(2972) ($F_{1,6} = 39.48$, $df=6$, $p=0.0008$), FcTPS1(2971) ($F_{1,6} = 15.2$, $df=6$, $p=0.008$), FcTPS2 ($F_{1,6} = 7.952$, $df=6$, $p=0.04$) and FcCYP1 ($F_{1,6} = 43.85$, $df=6$, $p=0.0006$) were significantly higher in receptive than in pollinated spring male figs. The expression of FcTPS1(2972) ($F_{1,6} = 24.23$, $df=6$, $p=0.003$), FcTPS1(2971) ($F_{1,6} = 7.091$, $df=6$, $p=0.04$) and FcTPS2 ($F_{1,6} = 8.759$, $df=6$, $p=0.03$) were significantly higher in receptive than in pollinated summer female figs, and FcCYP1 ($F_{1,6} = 2.041$, $df=6$, $p=0.21$) was no difference between receptive and pollinated summer female figs (Figure 4).

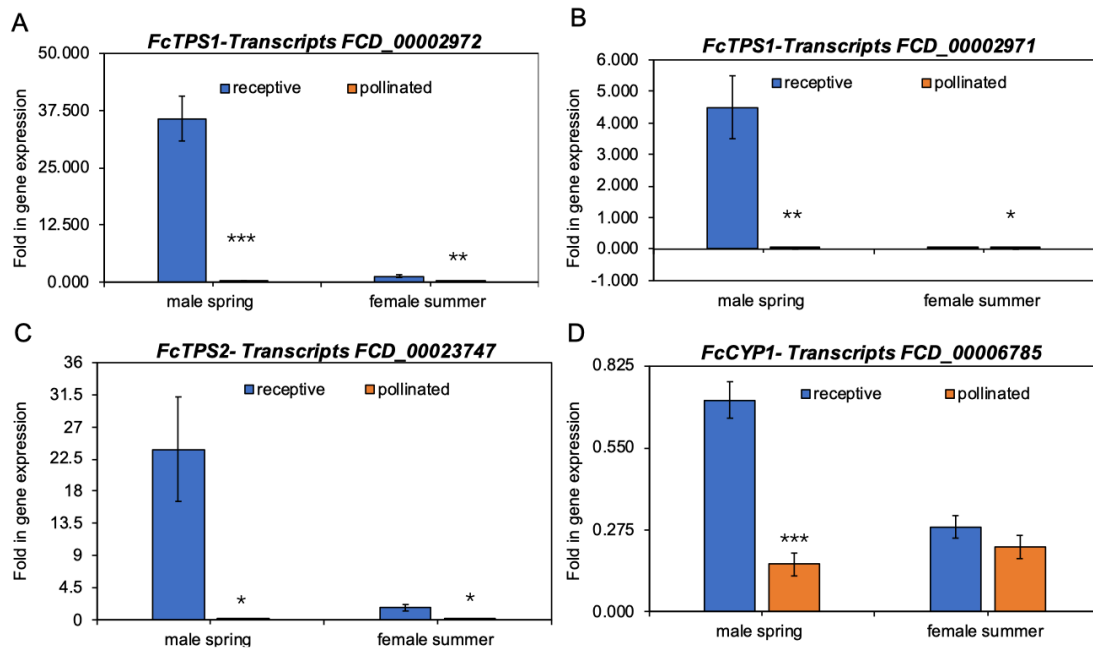


Figure 4. Transcriptional activity of FcTPS1, FcTPS2 and FcCYP1 in receptive and pollinated figs of *F. carica* (2022) measured by quantitative RT-PCR. The analyses were performed in receptive figs (blue bars) and pollinated figs (orange bars). Results of comparisons between receptive and pollinated figs was performed using analysis of variance (ANOVA). Transcript levels of synthases are normalized to those of Actin, Cap and Perixon4. Values are the means and standard deviation of three technical replicates.

3. Localization of the fig tissue producing the pollinator-attractive VOCs.

3.1 VOCs analyses in different tissues

There was a significant different of the quantity of pollinator-attractive VOCs among the three different tissues ($F_{2,6} = 37.56$, $df=6$, $p < 0.001$). Post hoc comparisons using the Tukey HSD test indicated that linalool and cis-linalool-oxide-furanoid were more abundant in flowers than ostiole and wall, and there was no difference between ostiole and wall; benzyl-alcohol was higher in ostiole than in flowers and wall, and there was no difference between flowers and wall; trans-linalool-oxide-furanoid was higher in flowers than in wall, and not found in ostiole. Linalool was the most present VOC in the three tissues, followed by benzyl-alcohol, the cis-linalool-oxide-furanoid and the trans-linalool-oxide-furanoid (Figure 5).

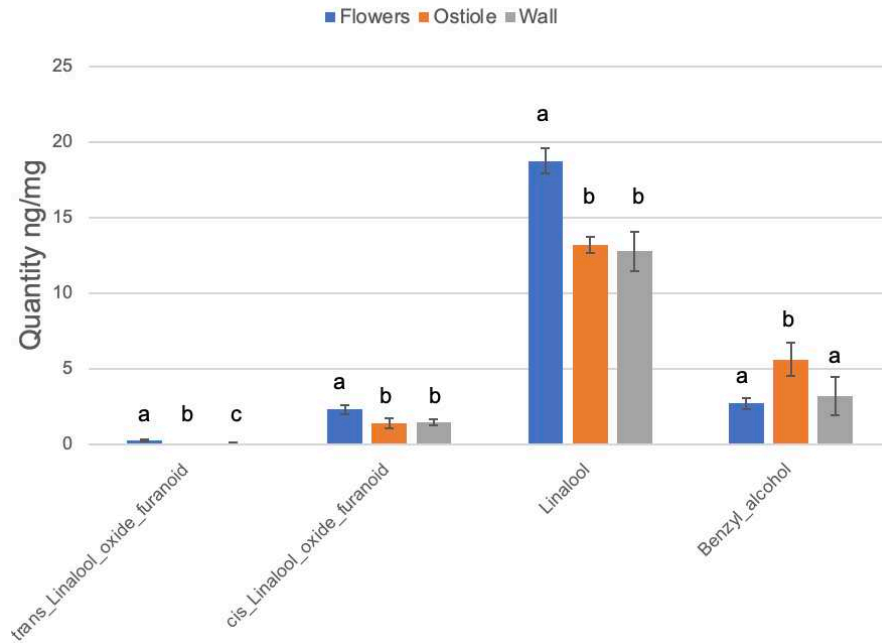


Figure 5. Quantification of the the four pollinator-attractive VOCs in flowers, ostiole and wall of figs in *F. carica*. GC-MS analysis of hexane-extracted compounds present in the different samples was used to quantify VOCs accumulation (ng/mg of dry weight). Results of pairwise comparisons among tissues were performed Tukey HSD test after using analysis of variance (ANOVA). Values are the means and standard deviation of three replicates. Different letters indicate statistically significant differences.

3.2 Transcript expression analysis in different fig tissues

The expression of FcTPS1, FcTPS2 and FcCYP1 genes was evaluated in different tissues of receptive figs using quantitative RT-PCR (Figure 4). We analysed the relative gene expression of FcTPS1, FcTPS2 and FcCYP1 genes in flowers, ostiole and wall of spring male figs (Figure 6). Relative gene expression of FcTPS1(2972) ($F_{2,9}=26.61$, $df=2$, $p=0.0001$), FcTPS1(2971) ($F_{2,9}=31.36$, $df=2$, $p=0.00001$), FcTPS2 ($F_{2,9}=8.719$, $df=2$, $p=0.007$) and FcCYP1 ($F_{2,9}=8.303$, $df=2$, $p=0.009$) are significant different among flowers, ostiole and wall. Post hoc comparisons using the Tukey HSD test indicated that the expression of FcTPS1(2972), FcTPS1(2971) and FcCYP1 were significantly higher in ostiole and wall than in flowers, the expression of FcTPS1(2972) and FcCYP1 were no difference in ostiole and wall, however the expression of FcTPS1(2971) was significantly higher in ostiole than in wall. of fig. The expression of FcTPS2 was significantly higher in wall than in ostiole and flowers, and was no difference in ostiole and flowers of fig.

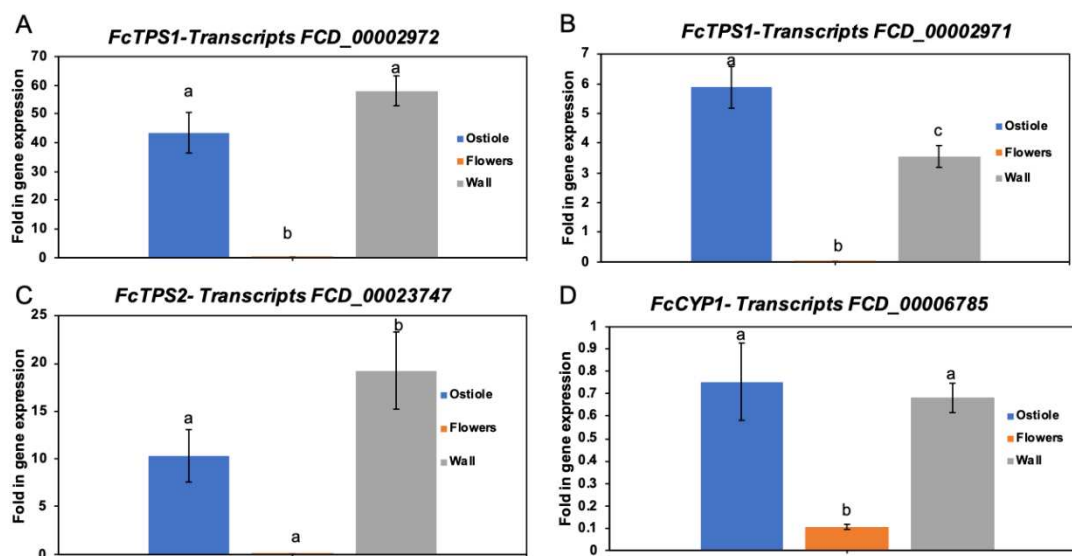


Figure 6. Transcriptional activity of FcTPS1, FcTPS2 and FcCYP1 in the ostiole, flower and wall in spring male figs (2022) measured by quantitative RT-PCR. The analyses were performed in ostiole (blue bar), flower (orange bar) and wall (gray bar). Results of pairwise comparisons among tissues were performed Tukey HSD test after using analysis of variance (ANOVA). Transcript levels of synthases are normalized to those of Actin, Cap and Perixon4. Values are the means and standard deviation of three technical replicates.

3.3 Cytological study of the different fig tissues

Semi-thin optical horizontal sections of different tissues from *F. carica* are shown in Figure 7. Histochemical tests were performed to detect terpenes in bracts of the ostiole, epiderme of wall and style of female flowers of fig (Nadi reagent; David and Carde 1964). Terpene droplets were found in the cell of bracts of the ostiole and in the cell of the wall, not in the cell of flowers.

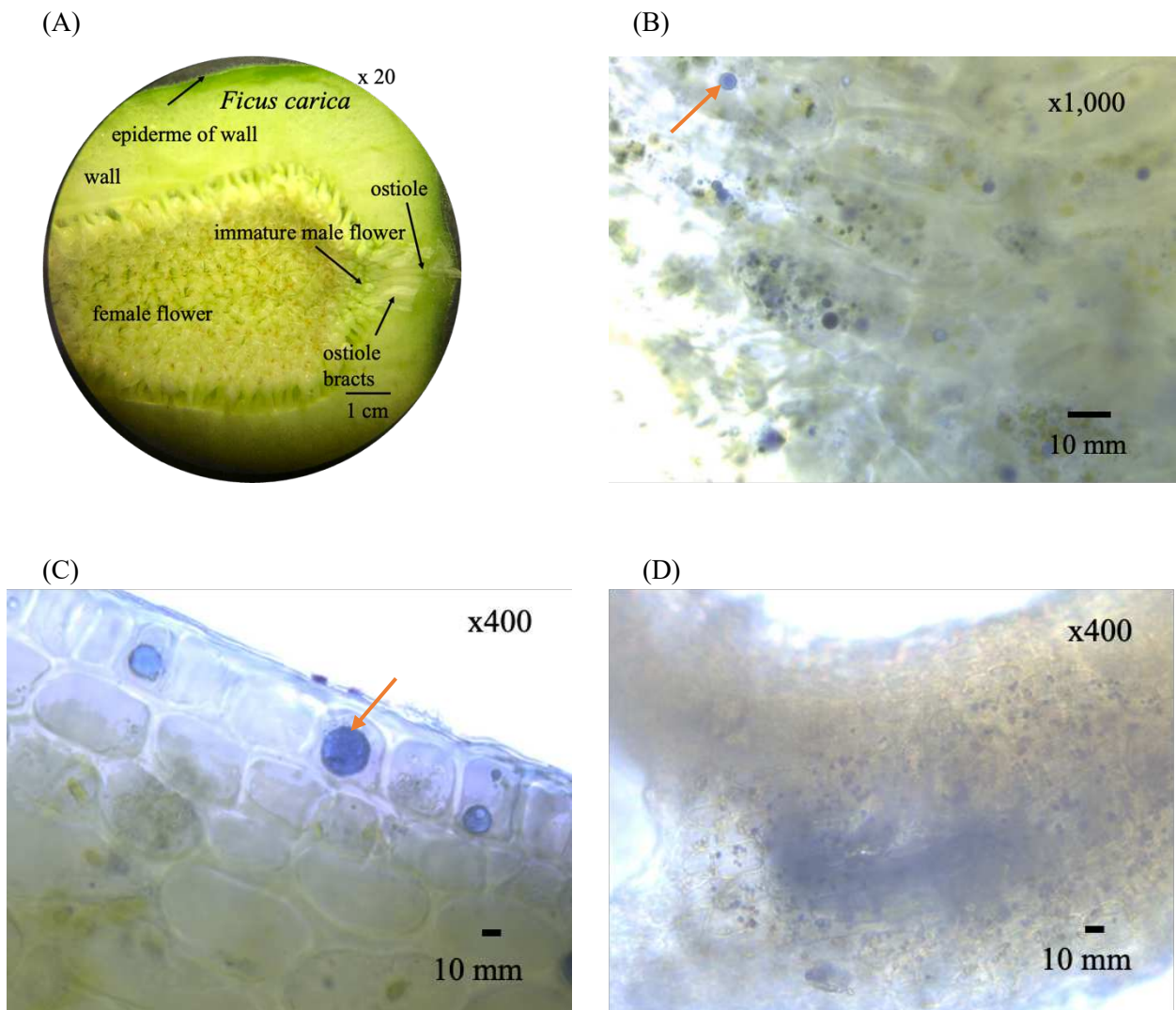


Figure 7. (A) Diagram showing the different tissues of a fig with a longitudinal sections of the fig. (B) Horizontal sections of the bracts of ostiole. (C) Horizontal sections of epiderme of wall. (D) Horizontal sections of the style of female flowers. Anatomic details showing droplets (orange arrows) of the terpenes (Nadi reagent) in ostiole bracts (B) and in epiderme of wall (C), starch grains (blue arrows) in the style of female flower. Scale bars: A= 1 cm, B= 10 mm, C= 10 mm, D= 10 mm. Magnification: A= x 20, B= x 1000, C= x 400, D= x 400.

Discussion

In this study, we integrated various methodologies, such as metabolomics, bioinformatics, biochemistry and molecular biology, to understand the mechanisms related to the production of VOCs in a highly specialized interaction, that between the Mediterranean fig tree and its pollinator. We were able to characterize genes potentially involved in the production of VOCs responsible for the attraction of the pollinator of *F. carica*. We have confirmed the products formed by some of these genes and also

identified for the first time in fig trees the biosynthetic site of the VOCs attractive to the pollinator.

A total of 60 genes were DE between male receptive and pollinated figs, only 5 genes were DE between female receptive and pollinated figs). It is possible due to the stress induced proteins high expression in the female samples (such as high temperature and drought induced proteins). Previous study used multi-omics (transcriptomic, proteomic and metabolomic) analysis to compare the differences in terpenoid synthesis pathways between pre-receptive and receptive phases of *F. hirta* syconia (Fan et al., 2022), but without characterized the synthases in vitro.

Previous study characterized two linalool synthases and cadinene in *F. carica* (Nawade et al., 2020). Our in vitro assays of heterologous expressions in *E. coli* indicated that FcTPS1 exclusively catalyzed the formation of *S*-linalool (Figure 5A), FcTPS2 converts FPP to Germacrene D. *S*-linalool synthase was present in higher expression in receptive figs than in pollinated figs (Figure 4). Heterologous expression in *E. coli* of FcTPS3 was also conducted but with low activity. Our selected gene cytochrome P450s FcCYP1, as candidate for a linalool oxide synthase, should be identified in a eukaryotic expression system such as *Saccharomyces cerevisiae* or *Nicotiana benthamiana*. Experiments conducted in *Arabidopsis* flowers reported the co-expression of two monoterpene synthases and two cytochrome P450s suggest that linalool oxide was produced by linalool (Ginglinger et al., 2013; Boachon et al., 2015). Verifying whether the linalool and linalool oxide synthase genes are co-expressed in *F. carica* is also important for further understanding of plant pollinator interactions. One study reported that benzyl-alcohol can be reduced from benzaldehyde in *Petunia*, but the enzyme is still unknown (Huang et al., 2022). Revealing the enzyme(s) of benzyl-alcohol should be an interesting future prospective.

The three complementary experiments (VOCs, transcript and cytological analyses in different tissues) all suggested the same results, with the ostiole as the source of attractive VOCs. It coincides with the pollinating wasps detecting the attractive volatile compounds at relatively long distances and getting a more precise guidance at short

distances (Okamoto and Su 2021). The results of transcript and cytological analyses in different tissues of receptive figs were indicating that the pollinator-attractive VOCs were produced and stored in the ostiole and the wall. The result of VOCs analysis in different tissues of receptive figs reveal that the pollinator-attractive VOCs are emitted from the ostiole and the flowers. The VOCs found in flowers were maybe transferred from the wall tissue (and not synthesized *de novo* by the flowers). In general, the VOCs are released through the epidermis (Castro-Cárdenas et al., 2022). VOCs production by vegetative structures and their liberation to the exterior through the epidermis have been reported previously (Maoz et al., 2020). Another possibility is that the VOCs found in the flowers (Figure 5) may have been released from the ostiole, as this is an enclosed system of inflorescence, the syconium. When figs reach receptivity, large quantities of pollinator-attractive VOCs are released from the ostiole and filled up the syconium chamber.

Our study contributes to novel direct evidence on the fig ostiole role which is consistent with the hypothesized one of the ostiole as a source of volatile compounds for attracting fig wasps. Previous studies found terpene-secreting cells in ostiolar bracts (Souza et al., 2015) and two types of ostiolar osmophores (Castro-Cárdenas et al., 2022) in *Ficus*. Here, we found the pollinator-attractive VOCs emitted from the ostiole and the related gene expressed in the ostiole, with terpene presence in the cells of the ostiole. This is thus the first time the role of the ostiole bracts of the syconium as source of volatile compounds attracting the pollinating fig wasps was proven. In other flowering plants, petals are generally the main floral tissue for spatial VOCs emission. VOC production was primarily localized in the corolla and more specifically the petal limb in *Petunia* (Verdonk et al., 2003). In the Orchidaceae family, scent is emitted by specialized areas of the petal, which have been termed osmophores by Vogel (Baudino et al., 2007). The ostiole of fig is composed of bracts as the petals in other plants. It is also reasonable that the pollinator-attractive VOCs are emitted from bracts of the ostiole with terpene presence in the bract cells of the ostiole. In *Petunia* flowers, expression of PhBSMTs (benzoic acid/salicylic acid carboxyl methyltransferases) was highest in the

petal limbs compared to the other floral organs (expression highest in petal limb > petal tube > ovary > stigma/ style) (Underwood et al., 2003).

Most studies focus on the changes in floral VOCs emission after pollination in *Ficus* (Chen et al., 2009; Gu et al., 2012; Proffit et al., 2018; Proffit et al., 2008). The floral VOC methylbenzoate (MeBA) (Negre et al., 2003) and related genes PhBSMTs (Underwood et al., 2003) decreases after pollination have been reported in petunia flowers. This is the first time that a simultaneous reduction in pollinator attracting VOCs and related genes has been observed after pollination in *Ficus*.

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Supplementary data

Table S1. DE genes expression levels between receptive and pollinated spring male figs.
 IFC = $\log_2(\text{receptive/pollinated})$, IFC>0 represent upregulated, IFC<0 represent downregulated.
 Gene expression is significant different between receptive and pollinated figs (p-value <0.05).
 F_BM_* represent individual of samples.

| GeneID | Normalised counts | | | | | | | |
|--------------|-------------------|---------|----------------|-----------|------------|-----------------|------------|-----------|
| | IFC | p-value | Male receptive | | | Male pollinated | | |
| | | | F BM 03 | F BM 08 | F BM 10 | F BM 04 | F BM 07 | F BM 09 |
| FCD_00023749 | 8.3153 | 0.0000 | 4004.3481 | 19.6304 | 5868.6111 | 6.1189 | 5495.1770 | 16.1927 |
| FCD_00002972 | 8.2955 | 0.0000 | 39627.2475 | 195.5767 | 64709.3988 | 166.0853 | 56664.3823 | 19.6017 |
| FCD_00023747 | 8.2062 | 0.0000 | 16954.6801 | 59.1682 | 26159.4915 | 29.5286 | 25383.2409 | 95.9125 |
| FCD_00005742 | 8.2029 | 0.0003 | 154.5019 | 0.0000 | 56.6920 | 0.0000 | 146.7019 | 0.0000 |
| FCD_00002262 | 8.0686 | 0.0003 | 155.3709 | 0.0000 | 129.6231 | 0.0000 | 18.7815 | 0.0000 |
| FCD_00002971 | 8.0165 | 0.0000 | 1193.6550 | 20.8338 | 5157.6140 | 1.7275 | 6560.9582 | 7.5790 |
| FCD_00006230 | 6.5098 | 0.0000 | 39845.8619 | 787.5975 | 75067.3082 | 247.8792 | 50200.6086 | 434.3829 |
| FCD_00021080 | 6.3323 | 0.0170 | 198.6409 | 1.4541 | 249.2956 | 0.0000 | 526.8414 | 1.7045 |
| FCD_00030386 | 6.3103 | 0.0230 | 1113.3440 | 2.0114 | 2008.4459 | 4.0304 | 338.2204 | 0.0000 |
| FCD_00007115 | 6.0944 | 0.0000 | 5096.1878 | 172.9531 | 24836.3909 | 318.9017 | 21548.3083 | 107.3302 |
| FCD_90000722 | 5.7115 | 0.0017 | 33154.1106 | 292.2748 | 12541.8157 | 338.2898 | 23652.4489 | 217.3229 |
| FCD_00020525 | 5.4401 | 0.0000 | 13900.1397 | 444.7010 | 24856.2417 | 329.6648 | 18241.0213 | 283.5278 |
| FCD_00002246 | 5.4040 | 0.0000 | 2854.8390 | 43.4857 | 4388.3847 | 53.9855 | 2274.4143 | 86.6215 |
| FCD_90002261 | 4.9065 | 0.0059 | 358.5284 | 12.6892 | 386.7784 | 4.3168 | 432.3944 | 7.5757 |
| FCD_90001016 | 4.5561 | 0.0379 | 30.4793 | 1.4541 | 153.0012 | 4.3707 | 97.1843 | 0.0000 |
| FCD_00018344 | 4.4355 | 0.0003 | 18993.0932 | 582.7244 | 22952.3027 | 1202.1815 | 19723.2410 | 395.8682 |
| FCD_00023653 | 4.4353 | 0.0004 | 7837.3816 | 648.5301 | 20658.3669 | 756.9999 | 20904.0079 | 322.1492 |
| FCD_00015945 | 4.0471 | 0.0279 | 4756.5424 | 108.7858 | 2277.5549 | 265.0741 | 5127.6145 | 57.8083 |
| FCD_90000023 | 4.0197 | 0.0064 | 1381.5595 | 102.7141 | 1436.9515 | 37.7815 | 1565.3215 | 42.4256 |
| FCD_00036144 | 3.9640 | 0.0002 | 2792.5915 | 228.0052 | 10030.4564 | 365.3727 | 4212.6217 | 270.1191 |
| FCD_00001547 | 3.8591 | 0.0019 | 8780.1376 | 1032.4134 | 21825.6695 | 941.4421 | 18100.1548 | 505.3822 |
| FCD_00037357 | 3.5943 | 0.0054 | 16014.2395 | 1325.4153 | 17296.6211 | 925.7077 | 16685.8671 | 739.7499 |
| FCD_00027571 | 3.5137 | 0.0084 | 5397.9874 | 466.7672 | 6548.0218 | 410.8429 | 6634.1091 | 278.6846 |
| FCD_00031599 | 3.2600 | 0.0021 | 5761.6368 | 757.5879 | 10690.8226 | 444.0600 | 7723.5966 | 790.8847 |
| FCD_00010705 | 3.2323 | 0.0034 | 587.5146 | 69.7970 | 937.2659 | 86.5392 | 1605.2464 | 103.1218 |
| FCD_00014518 | 3.1495 | 0.0000 | 1229.6817 | 206.4827 | 1659.4742 | 106.6443 | 1421.1077 | 133.8027 |
| FCD_00003269 | 3.1365 | 0.0016 | 1749.1974 | 200.3958 | 1540.4953 | 77.4950 | 1734.6436 | 185.9112 |
| FCD_00009134 | 3.1117 | 0.0010 | 32524.5556 | 2162.9789 | 53847.8502 | 5764.9124 | 32729.8061 | 3435.4056 |
| FCD_00018434 | 2.7491 | 0.0022 | 5014.8182 | 835.3794 | 11635.1009 | 934.3079 | 5374.4770 | 1040.9435 |
| FCD_00031634 | 2.6586 | 0.0001 | 2700.0358 | 777.9428 | 4301.1374 | 500.0029 | 5288.8560 | 489.1879 |
| FCD_00001944 | 2.5253 | 0.0024 | 10657.2415 | 1103.6645 | 10397.6595 | 1712.4281 | 8306.7025 | 1710.4587 |
| FCD_00007457 | 2.4524 | 0.0000 | 9831.5744 | 1961.7457 | 13693.6601 | 1601.0187 | 6928.6257 | 1640.0604 |
| FCD_00010755 | 2.4041 | 0.0358 | 9933.0956 | 1983.3971 | 14991.9742 | 2010.5077 | 11449.8483 | 1882.6125 |
| FCD_00006785 | 2.3072 | 0.0067 | 2212.3761 | 295.1830 | 2319.6260 | 479.0253 | 1579.6716 | 340.8986 |
| FCD_00020401 | 2.2163 | 0.0084 | 18516.5597 | 4033.1381 | 25085.6504 | 4067.5537 | 18722.1768 | 4137.9568 |
| FCD_00032174 | 2.2002 | 0.0117 | 458.1656 | 108.5297 | 859.4065 | 132.0463 | 512.1836 | 122.0323 |
| FCD_00037317 | 2.1639 | 0.0488 | 341.5783 | 69.0699 | 281.3937 | 52.4480 | 317.1278 | 65.6230 |
| FCD_00016208 | 2.0783 | 0.0036 | 560.9906 | 124.9669 | 806.2999 | 173.2268 | 551.3675 | 128.1522 |
| FCD_00032175 | 1.8859 | 0.0253 | 3815.1663 | 1227.9904 | 6871.1432 | 1451.0621 | 4729.6373 | 1257.9158 |
| FCD_00004246 | -2.1650 | 0.0314 | 90.3869 | 578.7332 | 109.1337 | 457.1720 | 98.0368 | 444.0204 |
| FCD_00024742 | -2.1918 | 0.0017 | 108.8023 | 752.6528 | 118.4221 | 569.7000 | 188.8329 | 719.8107 |
| FCD_00025193 | -2.2131 | 0.0003 | 179.3315 | 1018.4813 | 266.4943 | 1009.1191 | 205.5034 | 1179.6823 |
| FCD_00011569 | -2.4731 | 0.0146 | 79.8768 | 810.6627 | 165.8404 | 457.1720 | 88.6594 | 880.3706 |
| FCD_00000232 | -2.6007 | 0.0000 | 151.3199 | 760.3678 | 112.3246 | 808.1356 | 93.7585 | 766.2194 |
| FCD_00026372 | -2.6867 | 0.0063 | 225.1197 | 4155.0280 | 512.9322 | 3709.5461 | 645.7724 | 2678.5305 |
| FCD_00015775 | -2.7089 | 0.0000 | 126.0090 | 820.9390 | 133.7956 | 888.3575 | 125.3446 | 890.9133 |
| FCD_00005614 | -2.8716 | 0.0022 | 56.7545 | 656.5277 | 128.3926 | 507.8717 | 52.0021 | 898.2678 |
| FCD_00036854 | -3.0469 | 0.0358 | 21.0202 | 90.1544 | 14.9791 | 142.4838 | 5.9675 | 247.1515 |
| FCD_00015477 | -3.1621 | 0.0001 | 280.6197 | 2587.5771 | 172.2601 | 3060.3424 | 242.1083 | 1655.0627 |
| FCD_00013146 | -3.5870 | 0.0488 | 3.1530 | 42.1690 | 3.2098 | 55.0704 | 0.8525 | 44.3168 |
| FCD_00000970 | -3.8605 | 0.0036 | 28.3773 | 207.2097 | 20.3288 | 312.0658 | 17.0499 | 815.5999 |
| FCD_00000828 | -4.1126 | 0.0488 | 2.1020 | 52.3477 | 3.2098 | 46.3291 | 0.0000 | 73.2932 |
| FCD_00003151 | -4.6437 | 0.0014 | 13.6631 | 201.3933 | 1.0699 | 187.9388 | 0.0000 | 114.2010 |
| FCD_00011706 | -4.8071 | 0.0002 | 22.0712 | 1033.8675 | 5.3497 | 250.0023 | 20.4599 | 463.6221 |
| FCD_00036893 | -5.0252 | 0.0131 | 12.6121 | 234.8377 | 0.0000 | 388.9895 | 1.7050 | 213.0616 |
| FCD_00033036 | -5.5345 | 0.0414 | 0.0000 | 81.4805 | 0.0000 | 51.4093 | 0.0000 | 22.9372 |
| FCD_00036151 | -5.5681 | 0.0026 | 3.1530 | 57.4371 | 0.0000 | 101.3995 | 0.0000 | 69.8842 |
| FCD_00019523 | -5.9313 | 0.0096 | 3.1530 | 502.3927 | 0.0000 | 50.6998 | 1.7050 | 86.9291 |
| FCD_00006514 | -6.8467 | 0.0006 | 3.1530 | 155.5891 | 0.0000 | 181.8198 | 0.0000 | 289.7638 |
| FCD_00029525 | -7.0287 | 0.0059 | 0.0000 | 37.1005 | 0.0000 | 41.0574 | 0.0000 | 139.9649 |

Table S2. DE genes expression levels between receptive and pollinated summer female figs
 IFC = $\log_2(\text{receptive/pollinated})$, IFC>0 represent upregulated, IFC<0 represent downregulated.
 Gene expression is significant different between receptive and pollinated figs (p-value <0.05).
 F_BM_* represent individual of samples.

| GeneID | IFC | p-value | Normalised counts | | | | | |
|--------------|---------|---------|-------------------|-----------|-----------|-------------------|-----------|-----------|
| | | | Female receptive | | | Female pollinated | | |
| | | | F_BM_16 | F_BM_18 | F_BM_20 | F_BM_15 | F_BM_17 | F_BM_19 |
| FCD_00000870 | 18.4199 | 0.0096 | 0.0000 | 0.0000 | 93.2294 | 0.0000 | 0.0000 | 0.0000 |
| FCD_00023747 | 6.4034 | 0.0000 | 324.3732 | 4216.0522 | 7035.0513 | 45.1322 | 14.7975 | 44.6177 |
| FCD_00023749 | 6.2803 | 0.0000 | 321.6143 | 1186.5194 | 1048.8309 | 19.3497 | 3.8248 | 7.0618 |
| FCD_00002246 | 4.3644 | 0.0168 | 331.2694 | 951.0282 | 371.2725 | 57.1425 | 5.2711 | 5.2136 |
| FCD_00010668 | -5.8427 | 0.0036 | 1.9316 | 22.0017 | 15.5382 | 29.0245 | 1075.5262 | 2561.6647 |



Discussion and Perspectives

During this thesis, I was interested in some internal (genetic and biochemical) and external (biotic-pollinators, human practices and abiotic-temperature, light, humidity) factors on the emission of floral VOCs, in particular on VOCs responsible for pollinator attraction in plants using the Mediterranean fig tree as a model. Through field monitoring and experiments presented in this thesis, we have been able to provide answers to each of these points:

1) **regarding the factors shaping the composition of floral VOCs along the evolution history in the genus *Ficus***, we reported a phylogenetic signal in VOCs emitted by receptive fig along the evolutionary history of the genus *Ficus*.

2) **regarding the effect of human-driven varietal diversification on the VOCs emitted by *Ficus carica***, we show that stabilizing selection has maintained the chemical signal responsible for pollinator attraction.

3) **regarding the effects of pollinator activity and abiotic factors on the temporal dynamics of floral VOC emission in *F. carica***, we demonstrated that temperature and humidity affected the emission of these VOCs in a non-linear manner. Moreover, the temporal dynamics of the emission of these VOCs follows a daily rhythm, which seems to follow that of the pollinator's activity with an important decrease of this emission in the 5h following the pollination.

4) **Regarding the genes encoding the biosynthesis of VOCs involved in pollinator attraction in *F. carica* and the localization of their production in the fig**, we have identified the function of some genes, and identified for the first time in the fig tree the site of biosynthesis of VOCs attractive to the pollinator.

These main results are detailed below along with the associated perspectives.

1. Evolutionary history shapes the composition of floral scents in a specialized pollination mutualism

In chapter 1 we combined chemical and molecular phylogenetic data from 32 fig species, half of which were analyzed in this study, to examine the effects of factors that could potentially have shaped fig VOC composition over the evolutionary history of the genus. Using the Kmult assay and pPCA we revealed a strong phylogenetic signal in the VOCs emitted by receptive figs.

Variation in floral odor is due to balancing selection by pollinators and other agents, but that it is also affected by phylogenetic constraints associated with VOC biosynthesis (Raguso 2001; Schäffler et al. 2012). In most studies of pollination ecology, pollinator attraction is considered the primary selection pressure acting on floral odor evolution (Majetic et al., 2009), often resulting in convergence in odor composition among unrelated species (Knudsen and Tollsten 1995). However, phylogenetic constraints can have an important influence on the evolution of floral odors. For example, a study on the evolution of floral odors in orchids secreting oil to attract their pollinators found that the phylogenetic signal had a greater effect on floral odor evolution than pollinator-mediated selection in this system (Steiner et al., 2011). Apart from this study, the presence of a phylogenetic signal in the composition of floral odors has rarely been demonstrated to date. This may be because most studies that have tested for the presence of this signal have involved plants pollinated by very different pollinator guilds. Therefore, pollinator effects could be so important that they mask or cancel out the effects of phylogeny. In fig-pollinator interactions, our results suggest the presence of a strong phylogenetic signal in shaping the composition of VOCs released from figs. This phylogenetic signal could be the result of different processes. It could either be due to constraints in the VOC biosynthetic pathways or to selection for attraction of pollinators that have co-evolved with the fig host.

Using the phylogeny available for wasps and the same analysis (Kmult and PCA), we found no significant effect of pollinator phylogeny on the evolution of receptive fig odors. However, in this analysis, we used all emitted VOCs because those responsible

for pollinator attraction are only known for two species (Chen et al., 2009; Proffit et al., 2020). In the future, in order to determine if the phylogenetic signal highlighted in this study is the result of selection pressures exerted by pollinator attraction, we should perform similar statistical analyses but this time using only the compounds recognized by fig wasps. Furthermore, in order to determine whether this signal could be the result of constraints related to the biosynthesis of the VOCs, the characterization of the biosynthetic pathways of some of these VOCs is necessary.

2. Floral scent of the Mediterranean fig tree: significant inter-varietal difference but strong conservation of the signal responsible for pollinator attraction

By characterizing the VOCs emitted by receptive figs of eight varieties of *F. carica* in Morocco, we revealed in chapter 2 a strong conservation of the signal responsible for the attraction of specific pollinators (Cao et al., 2023). In addition to the four VOCs important for pollinator attraction, thirty other compounds were identified in the chemical profiles of receptive figs of these varieties, and these showed substantial inter-varietal differences. Some varieties release large amounts of monoterpenes, while others release large amounts of sesquiterpenes, and one variety is intermediate. The effect of abiotic factors such as temperature was not tested in this study. However, as the sampling was done at the same time for all varieties present in a limited geographical zone, the inter-varietal differences found in this study are most likely the result of human-induced diversification.

Based on the quantitative differences in VOC emissions found in this study, we hypothesize that the varieties with the highest levels of pollinator attracting VOCs are more effective at attracting pollinators, and are pollinated at the beginning of the flowering season when pollinator density is low. These female varieties with very different chemical profiles from wild females were probably selected during the domestication process because their early season pollination was facilitated by their

high VOC emissions. This hypothesis would be interesting to test in the future by conducting behavioral tests of choice between the different varieties studied.

In the present study, the lack of correlation between chemical and genetic distances is most likely explained by the fact that only neutral markers (microsatellites) were used. However, such a correlation could occur at the level of VOC biosynthetic pathways (Levin et al., 2003), or when specific loci are selected. Several terpene synthase genes involved in the synthesis of some monoterpenes (including linalool) and sesquiterpenes in *F. carica* figs have been identified (Nawade et al., 2020; Chapter 4). In the present study, since significant differences in the emission of mono- and sesquiterpenes were found between some varieties, comparison of the expression of these genes in receptive figs of these varieties would be of interest to explore the links between chemical and genetic differences.

Our results suggest that in *F. carica*, a stabilizing selection during human-induced varietal diversification has maintained the chemical signal responsible for pollinator attraction. Future studies should examine whether the conservation of the pollinator attraction signal highlighted here is the result of stabilizing selection driven by dependence on a specific pollinator for fruit production. This hypothesis could be tested by comparing the chemical profiles and expression of related genes of varieties that do not require the presence of pollinators (i.e., parthenocarpic varieties) versus those that depend exclusively on pollinators for fruit production.

3. The temporal dynamic of floral odour emission of *Ficus carica* is influenced by pollinator activity and abiotic factors

In chapter 3 we investigated the emission kinetics of floral VOCs from receptive and pollinated male spring figs of *F. carica* under controlled conditions by performing real-time PTR-ToF-MS measurements and GC-MS identifications for three consecutive days. In parallel, the effects of several abiotic factors, such as light intensity, temperature or humidity were tested. In addition, we tested with behavioral olfactometers whether pollination-induced changes modified pollinator attraction.

VOCs emitted from receptive figs followed a diurnal emission pattern, with a peak at midday corresponding to the period when the pollinator is most active. In addition, we showed that factors such as temperature and humidity affected the emission of these VOCs in a nonlinear manner. After pollination, the emission of pollinator-attracting VOCs decreased very rapidly (after 5 hours following the pollination) and the induced change altered pollinator attraction. Through the study of small-scale variation in floral VOCs, this study shows that the floral odor emission pattern in *F. carica* can be optimized to attract its exclusive pollinators, but also that abiotic factors can influence the emission of these VOCs.

3.1. Daily kinetic of emission of VOCs of fig tree with receptive figs

We showed that fig trees bearing receptive figs exhibited a rhythm of VOC emission throughout the day. The largest emission peak occurred between 11 am and 3 pm, which corresponds to the main activity period of *B. psenes*. Several studies have shown that gene expression of key enzymes, as the final enzymes in biosynthetic pathways, and their enzymatic activity play a role in controlling the circadian rhythmic emission of plant VOCs (Zeng et al, 2017). Therefore, in the future, it would be interesting to conduct experiments by keeping the plants in darkness and compare their VOCs emission with those of plants maintained under natural light conditions to determine whether the diurnal rhythm of floral VOCs emission in fig trees is due to an internal clock (i.e., a circadian rhythm). Furthermore, monitoring VOC emissions and the expression of certain genes in fig trees under these conditions (in the dark) would allow us to deepen our knowledge of the factors regulating this circadian rhythm, whether at the level of gene expression, transcription factors or substrate availability.

3.2. Effects of abiotic factors on the emission of VOCs by receptive figs

The hypothesis that some abiotic factors can influence VOC emissions was also confirmed and the results observed for temperature and humidity (climatic factors) on receptive fig trees are consistent with our hypotheses, but not in those concerning light. For the temperature factor, the VOC emission of the four attractive VOCs increases with temperature up to a threshold value around 22°C and decreases thereafter. This

emission pattern is consistent with what has been reported in other Mediterranean plants (Farré-Armengol et al., 2014). For the humidity factor, there is a significant and positive effect above 60% of humidity on the emission of pollinator-attractive VOCs, but negative on the concentration of sesquiterpenes. Stomatal conductance increases with humidity and thus photosynthesis as well (Lange et al., 1971). This results in increased VOC emissions from plants. The decrease of sesquiterpenes with increasing humidity may be due to the fact that they are mainly involved in aerosol formation (Ormeño et al., 2007). Regarding the light factor, a nonlinear effect on sesquiterpenes and no effect for the four attractive VOCs was expected. The light responses of sesquiterpene emissions are not exactly known, although evidence of light dependence has been reported in some studies (Duhl et al., 2008; Geron and Arnts, 2010). Therefore, a study to experimentally test the molecular mechanisms of the effect of abiotic factors on floral odor would be very interesting.

3.3. Effects of pollination on the emission of VOCs by figs

Floral odor decreases significantly shortly after pollination. In fig flowers, little is known about the signals involved in the cessation of stigma receptivity and the cessation of VOC emission. In snapdragon and petunia, it has been shown that these changes occur after the pollen tubes reach the ovary, which induces the decrease in methyl benzoate emissions between 35 and 40 hours, and, 32 hours after pollination, respectively (Negre et al., 2003). The effect of pollination on the variation of attractive VOC emissions in *F. carica* is much faster: the pollination-induced decrease in VOC emissions attracting pollinators occurs between 5 and 12 hours. Unlike other plants, the fig is a fleshy structure called a syconium, with hundreds of small flowers surrounded by a thick wall with a single entrance (i.e., ostiole) with a central cavity. Only the specific pollinator can access the flowers through the ostiole. Thus, different signals can initiate the post-pollination change in VOC emission: the passage of sufficient numbers of pollinators, the contact of pollinators with the stigma of female flowers, the arrival of the pollen tube at the flower ovaries. Thus, an experiment to characterize this signal could be an interesting perspective.

In Chapter 3, the results also show that the proportion of VOCs responsible for pollinator attraction varies from the first five hours after pollination. This suggests that the selection pressures that maintain the proportions of the 4 attractive VOCs stable at receptivity disappear rapidly after pollination. Behavioral tests of whether pollination-induced changes alter the attraction of figs to pollinators after 5 h of pollination and analysis of gene expression of genes related to biosynthesis of pollinator-attracting VOCs after 5 h of pollination could be an interesting prospect. Moreover, knowing that *F. carica* figs flower a second time in summer, and that abiotic conditions at this season are very different from those in spring, the comparison of fig VOC emission kinetics between these two seasons should also be performed.

4. Localization of the production of pollinator-attractive volatile organic compounds in *Ficus carica*

In chapter 4, analyses of VOCs emitted by *F. carica* figs allowed characterization of changes occurring after pollination. Comparative transcriptomics between receptive and pollinated figs revealed some genes whose expression is down-regulated after pollination. Heterologous expression in *Escherichia coli* of two genes encoding terpene synthases (TPS) revealed that a monoterpene synthase converts GPP to S-linalool and a sesquiterpene synthase catalyzes the formation of Germacrene D. Three complementary experiments (VOC, transcript and cytological analyses in different tissues) showed that the ostiole bracts of syconium are the source of volatile compounds that attract fig wasps.

A previous study characterized two linalool synthases in *F. carica* (Nawade et al., 2020). Our *in vitro* assays indicated that one of these linalool synthases, FcTPS1, exclusively catalyzes the formation of S-linalool. Furthermore, this S-linalool synthase was present in greater amounts in receptive figs than in pollinated figs. We selected a candidate gene for linalool oxide biosynthesis expressed more prominently in receptive than in pollinated figs. This gene encoding a cytochrome P450s FcCYP1 could not be successfully cloned in *E. coli* and tests conducted in a eukaryotic expression system

such as *Saccharomyces cerevisiae* or *Nicotiana benthamiana* were inconclusive. In *Arabidopsis thaliana* flowers, the coexpression of two monoterpene synthases and two cytochrome P450s suggests that the linalool oxide is produced from the linalool (Ginglinger et al., 2013; Boachon et al., 2015). The function of our selected cytochrome P450s gene FcCYP1 as a candidate for a linalool oxide synthase should be identified in a eukaryotic expression system, such as *S. cerevisiae* or *N. benthamiana*. It is also important to test whether the linalool and linalool oxide synthase genes are co-expressed in *F. carica* to better understand pollinator-plant interactions. The genes involved in benzyl alcohol biosynthesis are not known. One study reported that benzyl alcohol can be reduced from benzaldehyde in *Petunia*, but the enzyme is still unknown (Huang et al., 2020). Revealing the enzyme or enzymes of benzyl alcohol should be an interesting prospective.

The three complementary experiments (VOC analyses, transcriptomic analyses, and histology of different tissues) all suggested the same results: the ostiole is the source of production of attractive VOCs. These results confirm those of histological studies (only performed on several fig species (Souza et al., 2015; Castro-Cárdenas et al., 2022). Like *Ficus*, their close relatives, i.e. Castilleae, a tribe from the Moraceae family, possess also involucre bracts (Clement et al., 2020). It will be thus interesting to study the site of biosynthesis of pollinator attractive VOCs in Castilleae trees.

In future, it will be of interest to identify the unknown linalool oxide candidate gene function in yeast *Saccharomyces cerevisiae* or tobacco *Nicotiana benthamiana*, and to perform a gene co-expression analysis of monoterpene linalool and cytochrome P450 linalool oxide synthase in other *Ficus* trees. For example to study the evolution of linalool synthase in different *Ficus* species from China, such as *F. curtipes*, *F. racemosa*, *F. tinctoria* and *F. hispida*. Also, investigating the biosynthetic site of the VOCs attractive to the pollinator in some Castilleae trees will be of interest.

Conclusion

To resume, in this study we integrated metabolomics, bioinformatics, biochemistry, and molecular biology methodologies to investigate the evolution, spatiotemporal variation, and biosynthesis of pollinator attractive signal emission in fig trees. We reveal a strong phylogenetic signal in VOCs emitted by receptive fig trees. Whether pollinator attraction-related selection impacts VOCs emitted by receptive figs should be tested in future work. We found that human domestication may have resulted in inter-varietal differences in VOCs, although the chemical signal responsible for pollinator attraction is highly conserved between varieties. Future studies should examine whether the conservation of the pollinator-attractive signal is the result of stabilizing selection driven by dependence on a specific pollinator for fruit production. We also demonstrated that VOCs emitted from receptive figs follow a diurnal emission pattern, with a peak at midday corresponding to the period when the pollinator is most active. The VOC emission pattern in *F. carica* can be optimized to attract its exclusive pollinators. In addition, abiotic factors such as temperature and humidity influence the emission of these VOCs. It would also be interesting to explore the molecular mechanisms involved in the variation of VOC emission from figs after pollination and under increased temperature, using comparative transcriptomics on different identified genes whose expression decreases after pollination. Heterologous expression of two terpene synthase (TPS) genes revealed that a monoterpene synthase converts GPP to *S*-linalool, and that a sesquiterpene synthase catalyzes the formation of Germacrene D. In the future, the function of the candidate linalool oxide gene should be identified in a eukaryotic expression system and an analysis of the co-expression of this gene and the monoterpene *S*-linalool gene should be performed. Finally, the ostiolar bracts of the syconium were found to be the source of volatile compounds that attract fig wasps. It would be also interesting to study the site of biosynthesis of attractive VOCs to the pollinator in the sister group of *Ficus*, i.e. Castilleae. The present study has examined some key factors affecting the emission of pollinator attractive signals in fig trees and elucidated some molecular mechanisms of the biosynthesis of these VOCs. Our work

thus provides a solid basis for future studies on the evolution of VOC biosynthesis in *Ficus*.



Discussion et perspectives

Au cours de cette thèse, je me suis intéressé à certains facteurs internes (génétiques et biochimiques) et externes (biotiques-pollinisateurs, pratiques humaines et abiotiques-température, lumière, humidité) sur l'émission des COVs floraux, en particulier sur les COVs responsables de l'attraction des pollinisateurs chez les plantes en utilisant le figuier méditerranéen comme modèle. Grâce aux suivis de terrain et aux expérimentations présentées dans cette thèse, nous avons pu apporter des réponses à chacun de ces points :

- 1) **En ce qui concerne les facteurs qui déterminent la composition des COVs floraux au cours de l'histoire évolutive du genre *Ficus***, nous avons démontré qu'il existait un signal phylogénétique des COVs émis par les figes réceptives au cours de l'histoire de l'évolution du genre *Ficus*.
- 2) **En ce qui concerne l'effet de la diversification variétale induite par l'homme sur les COVs émis par *Ficus carica***, nous montrons qu'une sélection stabilisatrice a maintenu le signal chimique responsable de l'attraction des pollinisateurs chez les différentes variétés étudiées.
- 3) **En ce qui concerne les effets de l'activité du pollinisateur et des facteurs abiotiques sur la dynamique temporelle de l'émission des COVs floraux chez *F. carica***, nous avons montré que la température et l'humidité affectaient l'émission de ces COVs de manière non linéaire. De plus, la dynamique temporelle de l'émission de ces COVs suit un rythme journalier qui semble suivre celui de l'activité du pollinisateur, avec une diminution importante de cette émission dans les 5h suivant la pollinisation.
- 4) **Concernant les gènes codant pour les voies de biosynthèse des COVs impliqués dans l'attraction des pollinisateurs chez *F. carica* et la localisation de leur production dans la figue**, nous avons identifié la fonction de certains gènes, et identifié pour la première fois chez ce figuier le site de biosynthèse des COVs attractifs pour le pollinisateur.

Ces principaux résultats sont détaillés ci-dessous, ainsi que les perspectives associées.

1. L'histoire de l'évolution façonne la composition des odeurs florales dans un mutualisme de pollinisation spécialisé

Dans le chapitre 1 nous avons combiné les données chimiques et phylogénétiques moléculaires de 32 espèces de figuiers, dont la moitié a été analysée dans cette étude, afin d'examiner les effets des facteurs qui pourraient potentiellement avoir façonné la composition des COVs des figuiers au cours de l'histoire évolutive du genre. En utilisant le test Kmult et la pPCA, nous avons révélé un signal phylogénétique fort dans les COVs émis par les figes réceptives.

La variation de l'odeur florale est due à une sélection équilibrante par les pollinisateurs et d'autres agents, mais elle est également affectée par des contraintes phylogénétiques associées à la biosynthèse des COVs (Raguso 2001 ; Schäffler et al., 2012). Dans la plupart des études sur l'écologie de la pollinisation, l'attraction des pollinisateurs est considérée comme la principale pression de sélection agissant sur l'évolution des odeurs florales (Majetic et al., 2009), ce qui entraîne souvent une convergence dans la composition des odeurs parmi des espèces non apparentées (Knudsen & Tollsten 1995). Cependant, les contraintes phylogénétiques peuvent avoir une influence importante sur l'évolution des odeurs florales. Par exemple, une étude sur l'évolution des odeurs florales chez les orchidées sécrétant de l'huile pour attirer leurs pollinisateurs a montré que le signal phylogénétique avait un effet plus important sur l'évolution des odeurs florales que la sélection médiée par les pollinisateurs dans ce système (Steiner et al., 2011). Hormis cette étude, la présence d'un signal phylogénétique dans la composition des odeurs florales a rarement été démontrée à ce jour. Cela peut s'expliquer par le fait que la plupart des études qui ont testé la présence de ce signal concernaient des plantes pollinisées par des guildes de pollinisateurs très différentes. Par conséquent, les effets des pollinisateurs pourraient être si importants qu'ils masqueraient ou annuleraient les effets de la phylogénie. Dans les interactions figuier-pollinisateur, nos résultats suggèrent la présence d'un signal phylogénétique fort dans l'élaboration de la composition des COVs libérés par les figes. Ce signal phylogénétique pourrait être le résultat de différents processus. Il pourrait être dû, soit

à des contraintes dans les voies de biosynthèse des COVs, soit à une sélection pour attirer les pollinisateurs qui ont évolué en même temps que la figue hôte.

En utilisant la phylogénie disponible pour les guêpes et la même analyse (Kmult et PCA), nous n'avons pas trouvé d'effet significatif de la phylogénie des pollinisateurs sur l'évolution des odeurs réceptives des figes. Cependant, dans cette analyse, nous avons utilisé tous les COVs émis car ceux responsables de l'attraction des pollinisateurs ne sont connus que pour deux espèces (Chen et al., 2007 ; Proffit et al., 2012). Dans le futur, afin de déterminer si le signal phylogénique mis en évidence dans cette étude est le résultat de pressions de sélection exercées par l'attraction des pollinisateurs, il conviendrait de réaliser des analyses statistiques similaires mais cette fois-ci en n'utilisant que les composés reconnus par les guêpes du figuier. De plus, afin de déterminer si ce signal pourrait être le résultat de contraintes liées à la biosynthèse des COVs, la caractérisation des voies de biosynthèse de certains de ces COVs est nécessaire.

2. Odeur florale du figuier méditerranéen : différence inter-variétale importante mais forte conservation du signal responsable de l'attraction des pollinisateurs

En caractérisant les COVs émis par les figes réceptives de huit variétés de *F. carica* au Maroc, nous avons mis en évidence, dans le chapitre 2, une forte conservation du signal responsable de l'attraction de pollinisateurs spécifiques. En plus des quatre COVs importants pour l'attraction des pollinisateurs, trente autres composés ont été identifiés dans les profils chimiques des figes réceptives de ces variétés, et ceux-ci ont montré des différences inter-variétales substantielles. Certaines variétés libèrent de grandes quantités de monoterpènes, tandis que d'autres libèrent de grandes quantités de sesquiterpènes, et une variété est intermédiaire. L'effet des facteurs abiotiques tels que la température n'a pas été testé dans cette étude. Toutefois, comme l'échantillonnage a été effectué au même moment pour toutes les variétés dans une zone géographique

limitée, les différences inter-variétales constatées dans cette étude sont plus probablement le résultat d'une diversification induite par l'homme.

Sur la base des différences quantitatives d'émissions de COVs constatées dans cette étude, nous émettons l'hypothèse que les variétés ayant les niveaux les plus élevés de COVs attirant les pollinisateurs sont plus efficaces pour attirer les pollinisateurs et sont pollinisées au début de la saison de floraison, lorsque la densité des pollinisateurs est faible. Ces variétés femelles aux profils chimiques très différents de ceux des femelles sauvages ont probablement été sélectionnées pour cela au cours du processus de domestication. Il serait intéressant de vérifier cette hypothèse à l'avenir en effectuant des tests comportementaux de choix entre les différentes variétés étudiées.

Dans la présente étude, l'absence de corrélation entre les distances chimiques et génétiques s'explique très probablement par le fait qu'uniquement des marqueurs neutres (microsatellites) ont été utilisés. Cependant, une telle corrélation pourrait se produire au niveau des voies de biosynthèse des COVs (Levin et al., 2003), ou lorsque des loci spécifiques sont sélectionnés. Plusieurs gènes de terpènes synthases impliqués dans la synthèse de certains monoterpènes (dont le linalool) et sesquiterpènes chez la figue *F. carica* ont été identifiés (Nawade et al., 2020 ; chapitre 4). Dans la présente étude, étant donné que des différences significatives dans l'émission de mono- et sesquiterpènes ont été trouvées entre certaines variétés, la comparaison de l'expression de ces gènes dans les figes réceptives de ces variétés serait intéressante pour explorer les liens entre les différences chimiques et génétiques.

Nos résultats suggèrent que chez *F. carica*, une sélection stabilisatrice au cours de la diversification variétale, induite par l'homme, a maintenu le signal chimique responsable de l'attraction des pollinisateurs. De futures études devraient examiner si la conservation du signal d'attraction des pollinisateurs mis en évidence ici est le résultat d'une sélection stabilisatrice motivée par la dépendance d'un pollinisateur spécifique pour la production de fruits. Cette hypothèse pourrait être testée en comparant les profils chimiques et l'expression des gènes apparentés des variétés qui ne nécessitent

pas la présence de pollinisateurs (c'est-à-dire les variétés parthénocarpiques) vis-à-vis de celles qui dépendent exclusivement des pollinisateurs pour la production de fruits.

3. La dynamique temporelle de l'émission d'odeurs florales de *Ficus carica* est influencée par l'activité des pollinisateurs et par des facteurs abiotiques.

Dans le chapitre 3 nous avons étudié la cinétique d'émission des COVs floraux de figes de printemps mâles réceptives et pollinisées de *F. carica* dans des conditions contrôlées en effectuant des mesures PTR-ToF-MS en temps réel et des identifications GC-MS pendant trois jours consécutifs. Parallèlement, les effets de plusieurs facteurs abiotiques, tels que l'intensité lumineuse, la température ou l'humidité, ont été testés. En outre, nous avons testé, par olfactométrie, si les changements induits par la pollinisation modifiaient le comportement d'attraction des pollinisateurs. Les COVs émis par les figes réceptives ont suivi un schéma d'émission diurne, avec un pic à midi correspondant à la période où le pollinisateur est le plus actif. De plus, nous avons montré que des facteurs tels que la température et l'humidité affectaient l'émission de ces COVs de manière non linéaire. Après la pollinisation, l'émission de COVs attirant les pollinisateurs a diminué très rapidement (après 5 heures suivant la pollinisation), et le changement induit a modifié l'attraction des pollinisateurs. En étudiant les variations à petite échelle des COVs floraux, cette étude montre que le schéma d'émission des odeurs florales de *F. carica* peut être optimisé pour attirer ses pollinisateurs exclusifs, mais aussi que des facteurs abiotiques peuvent influencer l'émission de ces COVs.

3.1. Cinétique journalière d'émission de COVs du figuier avec des figes réceptives

Nous avons montré que les figuiers portant des figes réceptives présentaient un rythme d'émission de COVs tout au long de la journée. Le pic d'émission le plus important se situe entre 11h et 15h, ce qui correspond à la principale période d'activité de *B. psenes*. Plusieurs études ont montré que l'expression génétique d'enzymes clés, telles les enzymes finales des voies de biosynthèse, et leur activité enzymatique jouent

un rôle dans le contrôle de l'émission rythmique circadienne de COVs végétaux (Zeng et al., 2017). Par conséquent, à l'avenir, il serait intéressant de mener des expériences en maintenant les plantes dans l'obscurité et de comparer leurs émissions de COVs avec celles des plantes maintenues dans des conditions de lumière naturelle pour déterminer si le rythme diurne de l'émission de COVs floraux chez les figuiers est dû à une horloge interne (c.-à-d. un rythme circadien). De plus, le suivi des émissions de COVs et de l'expression de certains gènes chez le figuier dans ces conditions (à l'obscurité) permettrait d'approfondir nos connaissances sur les facteurs régulant ce rythme circadien, que ce soit au niveau de l'expression des gènes, des facteurs de transcription ou de la disponibilité des substrats.

3.2. Effets des facteurs abiotiques sur l'émission de COVs par les figuiers réceptifs

L'hypothèse selon laquelle certains facteurs abiotiques peuvent influencer l'émission de COVs a également été confirmée et les résultats observés pour la température et l'humidité (facteurs climatiques) sur les figuiers réceptifs sont cohérents avec nos hypothèses, mais pas dans celles concernant la lumière. Pour le facteur température, l'émission des quatre COVs attractifs augmente avec la température jusqu'à une valeur seuil autour de 22°C et diminue ensuite. Ce schéma d'émission est cohérent avec ce qui a été rapporté chez d'autres plantes méditerranéennes (Farré-Armengol et al., 2014). Pour le facteur humidité, il y a un effet significatif et positif au-dessus de 60% d'humidité sur l'émission de COVs attractifs pour les pollinisateurs, mais négatif sur la concentration de sesquiterpènes. La conductance stomatique augmente avec l'humidité et donc la photosynthèse (Lange et al., 1971). Il en résulte une augmentation des émissions de COVs par les plantes. La diminution des sesquiterpènes avec l'augmentation de l'humidité peut être due au fait qu'ils sont principalement impliqués dans la formation d'aérosols (Ormeño et al., 2007). En ce qui concerne le facteur lumière, on s'attendait à un effet non linéaire sur les sesquiterpènes et à aucun effet pour les quatre COVs attractifs. Les réactions à la lumière des émissions de sesquiterpènes ne sont pas exactement connues, bien que des preuves de dépendance à la lumière aient été rapportées dans certaines études (Duhl et al., 2008 ; Geron & Arnts, 2010). Par

conséquent, une étude visant à tester expérimentalement les mécanismes moléculaires de l'effet des facteurs abiotiques sur l'odeur florale serait très intéressante.

3.3. Effets de la pollinisation sur l'émission de COVs par les figes

L'odeur florale diminue considérablement peu après la pollinisation. Au niveau des fleurs de la figue, on sait peu de choses sur les signaux impliqués dans l'arrêt de la réceptivité du stigmate et l'arrêt de l'émission de COVs. Chez le muflier et le pétunia, il a été démontré que ces changements se produisent après que les tubes polliniques aient atteint l'ovaire, ce qui induit la diminution des émissions de benzoate de méthyle entre 35 et 40 heures, et 32 heures après la pollinisation, respectivement (Negre et al., 2003). L'effet de la pollinisation sur la variation des émissions de COVs chez *F. carica* est beaucoup plus rapide : la diminution des émissions de COVs attirant les pollinisateurs, induite par la pollinisation, se produit entre 5 et 12 heures. Contrairement à d'autres plantes, la figue est une structure charnue, appelée un sycone (ou syconium), avec des centaines de petites fleurs entourées d'une paroi épaisse avec une cavité centrale, avec une entrée unique (l'ostiole). Seul le pollinisateur spécifique peut accéder aux fleurs par l'ostiole. Ainsi, différents signaux peuvent initier le changement post-pollinisation dans l'émission de COVs : le passage d'un nombre suffisant de pollinisateurs, le contact des pollinisateurs avec le stigmate des fleurs femelles, l'arrivée du tube pollinique dans les ovaires de la fleur. Une expérience visant à caractériser ce signal pourrait donc être une perspective intéressante.

Dans le chapitre 3, les résultats ont également montré que la proportion de COVs responsables de l'attraction des pollinisateurs varie à partir des cinq premières heures après la pollinisation. Ceci suggère que les pressions de sélection qui maintiennent les proportions des 4 COVs attractifs stables à la réceptivité disparaissent rapidement après la pollinisation. Des tests comportementaux visant à déterminer si des changements induits par la pollinisation modifient l'attraction des figes pour les pollinisateurs après 5 heures de pollinisation et l'analyse de l'expression des gènes liés à la biosynthèse des COVss attractifs pour les pollinisateurs après 5 heures de pollinisation pourraient constituer une perspective intéressante. De plus, sachant que les figes *F. carica*

fleurissent une seconde fois en été, et que les conditions abiotiques à cette saison sont très différentes de celles du printemps, la comparaison de la cinétique d'émission des COVs des figes entre ces deux saisons devrait également être effectuée.

4. Localisation de la production de composés organiques volatils attractifs pour les pollinisateurs chez *Ficus carica*

Dans le chapitre 4, l'analyse des COVs émis par les figes *F. carica* a permis de caractériser les changements survenant après la pollinisation. La transcriptomique comparative entre les figes réceptives et les figes pollinisées a révélé certains gènes dont l'expression est régulée à la baisse après la pollinisation. L'expression hétérologue dans *Escherichia coli* de deux gènes codant pour des terpènes synthases (TPS) a révélé qu'une synthase monoterpénique convertissait le GPP en S-linalool, et qu'une synthase sesquiterpénique catalysait la formation de Germacrène D. Trois expériences complémentaires (analyses de COVs, de transcription et de cytologie dans différents tissus) ont montré que les bractées ostiolées du syconium sont la source des composés volatils qui attirent les guêpes du figuier.

Une étude précédente a caractérisé deux linalool synthases chez *F. carica* (Nawade et al., 2020). Nos essais *in vitro* ont indiqué que l'une de ces linalool synthases, FcTPS1, catalyse exclusivement la formation de S-linalool. De plus, cette S-linalool synthase était présente en plus grande quantité dans les figes réceptives que dans les figes pollinisées. Nous avons sélectionné un gène candidat pour la biosynthèse de l'oxyde de linalool, exprimé de manière plus importante dans les figes réceptives que dans les figes pollinisées. Ce gène codant pour un cytochrome P450s (FcCYP1) n'a pas pu être cloné avec succès dans *E. coli* et les tests effectués dans un système d'expression eucaryote tel que *Saccharomyces cerevisiae* ou *Nicotiana benthamiana* n'ont pas été concluants. Dans les fleurs d'*Arabidopsis thaliana*, la coexpression de deux monoterpènes synthases et de deux cytochromes P450 suggère que l'oxyde de linalool est produit à partir du linalool (Ginglinger et al., 2013 ; Boachon et al., 2015). La fonction du gène FcCYP1 du cytochrome P450s, que nous avons sélectionné comme

candidat pour une synthèse d'oxyde de linalool, devrait être identifiée dans un système d'expression eucaryote, tels ceux donnés plus haut. Il est également important de vérifier si les gènes du linalool et de l'oxyde de linalool synthase sont co-exprimés chez *F. carica*, afin de mieux comprendre les interactions entre pollinisateurs et plantes. Les gènes impliqués dans la biosynthèse de l'alcool benzylique ne sont pas connus. Une étude a rapporté que l'alcool benzylique pouvait être réduit à partir du benzaldéhyde chez *Petunia*, mais l'enzyme est encore inconnue (Huang et al., 2020). La découverte de l'enzyme ou des enzymes de l'alcool benzylique devrait constituer une perspective intéressante.

Les trois expériences complémentaires (analyses de COVs, analyses de transcriptomique et histologie de différents tissus) ont toutes suggéré les mêmes résultats : l'ostiole est la source de production de COVs attractifs. Ces résultats confirment ceux d'études histologiques réalisées sur plusieurs espèces de figuiers (Souza et al., 2015 ; Castro-Cárdenas et al., 2022). Comme les *Ficus*, leurs proches parents, les Castilleae, une tribu de la famille des Moraceae, dont *Castilla* l'arbre à caoutchouc, possèdent des bractées involucales (Clement et al., 2020). Il serait intéressant d'étudier le site de biosynthèse des COVs attractifs pour les pollinisateurs chez les Castilleae.

À l'avenir, en résumé, il faudrait ainsi identifier la fonction inconnue du gène candidat de l'oxyde de linalool dans la levure *Saccharomyces cerevisiae* ou le tabac *Nicotiana benthamiana*, et effectuer une analyse de la co-expression des gènes du monoterpène linalool et du cytochrome P450 de l'oxyde de linalool synthase, chez les figuiers de Chine. Par exemple l'étude de l'évolution de la linalool synthase pourrait se faire chez différentes espèces du genre *Ficus* de Chine, telles que *F. curtipes*, *F. tinctoria*, *F. racemosa* et *F. hispida*. De plus, étudier le site de biosynthèse des COVs attractifs pour le pollinisateur chez les Castilleae.

Conclusion

Pour résumé, dans cette étude, nous avons intégré des méthodologies de métabolomique, de bioinformatique, de biochimie et de biologie moléculaire pour étudier l'évolution, la variation spatio-temporelle et la biosynthèse de l'émission de signaux attractifs pour les pollinisateurs chez les figuiers. Nous avons mis en évidence un signal phylogénétique fort dans les COVs émis par les figuiers réceptifs. La question de savoir si la sélection liée à l'attraction des pollinisateurs a un impact sur les COVs émis par les figuiers réceptifs devrait être testée dans le cadre de travaux futurs. Nous avons constaté que la domestication humaine peut avoir entraîné des différences inter-variétales dans les COVs, bien que le signal chimique responsable de l'attraction des pollinisateurs soit fortement conservé entre les variétés. Les études futures devraient examiner si la conservation du signal chimique d'attraction des pollinisateurs est le résultat d'une sélection stabilisatrice motivée par la dépendance à l'égard d'un pollinisateur spécifique pour la production de fruits. Nous avons également démontré que les COVs émis par les figues réceptives suivent un schéma d'émission diurne, avec un pic à midi correspondant à la période où le pollinisateur est le plus actif. Le schéma d'émission des COVs chez *F. carica* peut être optimisé pour attirer ses pollinisateurs exclusifs. En outre, des facteurs abiotiques tels que la température et l'humidité influencent l'émission de ces COVs. Il serait également intéressant d'explorer les mécanismes moléculaires impliqués dans la variation de l'émission de COVs par les figues après la pollinisation et sous l'effet de l'augmentation de la température. En utilisant la transcriptomique comparative, différents gènes dont l'expression diminue après la pollinisation ont été identifiés. L'expression hétérologue de deux gènes de terpène synthase (TPS) a révélé qu'une synthase de monoterpène convertit le GPP en *S*-linalool, et qu'une synthase de sesquiterpène catalyse la formation du Germacrène D. A l'avenir, la fonction du gène candidat de l'oxyde de linalool devrait être identifiée dans un système d'expression eucaryote et une analyse de la co-expression de ce gène et du gène du monoterpène *S*-linalool devrait être effectuée. Enfin, les bractées

ostiolaires du syconium se sont révélées être la source de composés volatils qui attirent les guêpes du figuier. Il serait intéressant d'étudier à l'avenir le site de biosynthèse des COVs qui attirent les pollinisateurs dans le groupe frère des *Ficus*, c'est-à-dire les Castilleae. Cette étude a examiné certains facteurs clés affectant l'émission de signaux attractifs pour les pollinisateurs chez les figuiers et a élucidé certains mécanismes moléculaires de la biosynthèse de ces COVs. Nos travaux fournissent donc une base solide pour de futures études sur l'évolution de la biosynthèse des COVs chez les *Ficus*.

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