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# *Parfum de figues :*

# *Approche évolutive de la communication entre une plante, ses insectes pollinisateurs et ses vertébrés disperseurs*

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**RESUME: Parfums de figues : *Approche évolutive de la communication entre une plante, ses insectes pollinisateurs et ses vertébrés disperseurs***

Pour l'ensemble des organismes vivants, la communication (intra ou interspécifique) est le véhicule de l'information, un élément central dans l'interaction entre individus. Elle a ainsi des impacts primordiaux sur les traits d'histoire de vie des espèces, et est donc façonnée par la sélection naturelle. Au cours de cette thèse, nous nous sommes intéressés à l'évolution de la médiation chimique, c'est-à-dire à la transmission d'informations par voie chimique entre deux individus, dans les interactions plantes/animaux. Nous avons choisi le modèle figuier et ses partenaires mutualistes, de la guêpe pollinisatrice aux vertébrés disperseurs de graines. La médiation chimique (ici émission de bouquets volatils constitués de terpènes et de dérivés benzéniques et d'acides gras) permet aux figuiers d'assurer leur reproduction, par attraction des pollinisateurs et des disperseurs de graines. Il existe environ 800 espèces de figuiers à travers le monde, à distribution principalement tropicale. Les figuiers, fructifiant toute l'année, sont souvent qualifiés d'espèce clef de voute des écosystèmes tropicaux, car ils assurent ainsi le maintien des populations de frugivores dans les forêts tropicales dans les périodes où très peu d'espèces d'arbres fructifient. L'interaction figuier/disperseur est plutôt généraliste, alors que le mutualisme figuier/pollinisateur, un exemple de '*nursery pollination*', est hautement spécifique et obligatoire. Au cours de cette thèse, nous avons mis en évidence que les contraintes phylogénétiques et géographiques exercées sur la médiation chimique étaient négligeables, alors que les pressions de sélection que représentent les mutualistes sont nettement plus importantes dans l'évolution de cette médiation. Ainsi, sexe et stade phénologique du figuier et identité du partenaire mutualiste sont des informations qui peuvent être portées par la médiation chimique. Notre approche, globale et multidisciplinaire, des interactions entre les figuiers et leurs partenaires mutualistes aux deux phases de dispersions des gènes de la plante, permet une meilleure compréhension de l'évolution de la communication, et montre que les pressions façonnant ce message façonnent l'interaction elle-même.

**Mots clefs** : communication interspécifique, médiation chimique, composés volatils, mutualisme, pollinisation, dispersion des graines, *Ficus*, Agaonidés, lémuriens, chauves-souris

**ABSTRACT: *« Parfums de figues » : An evolutionary approach to communication between a plant, its pollinating insects and its vertebrate seed dispersers***

For all living organisms, communication (intra-or interspecific) is the vehicle of information, an element central to interactions among individuals. It thus has fundamental impacts on life history traits of species, and is shaped by natural selection. This thesis is concerned with the chemical mediation of communication, i.e., the transmission of information by chemical signals, in the context of animal-plant interactions. We chose as a model system the interactions between figs and their mutualist partners, including both pollinating fig wasps and vertebrate seed dispersers. Chemical mediation (in this case the emission of bouquets of volatile compounds comprising terpenes, benzenoids and fatty acid derivatives) enables figs to ensure their reproduction by attracting pollinators and seed dispersers. There exist about 800 species of *Ficus* worldwide, principally in tropical regions. Figs, which fruit throughout the year, are often considered keystone species of tropical ecosystems, because their year-round fruiting maintains populations of seed-dispersing frugivores during fruit-poor 'lean seasons'. Interactions between fig species and seed-dispersing animals are 'generalist' (i.e., broad food species ranges), while fig/pollinator mutualisms, examples of 'nursery pollination' systems, are highly species-specific and obligatory. In this thesis, we showed that phylogenetic and geographic constraints acting on chemical mediation are negligible, whereas selection pressures grounded in pollination and seed-dispersal mutualisms play a strong role in shaping the evolution of chemical mediation. Thus, chemical mediation carries information such as the sex and developmental stage of the fig tree, as well as the identity of the mutualistic partner. Our comprehensive, multidisciplinary approach to interactions between figs and their mutualist partners in the two phases of dispersal of the plant's genes leads to an improved understanding of the evolution of communication, and showed that the selective pressures shaping the message are also those shaping the interaction itself.

**Keywords** : interspecific communication, chemical mediation, mutualism, volatile compounds, pollination, seed dispersal, *Ficus*, Agaonidae, lemurs, bats

# SOMMAIRE


**I) INTRODUCTION** ……..p 1

**II) CONTEXTE GENERAL** ……..p 31

Manuscrit 1 : Hossaert-McKey M., **Soler C.**, Schatz B. and Proffit M. 2010. Floral scents: their roles in nursery pollination mutualisms. *Chemoecology* 20 :75-88.

Manuscrit 2 : **Soler C.**, Proffit M., Chen C. and Hossaert-McKey M. 2010**.** Private channels in plant-pollinator mutualisms. *Plant signaling & behavior* 5: 1-3.

**III) TRAVAUX EXPERIMENTAUX**……..p 35

**A) CONTRAINTES ET MEDIATION CHIMIQUE**……..p 36

Manuscrit 3 : **Soler C.**, Alvarez N., Bastelica F. , Proffit M., Bessière J.-M., Schatz B. and Hossaert-McKey M. No phylogenetic conservatism of fig odours**.** *In prep.*

Manuscrit 4 : **Soler C.**, Hossaert-McKey M., Buatois B., Bessière J.-M., Schatz B., Proffit M. 2010. Geographic variation of floral scent in a highly specialized pollination mutualism . *Phytochemistry* (in press).

**B) POLLINISATION ET MEDIATION CHIMIQUE**……..p 43

Manuscrit 5 : **Soler C.**, Bard Y., Bessière J.-M., Hossaert-McKey M. and Schatz B. Scent and hybridization: the case of the highly specific figs / fig wasps interactions**.** *In prep.*

Manuscrit 6 : Hossaert-McKey M., **Soler C.**, Proffit M., Chen C., Bessiere J.-M., Schatz B. and Borges R. How to be a dioecious fig ? Case of chemical mimicry and pollination by deceit in a nursery pollination mutualism**.** *In prep.*

Manuscrit 7 : **Soler C.**, Bessière J.-M., Proffit M., Hossaert-McKey M. and Schatz B.. When males change their scents in presence of females, the case of the plant *Ficus carica***.** *In prep.*

Manuscrit 8 : Proffit M., Schatz B., Bessiere J.-M., Chen C., **Soler C.**, Hossaert-McKey M. 2008. Signalling receptivity: Comparison of the emission of volatile compounds by figs of *Ficus hispida* before, during and after the phase of receptivity to pollinators. *Symbiosis* 45: 15-24.

**C) DISPERSION ET MEDIATION CHIMIQUE**.....p 50

Manuscrit 9 : **Soler C.**, Bessière J.-M., Schatz B. and Hossaert-McKey M. Scent as a component of dispersal syndromes: a comparative analysis in the genus *Ficus*. *In prep.*

Manuscrit 10 : **Soler C.**, Chen J., Bessière J.-M., Schatz B. and Hossaert-McKey M. Mutualistic partners require convergence and then divergence of fig scents. *In prep.*

**IV) DISCUSSION**.....p 57

**V) CONCLUSION**.....p 74

VI) **BIBLIOGRAPHIE**.....p 77

**VII) ANNEXES** .....p 86

**Annexe 1 :** Manuscrit 11

Proffit M., Chen C., **Soler C.**, Bessiere J.-M., Schatz B. and Hossaert-McKey M. 2009. Can chemical signals, responsible for mutualistic partner encounter, promote the specific exploitation of nursery pollination mutualisms? – The case of figs and fig wasps. *Entomologia Experimentalis et Applicata* 131: 46-57.

**Annexe 2 :** Matériel & Methodes : prélèvement d'odeurs.

**Annexe 3 :** Matériel & Methodes : tests comportementaux.

# AVANT PROPOS :

Cet avant-propos a pour objectif d'expliciter très rapidement le contexte dans lequel s'est déroulée cette thèse…

Le titre initial de la thèse était : « *Les interactions plantes-frugivores à Madagascar : Intérêt en biologie évolutive et conséquences en biologie de la conservation* ».

Cependant…

1 crise politique majeure à Madagascar

15 mois de missions

1 vol de caméra

1 vol de filet à captures de chauves-souris

des vols de chauves-souris

50 nuits avec les chauves-souris

500 extractions d'odeurs

…. soit 3 ans plus tard, il est devenu : « *Parfum de figues : Approche évolutive de la communication entre une plante, ses insectes pollinisateurs et ses vertébrés disperseurs* ».

Ceci est donc à nos yeux un bon exemple de thèse en écologie, avec les aléas propres au terrain. C'est également une thèse en biologie évolutive, notre objectif étant de comprendre pourquoi nous observions telles et telles choses sur le terrain.

## L'anti-thèse…

Chante à présent la figue, Simiane,

Parce que ses amours sont cachées.

Je chante la figue, dit-elle,

Dont les belles amours sont cachées.

Sa floraison est repliée.

Chambre close où se célèbrent des noces ;

**Aucun parfum ne les conte en dehors**.

Comme rien ne s'en évapore,

Tout le parfum devient succulence et saveur.

Fleur sans beauté ; fruit de délices ;

Fruit qui n'est que sa fleur mûrie.

J'ai chanté la figue, dit-elle,

Chante à présent toutes les fleurs.

Extrait de *'Les Grenades'*, d'André Gide

# MERCI, MERCI, MERCI…

**Merci :** Du latin *mercēdem*, accusatif de *merces* (« salaire, prix, récompense » et en latin tardif « faveur, grâce »), c'est un mot prononcé par politesse, pour exprimer de la reconnaissance, pour rendre grâce en réponse à un service ou une proposition…

Et comme je tiens tant à la politesse, et qu'il y a eu moult services et propositions : Soyez prévenus, ça va être long !! Bien pire encore qu'à la soutenance… ;-p

Alors voilà le retour de la page blanche…3 mois après la soutenance ! Et puis petite pression pour les remerciements, puisque ce sera l'unique section que liront la majorité des gens qui auront cette thèse entre leurs mains… ;-p

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Chose promise, chose due…donc : Merci à Air France et à la FRAM ! ;-p Forcément, 15 mois de mission, ça en a fait des heures de vol / au téléphone …

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MERCI, MERCI, MERCI…

# INTRODUCTION

## La communication comme véhicule d'information

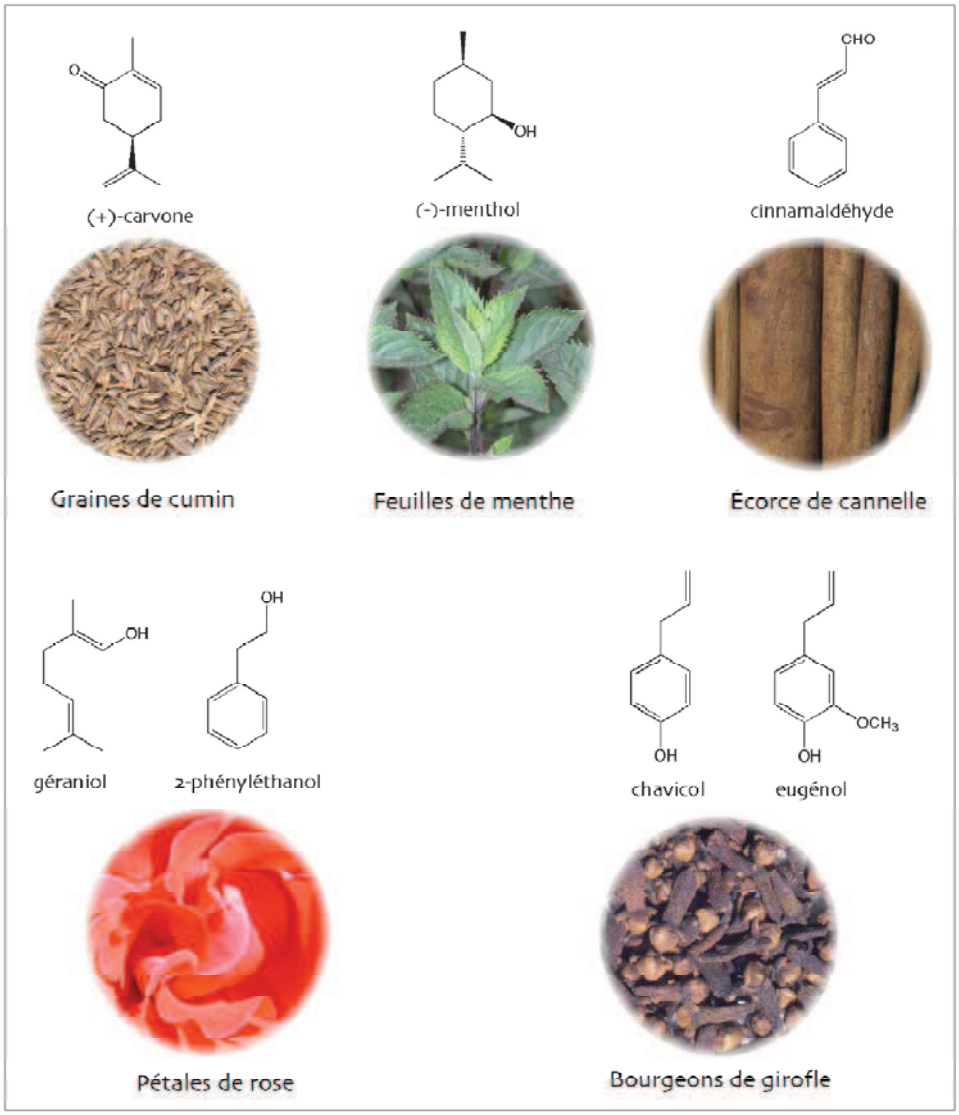
"*Nothing would work in the absence of communication*" : la première phrase du livre de Marc Hauser, " *The evolution of communication*" (1997) est l'idée principale de cette thèse. Pour l'ensemble des organismes vivants, la communication est le véhicule de l'information, un élément central et indispensable à l'interaction entre individus, qu'ils soient de même espèce ou de genres et de règnes différents. En conséquence, la communication a des impacts primordiaux sur les traits d'histoire de vie des espèces, et est donc fortement façonnée par la sélection naturelle. De nombreux chercheurs, qu'ils soient linguistes, psychologues ou biologistes, ont travaillé sur la communication, ce qui se traduit par une multitude de définitions différentes. Ici, nous opterons pour la définition la plus communément utilisée en écologie comportementale (Krebs & Davies, 1993), c'est-à-dire celle où la communication est « l'émission d'un message par un individu *émetteur* qui modifie le comportement d'un ou plusieurs individus *récepteur(s)* ». Emetteurs et récepteurs présentent très souvent des caractéristiques différentes (physiologie, âge, sexe, expérience lorsqu'il y a un acquis, etc.), notamment dans le cas de la communication interspécifique, ce qui engendre des différences entre l'information émise et l'information reçue. Une distinction formelle a dès lors été proposée pour distinguer ces deux types d'informations (Lindström & Kotiaho, 2002) : celle qui est émise est nommée *message*, et celle qui est perçue/interprétée est appelée *signal*. L'étude de la communication a contribué à notre compréhension de la sélection naturelle, en définissant le degré avec lequel les échanges d'informations sont influencés par les caractéristiques des individus communicants, par l'environnement dans lequel ces messages sont émis, ainsi que par le contexte social chez les animaux (Bradbury & Vehrencamp, 1998). La réception permanente d'informations concernant l'environnement abiotique, la communication intraspécifique, ainsi que l'échange interspécifique d'informations sont responsables de la formation et du

fonctionnement des sociétés animales (Danchin *et al.*, 2005), mais aussi de l'histoire de vie d'espèces solitaires (Bradbury & Vehrencamp, 1998), et ainsi de l'ensemble des communautés de l'écosystème.

La nature du message employé a un rôle important, car l'efficacité du message dépend directement du canal sensoriel utilisé (Bradbury & Vehrencamp, 1998). Dans cette définition, le canal correspond à l'audition, la vision, le toucher, l'olfaction, la gustation, ou la perception d'un signal électrique. Ces différents types de message ne sont pas exclusifs et peuvent se combiner entre eux, comme cela a été mis en évidence chez des orchidées, qui émettent des messages aussi bien visuels qu'olfactifs, mimétiques de ceux d'une abeille femelle, pour attirer l'abeille mâle qui les pollinise (Ayasse *et al.*, 2003). La voie de communication utilisée est fortement dépendante du milieu de vie de l'émetteur, et les propriétés physiques du milieu constituent un cadre rigide très contraignant (Endler, 1992). Par exemple, la communication électrique, utilisée notamment par certaines raies (Hopkins, 1974) ou par les poissons-éléphants (Leal & Losos, 2010), ne se rencontre qu'en milieu aquatique, où ce message peut être diffusé efficacement (Danchin *et al.*, 2005). De même, dans un milieu fermé, un message visuel aura une portée moindre qu'un message acoustique, mais cette situation s'inverse dans un milieu ouvert, révélant ainsi de remarquables adaptations du message à l'environnement. Certaines sont issues de la plasticité comportementale des communicants : c'est par exemple le cas du Jardinier satiné (*Ptilonorhynchus violaceus*), un oiseau de l'ouest de l'Australie dont les populations en forêts denses chantent sur des fréquences plus basses que celles des populations des forêts d'eucalyptus plus ouvertes, maximisant ainsi la distance à laquelle le message peut être perçu (Nicholls & Goldizen, 2006). Les caractéristiques physiques des types de communication ont également une influence forte sur le message. Ainsi, lumière et son se propageant par trains d'ondes diffusés en ligne quasi-droite et dans toutes les directions, ils assurent une bonne conservation du message lors de

l'éloignement de la source émettrice (Endler, 1992). *A contrario*, une odeur est très sensible à cet éloignement, car elle se déplace selon des constantes de diffusion propres à chacune des molécules qui la constituent et est influencée par la convection. Dans ce cas, la conservation de l'information varie en fonction de la nature du bouquet d'odeurs, des mouvements d'air et de la présence d'obstacles physiques (Wheeler, 1977; Raguso, 2004). Néanmoins, la diffusion de l'information olfactive permet d'assurer efficacement la localisation de la source : il a été démontré que de nombreux insectes remontent le gradient de concentration d'une odeur attractive jusqu'à parvenir à la source émettrice. C'est le cas par exemple des papillons *Darnio* mâles qui s'orientent vers les femelles distantes de plusieurs kilomètres (Brower *et al.*, 1965). Cet exemple illustre également que l'efficacité d'un message dépend directement de la nature du signal, et donc de la capacité de réception du receveur. La nature et les propriétés physiologiques des récepteurs de ce dernier vont donc être importantes pour la réception du signal. Ainsi, la mésange bleue possède des cônes sensibles aux ultraviolets, qui constituent le signal utilisé dans le cadre de la sélection d'un partenaire sexuel (Doutrelant *et al.*, 2008). Similairement, les insectes sociaux comme les termites et les fourmis possèdent des récepteurs antennaires sensibles aux composés cuticulaires qui leur permettent de repérer leurs conspécifiques (Bagneres *et al.*, 1991; Dietemann *et al.*, 2003). Si l'acquisition au cours de l'évolution de telles structures spécialisées pour la réception peut avoir des coûts physiologiques importants, l'émission du message peut également être coûteuse et complexe. L'émetteur doit être capable de mettre en œuvre des ressources suffisantes pour produire ce message, comme par exemple pour synthétiser tous les composés constituant un message olfactif. De plus, il existe également des coûts indirects liés à l'émission du message. C'est le cas notamment chez le criquet, dont le chant attirant les femelles peut indirectement signaler sa présence aux prédateurs. Cette notion de coûts associés, directs et indirects, est un point fréquemment discuté dans les études sur la communication (Maynard-Smith & Harper, 2003)

**<u>Figure 1 :</u> Exemples de COVs caractéristiques de l'odeurs de quelques espèces de plantes aromatiques** *(d'après Caissard, 2007)*.



En effet, de la notion de coûts associés à la production et/ou l'émission d'un message découle celle de signal honnête (Zahavi, 1977). Du fait de leur coût élevé, certains signaux ne peuvent pas provenir d'individus tricheurs, et constituent donc un signal honnête de la qualité de l'émetteur (comme discuté dans Maynard-Smith & Harper, 1995). Un signal honnête se définit donc comme un signal coûteux à produire, ce coût étant censé empêcher l'intrusion de tricheurs dans le système (Zahavi, 1977; Grafen, 1990). Dans le cas particulier où deux espèces sont impliquées dans un mutualisme obligatoire (voir ci-après), elles ont un intérêt réciproque à se rencontrer ; les coûts associés au message sont généralement compensés par des bénéfices directs, comme une augmentation de la valeur sélective. Cette notion de signal honnête semble donc particulièrement applicable aux cas des mutualismes, mais, étonnamment, elle n'a été que peu étudiée pour ce type particulier d'interactions.

Au cours de cette thèse, ces différents concepts liés à la communication ont été abordés dans le contexte de la médiation chimique. Étudiée ici au niveau interspécifique, la médiation chimique est certainement celle qui offre la plus grande possibilité de codages différents, du fait de la diversité des molécules impliquées, et des possibilités infinies de combinaisons.

## La communication par médiation chimique

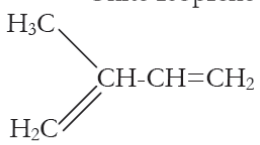
La médiation chimique est le mode de communication impliquant la transmission d'informations par voie chimique entre deux individus, d'espèce identique ou non. Dans le cadre de cette thèse, elle correspondra toujours à un message émis par une plante et perçu par un animal. Dans le cas des odeurs florales par exemple, plus de 1700 Composés Organiques Volatils (COVs) différents ont été identifiés à l'heure actuelle (Knudsen *et al.*, 2006), et

## **Encadré 1 : Les composés volatils de plantes** *(d'après Dufaÿ, 2003)*.

Parmi ces métabolites, certains sont des composés volatils. Ces molécules sont par définition des produits légers pouvant être dispersés par l'air, et sont en général composées de 6 à 20 atomes de carbone. Les composés volatils émis par les plantes appartiennent généralement aux grandes familles chimiques suivantes :

- **les terpènes :**

Unité Isoprène :



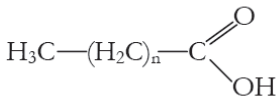

Il existe plus de 2500 terpènes naturels. Les terpènes sont dérivés de l'union d'unités Isoprène à 5 carbones. Parmi les terpènes du métabolisme primaire, on peut citer les pigments caroténoïdes, qui jouent un rôle dans la photosynthèse, et certaines hormones végétales (les gibbérellines, l'acide abcissique).

On trouve également des terpènes « secondaires » non volatils dans certaines résines.

Parmi tous les terpènes existants, certains monoterpènes (molécules en $C_{10}$), certains sesquiterpènes (molécules en $C_{15}$) et quelques diterpènes (molécules en $C_{20}$) sont des composés volatils. On détecte fréquemment des terpènes volatils dans les parfums floraux (ex : le géraniol, le linalol) et dans les bouquets émis par certaines feuilles (ex: le menthol).

- **les dérivés d'acide gras :**

Acide gras :

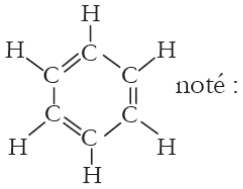



Un acide gras est une chaîne d'hydrocarbones terminée par un groupe carboxylate. Les processus d'oxydations successives d'acides gras conduisent à la formation de dérivés d'acides gras volatils.

Ces composés sont très communs dans les odeurs de feuilles (on les appelle les « green-leaf volatiles »). On les trouve également dans les émissions volatiles de certains champignons (par exemple : le octène-3-ol) et dans certains parfums floraux.

- **Les composés aromatiques (dérivés benzéniques) :**

Benzène :




noté :



Les composés aromatiques sont les molécules contenant un cycle benzénique, dérivés de l'acide shikimique. Ce chemin de biosynthèse produit une partie des protéines.

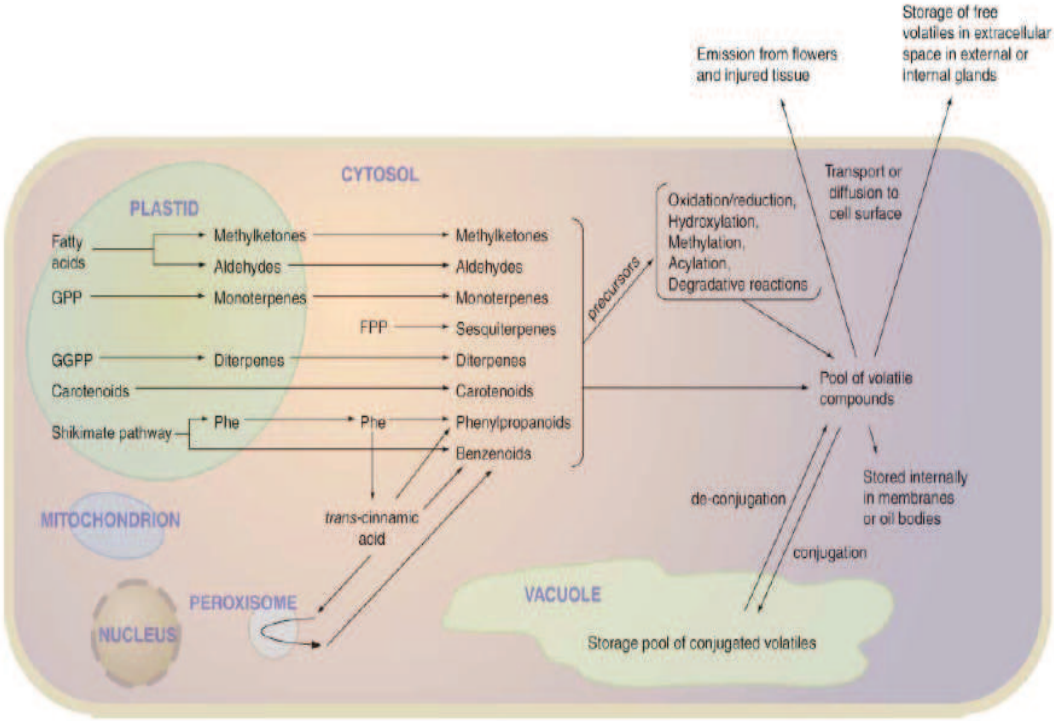
Le composé aromatique le plus abondant dans la biosphère est la lignine, polyphénol complexe stocké au niveau des parois secondaires des cellules conductrices, qui permet le port dressé des plantes vasculaires.

De nombreux composés aromatiques « secondaires » sont les composants caractéristiques d'odeurs « célèbres » comme la vanilline (odeur de la vanille) ou le cinnamaldehyde (odeur de la cannelle).

cette liste ne cesse de croître. La diversité des COVs émis est donc impressionnante (pour quelques exemples, Cf. Fig. 1), et leur combinaison permet une infinité de messages chimiques différents, sachant que leur codage peut varier en termes de présence / absence, de proportions relatives et de quantité totale (Raguso, 2008b). Les COVs sont des métabolites secondaires (c'est-à-dire ne participant pas aux fonctions primaires : respiration, nutrition azotée, photosynthèse des végétaux…), émis par différentes cellules spécialisées dans la sécrétion (trichomes, idioblastes, osmophores, canaux, etc.; Caissard, 2007) des appareils végétatifs ou reproducteurs des plantes (Croteau & Karp, 1991). L'hypothèse généralement admise dans la littérature est que les COVs seraient à l'origine des composés répulsifs « anti-herbivores ». Les herbivores, explorant la plante et visitant ainsi de plus en plus de fleurs, auraient alors propagé le pollen d'une plante à l'autre, devenant ainsi progressivement des pollinisateurs (Pellmyr & Thien, 1986). En outre, les herbivores utilisent souvent les COVs pour localiser leur plante hôte. Si cette pollinisation « accidentelle » par les herbivores augmente la valeur sélective de la plante, alors une pression de sélection s'exerce sur ces COVs pour qu'ils deviennent attractifs pour des pollinisateurs potentiels, plutôt que de rester répulsifs pour les herbivores (Pellmyr & Thien, 1986; Pellmyr *et al.*, 1991).

Les COVs peuvent être regroupés en trois grandes catégories, correspondant aux voies de biosynthèse dont ils sont issus : les terpènes, les composés aromatiques et les dérivés d'acides gras (Cf. encadré 1). Ces voies de biosynthèse nécessitent la mise en place de toute une 'machinerie enzymatique', et sont localisées dans différents compartiments cellulaires, comme les plastes et le cytosol (Cf. Fig.2 et Dudareva & Pichersky, 2000). Le fonctionnement de ces voies de biosynthèse doit être perçu comme un processus « en cascade », où un composé précurseur peut engendrer différents composés selon différentes réactions enzymatiques, mais le retour en arrière n'est jamais possible (après transformation enzymatique, le

**<u>Figure 2 :</u> Schéma global de présentation des voies de biosynthèses des principales familles de COVs** *(d'après Pichersky et al., 2006)*.

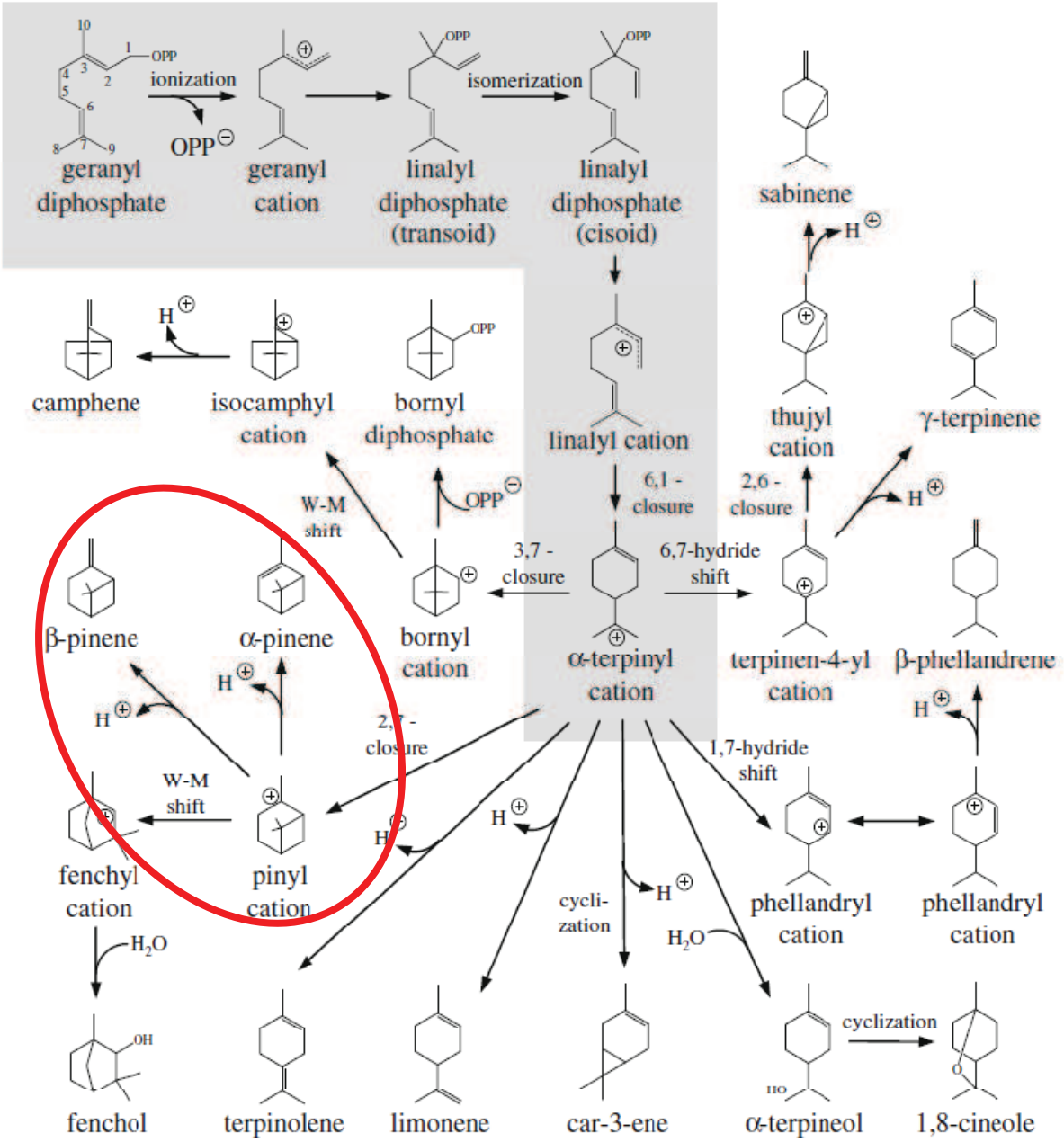


composé A peut engendrer les composés B et C, mais B ou C ne peuvent pas engendrer A). De par ce fonctionnement en cascade des voies de biosynthèse, les COVs d'une même voie ne sont pas indépendants les uns des autres, puisqu'ils sont issus des mêmes précurseurs. Ceci est représenté sur la figure 3, où l'on voit par exemple qu'à partir du pinyl-cation il peut y avoir production d'α-pinène et/ou de β-pinène, des COVs floraux très fréquents, mais que le contraire n'est pas possible (Degenhardt *et al.*, 2009). De plus, c'est la concentration des enzymes et de leur activité (parfois *via* des activateurs ou des inhibiteurs) qui détermine celle des COVs engendrés. Cependant, tous les composés issus de ces voies de biosynthèse ne sont pas volatils, puisque cette caractéristique nécessite d'être relativement « petit » (en général, avec un faible nombre de carbone) et peu fonctionnalisé (nombre de fonctions chimiques liées au composé initial). Les COVs sont donc de faible masse moléculaire (moins de 400 Da) et ont une pression de vapeur partielle relativement élevée à température ambiante (Croteau & Karp, 1991). Le degré de volatilité d'un composé est une caractéristique importante, puisqu'elle influence sa détectabilité à plus ou moins longue distance. Ainsi, un monoterpène (comme le linalol) pourra être perçu à plus longue distance qu'un sesquiterpène (comme le β-caryophyllène). Beaucoup de COVs sont chiraux, c'est-à-dire possèdent un énantiomère[1], souvent synthétisé simultanément (généralement en mélange non racémique[2]). Un récepteur pouvant être lui-même chiral, la discrimination entre deux molécules énantiomères est possible, et donc la forme chirale dans laquelle un COV est synthétisée est également très importante dans sa perception par l'individu receveur, comme cela a été montré dans le cas de l'‘interaction entre les silènes et leur papillons pollinisateurs (Dötterl *et al.*, 2007).

[1] Deux composés énantiomères ont une formule chimique identique, mais leurs configurations géométriques sont l'image en miroir l'une de l'autre.

[2] Se dit de tout mélange équimoléculaire de deux énantiomères.

**<u>Figure 3 :</u> voie de biosynthèse des monoterpènes** *(d'après Degenhardt et al., 2009)*.



Puisque la proportion relative d'un COV au sein d'un bouquet peut varier de 0 à 100%, il y a donc une diversité combinatoire de messages chimiques potentiellement très élevée. De plus, il existe plusieurs origines de ces variations chez les individus d'une même espèce. Un même individu pourra émettre un message différent en fonction de son sexe, de son âge, de son stade phénologique ou de son rythme circadien (Dudareva *et al.*, 2000; Dufaÿ *et al.*, 2004; Dötterl *et al.*, 2005). Des variations interindividuelles de ce message ont également été mises en évidence (Dötterl & Jürgens, 2005; Füssel *et al.*, 2007; Majetic *et al.*, 2009; Ibanez *et al.*, 2010), mais les variations les plus étudiées restent néanmoins les interspécifiques. Ces variations intra et interspécifiques peuvent être examinées selon trois approches différentes que sont l'approche mécanistique (pour comprendre les mécanismes de l'expression d'un caractère), ontogénique (pour déterminer les facteurs génétiques et environnementaux guidant l'expression d'un caractère) ou évolutive (pour comprendre l'impact du caractère sur la valeur reproductive de l'individu, et en tenant compte de l'histoire évolutive de son espèce). En effet, « rien n'a de sens en biologie, si ce n'est à la lumière de l'évolution », puisque les traits actuels résultent d'une longue évolution. Cependant, une récente revue de Raguso (2008b) souligne que la majorité des travaux réalisés en écologie chimique sont encore très descriptifs, et que l'approche évolutive reste encore très faiblement invoquée dans l'interprétation des variations intra et interspécifiques des messages chimiques. Une des spécificités de cette thèse consiste à mobiliser largement cette approche dans l'interprétation des résultats.

Il est cependant important de mentionner ici que d'une part, variations du message ne signifient par forcément variations du signal, et d'autre part, que plusieurs signaux peuvent exister au sein d'un même message. Imaginons ainsi un message émis par une plante, constitué de 10 COVs différents. Un insecte 'A' pourrait n'en percevoir que 5 d'entre eux, alors qu'un insecte 'B' ne percevrait que les 5 autres. Si les 5 derniers composés ne sont plus

synthétisés par la plante, le message émis sera donc différent, mais le signal perçu par l'insecte 'A' restera, lui, identique. Imaginons maintenant que l'insecte 'A' soit un pollinisateur de la plante, alors que l'insecte 'B' est un herbivore. Cette perte des 5 derniers composés dans le bouquet d'odeurs serait alors un gain du point de vue de la valeur reproductive de la plante, et pourrait ainsi être sélectionnée. Cet exemple est cependant hypothétique et simpliste, notamment parce qu'herbivores et pollinisateurs perçoivent généralement les mêmes COVs, ce qui contraint fortement l'évolution d'un message. Pour une plante, « perdre » un COV de son bouquet perçu par un herbivore pourrait être plus coûteux que de le garder, s'il s'agit d'un élément « clef » pour un pollinisateur majeur. Toutefois, cet exemple souligne le rôle que peuvent exercer les partenaires en tant que pression de sélection dans l'évolution du message émis par la plante. Les contraintes liées à la production et à la diffusion de ce message sont également des éléments importants dans son évolution. Pourtant, très peu d'études existent sur ce sujet, comme explicité dans la revue de Majetic et al. (2009) sur les sources de variations des odeurs florales. Au cours de cette thèse, nous nous sommes intéressés à l'évolution de la médiation chimique dans les interactions plantes/animaux. Nous avons ainsi testé l'effet de différentes pressions exercées sur cette médiation chimique telles que i) les contraintes environnementales et phylogénétiques, ii) les interactions liées à la pollinisation et iii) les interactions liées à la dispersion des graines.

## Contraintes liées à la médiation chimique

Comprendre l'importance des pressions de sélection dans l'évolution d'un message nécessite forcément la compréhension des contraintes liées à l'existence même de ce message. Il est possible de classer ces contraintes en deux grands groupes : les contraintes liées à la synthèse du message chimique, et celles liées à sa diffusion dans le milieu. Ces deux catégories ne sont pas exclusives, et par exemple, certaines contraintes sont sous une

influence commune de facteurs environnementaux, comme la température. Ainsi, le limonène, un composé extrêmement fréquent dans les bouquets floraux (Knudsen *et al.*, 2006), a sa diffusion facilitée par augmentation de la température (Kigathi *et al.*, 2009), ainsi qu'une synthèse augmentée du fait de la thermo-sensibilité de l'une des enzymes de biosynthèse, une monoterpène synthétase (Fischbach *et al.*, 2002). Evidemment, cette relation est valide pour une certaine gamme de température, et des températures extrêmes seront un facteur limitant. De surcroît, la température n'est bien sûr pas le seul facteur abiotique environnemental influençant la production des COVs. Ainsi, les périodes de sécheresse induisent par exemple une forte diminution des émissions foliaires chez *Quercus ilex* (Lavoir *et al.*, 2009). Par ailleurs, Ormeno *et al.* (2007) ont montré que, chez certains arbres méditerranéens, les émissions de monoterpènes sont plus fortes sur sol calcaire que sur sol siliceux. L'environnement abiotique d'une plante peut donc avoir une influence forte sur le message qu'elle émet. Majetic et al (2009) ont ainsi montré que l'expression phénotypique d'un caractère (ici, la présence/quantité d'un COV) est sous l'influence des conditions environnementales, tout comme des variations génétiques entre individus ou entre espèces. En effet, le bouquet de COVs peut aussi être contraint par l'histoire évolutive de l'espèce. Ainsi, les chênes sont considérés comme de grands émetteurs d'isoprène, mais il existe des particularités chez les chênes sempervirents méditerranéens, qui sont des émetteurs de monoterpènes ne possédant pas de structures spécifiques de stockage (Lavoir, 2008). Les chênes sempervirents forment par ailleurs un groupe distinct dans la phylogénie du genre *Quercus* (Loreto *et al.*, 1998). Similairement, dans le cas des Nyctaginacées, les dendrogrammes basés sur les voies de biosynthèse reconstituent bien la phylogénie des espèces, telle que construite à partir des marqueurs moléculaires (Levin *et al.*, 2003). En revanche, il ne semble pas y avoir d'inertie phylogénétique forte dans la composition du bouquet chez toutes les plantes, comme par exemple chez les palmiers (Meekijjaroenroj, 2004), ou chez les orchidées (Barkman *et al.*, 1997).

Au cours de cette thèse, nous nous somme intéressés à deux types de contraintes possibles, la contrainte géographique, c'est-à-dire la variation géographique du message chimique dans le cas d'espèces à large aire de distribution, et la contrainte phylogénétique, c'est-à-dire la variation liée à l'histoire évolutive des taxa. Ces deux études constituent la première section du deuxième chapitre de ce manuscrit. Etudier de telles contraintes nous a aidés à déterminer l'importance des pressions de sélection dans l'évolution du message émis, comme par exemple l'interaction de la plante avec son pollinisateur.

## Médiation chimique et pollinisation

Beaucoup d'angiospermes sont pollinisées par des insectes et sont dites entomogames. Il s'agit du mode de pollinisation le plus répandu sous tous les climats, même s'il existe aussi des cas de pollinisation par les oiseaux, les chauves-souris ou d'autres mammifères, et bien sûr par des facteurs abiotiques tels que le vent ou l'eau. Chez les plantes entomogames, l'interaction est parfois mutualiste, et le degré de spécificité de l'interaction peut varier de généraliste (Dobson, 1994; Dobson & Bergström, 2000; Gimenez-Benavides *et al.*, 2007; Karban, 2008; Raguso, 2008a) à hautement spécifique (Dufaÿ *et al.*, 2003; Hossaert-McKey *et al.*, 2010), en passant par des systèmes oligospécifiques (Meekijjaroenroj, 2004). La spécificité de l'interaction dépend notamment de différentes forces écologiques (favorisant ou non la spécialisation) et de l'histoire évolutive des espèces impliquées.

L'ensemble des traits impliqués dans l'attraction des pollinisateurs par la plante est regroupé sous le terme de syndrome de pollinisation, allant de la phénologie de la production des fleurs, à l'ensemble des caractéristiques florales, telles que la morphologie, la couleur, et l'odeur (Fenster *et al.*, 2004). Nous nous intéressons ici aux cas où la communication s'effectue par voie

chimique, ce qui permet notamment à l'insecte une détection de la plante hôte à distance (Faegri & Van Der Pijl, 1971; Dobson, 1994). Les pressions de sélection s'exerçant sur le message vont être différentes selon le degré de spécificité de l'interaction. Dans ce contexte, un cas de pollinisation spécifique, très intéressant en biologie évolutive, est celui des mutualismes « *nursery pollination*[3] ». Ces interactions particulières sont caractérisées par le fait qu'un pollinisateur spécifique utilise la plante comme site de reproduction, en pondant ses œufs dans les inflorescences (le plus souvent dans les ovaires), les larves se développant aux dépens des graines. Ces *nursery pollination* engendrent ainsi des conflits d'intérêts importants entre mutualistes, bien que chacun soit totalement dépendant de l'autre pour sa reproduction. Ces mutualismes sont à transmission horizontale, puisque la rencontre entre partenaires doit être renouvelée à chaque génération. L'utilisation par l'insecte du message émis par la plante pour la localiser est donc cruciale au maintien de l'interaction. Dans ces systèmes, un message spécifique est émis par l'inflorescence (Svensson *et al.*, 2005; Dötterl *et al.*, 2006; Proffit *et al.*, 2009; Ibanez *et al.*, 2010) ou, dans des cas très rares, par les feuilles (Dufaÿ *et al.*, 2003). Notons qu'il existe de nombreux débats dans la littérature, sur la médiation chimique d'interactions de pollinisation spécifiques, autour de l'existence de « canaux privés »[4], et de leur rôle comme mécanismes structurants des interactions plantes / pollinisateurs (Raguso, 2008b; Svensson *et al.*, 2010). Les mutualismes *nursery pollination* peuvent également être des exemples de pollinisation par tromperie. Ainsi, chez le palmier nain, les plantes mâles «coopèrent» avec le charançon pollinisateur, tandis que les plantes femelles « trichent » et sont pollinisées par tromperie (Dufaÿ & Anstett, 2004), c'est-à-dire pollinisées sans offrir le bénéfice du développement de ses larves au pollinisateur. La tromperie est

[3] Pour ne pas froisser les « pourfendeurs des anglicismes », nous aurions pu traduire ce terme par « pollinisation-pouponnière » (M-A Selosse, comm. pers.). Toutefois, le terme étant utilisé en anglais par l'ensemble de la communauté scientifique française, nous préférons le conserver ainsi.

[4] *private channels* en anglais, message qui ne peut être usurpé par un receveur non désiré.

possible grâce aux bouquets de COVs émis par les plantes, les plantes mâles et femelles palmiers émettant des bouquets similaires non discriminables par l'insecte.

Bien que le rôle des odeurs ait été nettement plus étudié dans le cadre de l'interaction plante/pollinisateur, de nombreuses questions demeurent sans réponse, comme en témoigne la récente revue sur les *nursery pollination* (Hossaert-McKey *et al.*, 2010). Mais le pollinisateur n'est évidemment pas le seul partenaire avec lequel une plante interagit ! Ainsi, la médiation chimique est également utilisée dans le cas d'interactions plantes/frugivores.

## Médiation chimique et dispersion des graines

La dispersion des graines est la seconde phase de dispersion des gènes d'une plante, après celle du pollen. La dispersion[5] permet la colonisation par la plante de nouveaux milieux, et influence le taux d'extinctions locales, constituant ainsi un déterminant important dans la structure d'une métapopulation (Olivieri *et al.*, 1995). Cette dynamique dépend essentiellement d'un flux de graines, qui varie selon la structuration de l'habitat et selon l'agent disperseur (Godoy & Jordano, 2001). Comme pour le pollen, les graines ne sont pas mobiles par elles-mêmes, et leur dispersion fait intervenir un vecteur abiotique comme le vent (anémochorie) ou l'eau (hydrochorie), ou biotique comme la plante elle-même (autochorie) ou bien des animaux (zoochorie) (Howe & Smallwood, 1982). Cependant, il existe une différence importante entre transport du pollen et transport des graines. Le pollen disséminé par le vecteur doit atteindre un lieu précis (les stigmates d'une fleur réceptive), ce qui n'est pas le cas lors de la dispersion des graines.

[5] Dans ce manuscrit, lorsque nous emploierons le terme de dispersion, il s'agira de celle des graines (sauf indication contraire).

En outre, cette dispersion dépend davantage des conditions environnementales : une graine pourra engendrer une plantule uniquement si les conditions microclimatiques et édaphiques requises pour sa germination existent là où elle a été déposée. Si une plante peut créer des mécanismes de guidage du pollinisateur vers les stigmates où déposer le pollen, *a contrario*, elle ne peut pas influencer le disperseur quant à l'endroit où déposer les graines – c'est ce qui permet du reste la colonisation d'espaces nouveaux. Chaque espèce animale présente cependant des particularités écologiques et comportementales qui l'amènent à visiter plus souvent certains biotopes, et donc à y déposer plus fréquemment les graines qu'il transporte (Charles-Dominique, 2001). Il peut donc y avoir une sélection permettant à la plante d'attirer plus spécifiquement un animal vivant dans des milieux favorables au développement de ses plantules.

Les premiers cas connus de zoochorie, correspondant à l'interaction entre Gymnospermes et Sauriens, sont apparus il y a près de 200 millions d'années (Tiffney, 2004). Nous nous intéresserons ici uniquement au cas de la frugivorie, c'est-à-dire lorsque les graines sont à l'intérieur d'un fruit charnu, dont la pulpe servira de ressource alimentaire aux frugivores. Dans ce cas, il y a échange de bénéfices entre deux individus d'espèces différentes (alimentation / dispersion), et donc mise en place d'un mutualisme de dispersion. Ce mutualisme a été particulièrement étudié dans les écosystèmes tropicaux, où il joue un rôle très important pour la dynamique forestière puisque jusqu'à 90% des plantes y sont zoochores (Charles-Dominique, 2001). Les frugivores peuvent être des frugivores stricts ou occasionnels, et les interactions de zoochorie sont le plus souvent peu spécifiques envers une espèce de plante. Dans ce travail, nous emploierons le terme de frugivore pour un animal dont le régime alimentaire est composé de fruits à hauteur d'au moins 50% (Terborgh, 1986).

La dispersion des graines est un processus complexe, comprenant plusieurs étapes, allant d'une plante adulte à l'établissement de sa

descendance. Dans ce travail, nous nous sommes concentrés sur la première phase, dite de pré-dispersion, où la plante doit attirer les frugivores. Comme dans le cadre de la pollinisation, la plante communique afin d'attirer son mutualiste, et le syndrome de dispersion correspond à l'ensemble des traits impliqués dans cette attraction, de la phénologie d'apparition à l'ensemble des caractéristiques du fruit, comme notamment la morphologie, la couleur, ou l'odeur (Van der Pijl, 1969). Cette communication présente des coûts physiologiques directs associés à la production d'un fruit, et des coûts indirects, du fait de l'utilisation de ces messages par d'autres frugivores non mutualistes, tels que des prédateurs de graines (Janzen, 1971). L'étude conjointe de la zoochorie et de la frugivorie a donné lieu à l'émergence de nombreux concepts et hypothèses quant à l'évolution d'une telle interaction, comme l'hypothèse de stratégie des plantes vers un fort ou faible investissement dans les fruits (McKey, 1975), ou la coévolution plantes/frugivores (Howe & Smallwood, 1982). La notion de syndromes de dispersion, fréquemment utilisée dans la littérature, permet de décrire des cas de coévolutions diffuses entre taxa, qui correspondent à des groupes plus ou moins diversifiés de frugivores qui ont des tailles, des anatomies et des comportements compatibles avec un ensemble de caractéristiques (ou syndromes) des fruits.

A l'heure actuelle, le rôle des fragrances des fruits dans l'attraction de vertébrés frugivores disperseurs de graines a été mis en évidence dans trois grands groupes : l'ensemble paraphylétique des reptiles (principalement les chéloniens, qui repèrent les fruits tombés à terre grâce à l'odorat et la vue) (Strong & Fragoso, 2006), les oiseaux (repérage des fruits principalement visuel, mais il existe des exemples de détection olfactive) (Leroy, 1987), et les mammifères (aériens, inféodés au sol ou arboricoles, et ayant une détection visuelle et/ou olfactive, selon qu'il s'agisse de petits mammifères terrestres, de primates ou de chauves-souris) (Thies *et al.*, 1998; Dominy *et al.*, 2001). Le rôle principal du message émis est d'attirer un disperseur, qui initiera ensuite une

**<u>Figure 4 :</u> Quelques exemples de la diversité des *Ficus*.**



séquence de comportements alimentaires, comme la collecte des fruits et leurs transports dans le cas de certaines chauves-souris par exemple (Sanchez *et al.*, 2004). Au cours de cette thèse, nous avons cherché à déterminer si les odeurs pouvaient constituer un élément pertinent dans le concept des syndromes de dispersion, et si les pressions de sélection s'exerçant sur le message émis étaient comparables à celles mises en œuvre lors de la phase de pollinisation.

## Modèle d'étude : les interactions figuiers / mutualistes

Pour étudier le rôle de la médiation chimique dans l'attraction des pollinisateurs et des frugivores disperseurs de graines, il est intéressant de proposer un modèle intégrant à la fois des interactions hautement spécialisées et des interactions plus généralistes. Du fait de son implication, d'une part dans un mutualisme de pollinisation spécifique, de type *nursery pollination*, et d'autre part, dans un mutualisme de dispersion des graines plus généraliste, de par son statut « d'espèce clef de voûte des écosystèmes tropicaux »[6] (Shanahan *et al.*, 2001), le genre *Ficus* correspond idéalement à nos pré-requis concernant le choix du modèle d'étude.

Il existe environ 800 espèces de figuiers à travers le monde (Cf. Fig. 4 pour quelques exemples). A l'exception de *Ficus carica*, le figuier méditerranéen, il s'agit d'un genre à distribution pantropicale. Toutes les espèces du genre *Ficus* sont caractérisées par la présence d'une inflorescence refermée sur elle-même : la figue, ou sycone (elle engendre une infrutescence que nous considérerons ici pour simplifier comme un fruit). Les figuiers présentent une phénologie particulière puisqu'ils fructifient tout au long de l'année, et ils sont, de ce fait, qualifiés d'espèce clef de voûte des écosystèmes tropicaux (Shanahan *et al.*, 2001). En effet, en saison sèche, les

[6] Généralement nommée « *keystone species*» dans la littérature scientifique, cette notion est expliquée dans le paragraphe suivant.

**<u>Figure 5 :</u> Quelques exemples de frugivores se nourrissant notamment de figues.**



frugivores forestiers tropicaux ont peu de fruits à leur disposition. Les figues étant presque l'unique ressource disponible en permanence sur l'année, elles assurent ainsi le maintien des populations de frugivores dans les forêts tropicales (Lambert & Marshall, 1991), profitant en retour à d'autres taxa plus saisonniers. En outre, les figues correspondent à une ressource intéressante, car ce sont des fruits riches énergétiquement. La défense principale contre l'herbivorie correspond à la production de latex, qui cesse à maturité des fruits, et joue donc seulement un rôle de protection du 'pré-fruit'. Il existe ainsi une importante guilde de frugivores associée aux figuiers (notamment pendant les périodes de faible production des autres espèces végétales à fruits charnus) qui sont, à quelques rares cas d'hydrochorie cités, les principaux agents disperseurs des graines de figuiers (Shanahan *et al.*, 2001).

Cette caractéristique de « ressource alimentaire essentielle », dans le fonctionnement des écosystèmes tropicaux, explique la présence fréquente des figuiers dans les études sur les frugivores (Shanahan *et al.*, 2001). De plus, le mutualisme hautement spécifique et obligatoire figuier/pollinisateur est lui aussi fréquemment cité dans la littérature scientifique (Ramírez, 1974). En effet, toutes les figues d'une espèce de figuier sont pollinisées par une ou quelques espèces de pollinisateurs spécifiques (Rasplus, 1996; Kerdelhue *et al.*, 1999; Lopez-Vaamonde *et al.*, 2001; Lopez-Vaamonde *et al.*, 2002). Le pollinisateur est un hyménoptère de la famille des Agaonidés, qui dépend obligatoirement et spécifiquement de la figue pour sa reproduction, car ses larves se nourrissent des ovules des fleurs femelles transformées en galles (Cf. Fig. 6 pour quelques exemples d'insectes). Cette interaction figuier-pollinisateur s'est mise en place il y a 60-100 millions d'années (Cook & Rasplus, 2003), et la pollinisation s'effectue activement ou passivement selon l'espèce d'agaonide considérée (Jousselin & Kjellberg, 2001). Il existe ainsi près de 800 réplicats de cette interaction mutualiste, non indépendants phylogénétiquement (Anstett *et al.*, 1997).

**<u>Figure 6 :</u> Quelques exemples d'insectes (pollinisateurs et parasites) associés aux *Ficus*.**



Près de la moitié des espèces de figuiers sont monoïques, les autres étant (gyno)dioïques[7]. Chez les figuiers monoïques, les femelles de pollinisateurs (les fondatrices) entrent dans les figues réceptives, à la fois pour y déposer le pollen qu'elles transportent et pondre leur œufs en insérant leur ovipositeur dans le style de la fleur (Verkerke, 1989; Kjellberg *et al.*, 2005). Graines et descendants de pollinisateurs se développent donc ensemble, à l'intérieur de la figue. Après plusieurs semaines (en moyenne 2 à 3 mois selon les espèces), les guêpes mâles sortent de leurs galles[8], et fécondent les femelles, avant de les aider à sortir de leurs propres galles. La forte protogynie des figuiers monoïques permet aux guêpes femelles, dès la sortie de leurs galles, de se charger en pollen. Utilisant l'ostiole, ou bien un tunnel creusé à travers la paroi de la figue par les agaonides mâles avant qu'ils ne meurent, les guêpes femelles quittent leur figue natale à la recherche d'une nouvelle figue réceptive où déposer leurs œufs. Dans le cas des figuiers monoïques, une même figue assurera donc les fonctions mâles et femelles, grâce au polymorphisme de longueur des styles des fleurs femelles. En effet, certains styles sont trop longs pour que le pollinisateur puisse atteindre l'ovule de la fleur et y déposer son œuf (un œuf par fleur). Ces fleurs seront donc uniquement pollinisées et produiront des graines, alors que les fleurs à styles plus courts permettront la ponte de la fondatrice, et donc le développement de larves, qui assureront, au stade adulte, le transport du pollen de la figue où elles sont nées.

Chez les figuiers dioïques, le même mécanisme est réalisé, mais chez des individus différents. En effet, les arbres femelles produisent des figues à fleurs femelles uniquement, de type « style long ». Ces figues n'assureront alors

[7] Coexistence au sein d'une même espèce végétale de pieds femelles (à fleurs femelles) et de pieds fonctionnellement mâles (à fleurs mâles et femelles).

[8] Excroissance du tissu végétal, provoquée dans notre cas par l'insecte.

**<u>Figure 7 :</u> Cycle de *F. sycomorus*, figuier monoïque** *(d'après Galil & Eisikowitch, 1968)*.

Galil & Eisikowitch (1968) ont été les premiers à distinguer cinq phases dans le cycle de développement des figues.

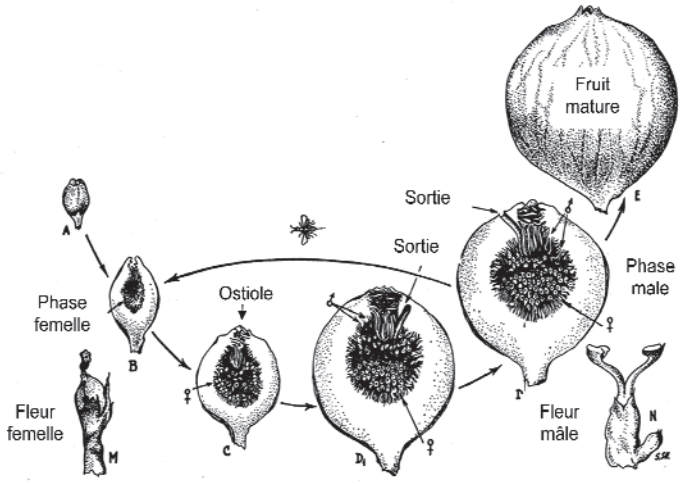
- En phase *pré-femelle*, **phase A**, les jeunes fleurs femelles à l'intérieur des figues ne sont pas encore réceptives.

- En phase *femelle*, **phase B**, les fleurs femelles sont à maturité et leur stigmate est réceptif. Une femelle de pollinisateur va pénétrer dans la cavité de la figue en passant par l'ostiole (bractées imbriquées au sommet de la figue). Une fois à l'intérieur de la figue, elle va polliniser les fleurs femelles et déposer un œuf dans les ovaires de certaines fleurs (voir figure 0-1).

- En phase *inter-florale*, **phase C**, les graines et les larves (dans les ovaires transformés en galles) vont se développer. La figue va augmenter en diamètre.

- En phase *mâle*, **phase D**, les fleurs mâles sont à maturité. Les pollinisateurs mâles, aptères, vont émerger dans la figue et féconder les femelles toujours enfermées dans leur galle. Après avoir émergé de leur galle, les femelles se chargent en pollen (activement ou passivement). Elles vont ensuite quitter leur figue natale, à travers le trou de sortie creusé par les mâles dans la paroi de la figue et chercher une autre figue réceptive pour se reproduire.

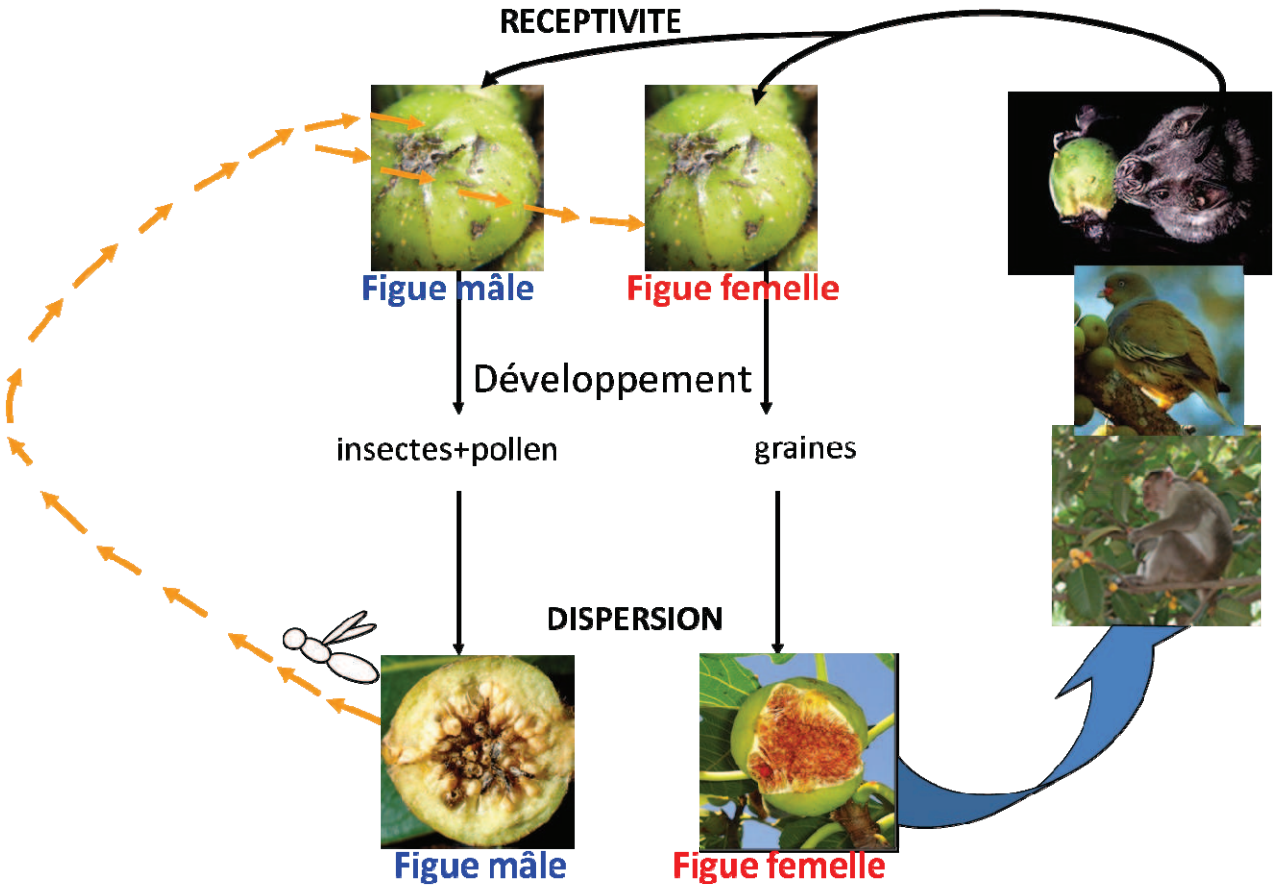
- En phase post-florale, **phase E**, la figue est alors mûre et attire les frugivores, disperseurs de graines.



que la production de graines. *A contrario*, les figuiers mâles (hermaphrodites fonctionnellement mâles en fait) produisent des figues contenant à la fois des fleurs mâles (production du pollen) et des fleurs femelles de type « style court », adéquates à la ponte des insectes. Ces figues assureront donc simultanément la production d'insectes et de pollen. Il apparait donc ici que la ressource pour l'insecte (les fleurs femelles où ses larves se développent) est indisponible dans le cas des figuiers dioïques femelles. Nous sommes donc en présence d'un cas de pollinisation par tromperie, où certains insectes sont leurrés, et il est alors très intéressant de comprendre pourquoi, au cours de l'évolution, le pollinisateur n'a pas appris à éviter les figuiers femelles (Grafen & Godfray, 1991; Patel *et al.*, 1995).

Le cycle de développement d'une figue est caractérisé par cinq phases successives (Cf. Fig. 7), à savoir (A) une phase de pré-réceptivité, (B) une phase dite réceptive (c'est-à-dire la phase où les fleurs femelles sont prêtes à recevoir le pollen), (C) une phase post-pollinisation, ensuite (D) la phase dite mâle, où le pollen est produit à l'intérieur des figues sur les fleurs mâles et où les insectes, chargés de pollen, émergent (Kjellberg *et al.*, 2005), et une dernière phase (E) correspondant à celle de la dispersion des graines. Chez les figuiers dioïques, la phase (D) est la dernière phase pour les arbres mâles, et est inexistante chez les arbres femelles, pour qui la dernière phase est la (E) (Cf. Fig. 8). La phénologie des figuiers est le plus souvent caractérisée par une synchronie intra-arbre des figues, et une asynchronie inter-arbres dans une population. Chez certaines espèces dioïques, il existe une asynchronie phénologique importante entre arbres mâles et femelles d'une même population, qui ne sont jamais simultanément au même stade phénologique. Les phases de réceptivité et de dispersion sont des phases très sensibles du cycle, puisqu'elles correspondent à la dispersion des gènes du figuier, et le succès de ces deux phases dépend tout particulièrement de la communication entre le figuier et ses partenaires mutualistes. Quel que soit le

**Figure 8 :** Cycle d'un figuier dioïque



type de mutualistes impliqués (pollinisateur ou disperseur de graines), la médiation chimique joue un rôle important, voire indispensable. De nombreux travaux se sont intéressés à l'utilisation de ces signaux dans des relations hautement spécifiques, notamment celles entre figuiers et pollinisateurs (Ware *et al.*, 1993; Hossaert-McKey *et al.*, 1994; Gibernau *et al.*, 1998; Song *et al.*, 2001; Proffit *et al.*, 2009) ; l'interaction entre figuiers et animaux disperseurs de graines est beaucoup plus négligée, alors qu'elle est aussi importante dans la valeur sélective des figuiers (Shanahan *et al.*, 2001). Comprendre le rôle de la médiation chimique dans les interactions figuiers / mutualistes, à savoir avec le pollinisateur, et avec le disperseur de graines, ont été des objectifs de cette thèse, et correspondent respectivement aux sections 2 et 3 du deuxième chapitre de ce manuscrit.

Deux éléments très importants doivent être considérés dans le cas particulier de la médiation chimique figuier / partenaires mutualistes. Tout d'abord, les figuiers présentent une forte variation interindividuelle des messages chimiques émis, susceptible de masquer d'autres types de variations (Grison-Pigé, 2001; Proffit, 2007), comme les variations intersexuelles. Nous en présenterons notamment l'impact dans le cas de la pollinisation d'espèces dioïques. Ensuite, il est important de comprendre que même s'il est capital pour le figuier d'être aisément repérable par son mutualiste, il y a toujours un compromis très important à envisager quant à sa détectabilité par d'autres partenaires non mutualistes. Les bouquets de COVs émis sont ainsi un élément structurant de la large communauté de fourmis prédatrices opportunistes (Schatz *et al.*, 2008; Schatz & Hossaert-McKey, 2010) et d'hyménoptères parasites spécifiques associés aux figuiers (Proffit, 2007; Proffit *et al.*, 2007). L'usurpation du message par des parasites peut avoir un coût très élevé, et ceci représente donc une pression importante sur la médiation chimique. Malgré l'importance de cette question, elle n'était pas au centre de cette thèse, ce pourquoi l'article correspondant à l'étude de la médiation

chimique des interactions figuiers/parasites figure en annexe 1 de ce manuscrit.

L'ensemble des éléments présentés explique la structure de ce manuscrit de thèse, qui commence par une partie sur le contexte général, correspondant à un article de revue et un autre de commentaire. Ensuite, ce manuscrit continue par la présentation de la partie expérimentale organisée en trois sections, la première sur les contraintes exercées sur la médiation chimique, la deuxième sur la relation entre cette médiation et la pollinisation, et la troisième sur la relation entre cette médiation et la dispersion des graines. L'ensemble de ces travaux seront ensuite discutés, pour retranscrire ce qu'ils apportent à la connaissance de ce modèle figuier-mutualistes et, de façon plus générale, à la communication interspécifique. Enfin, ce manuscrit se terminera par la présentation d'un article supplémentaire, concernant l'intervention des parasites du mutualisme figuier/pollinisateur, puis par celle de quelques aspects méthodologiques communs à plusieurs de ces chapitres.

# CHAPITRE 1 : Contexte général

**Articles associés :**

Manuscrit 1 : Hossaert-McKey M., **Soler C.**, Schatz B. and Proffit M. 2010. Floral scents: their roles in nursery pollination mutualisms. *Chemoecology* 20 :75-88.

Manuscrit 2 : **Soler C.**, Proffit M., Chen C. and Hossaert-McKey M. 2010**.** Private channels in plant-pollinator mutualisms. *Plant signaling & behavior* 5: 1-3.

Ce premier chapitre correspond à un travail assez « théorique ». En effet, les deux articles associés ne correspondent pas à une démarche expérimentale, mais sont des travaux de revue et de réflexion autour des théories de la communication, pouvant s'appliquer aux *Ficus*. Dans le cadre de la médiation chimique d'interactions plante/pollinisateur, nous nous sommes particulièrement penchés sur le fait que les pressions de sélection s'exerçant sur le message chimique vont être différentes selon le degré de spécificité de l'interaction.

Comme explicité dans l'introduction, l'interaction figuier/pollinisateur est un exemple de mutualisme *nursery pollination*, un cas de pollinisation souvent considéré comme un « cas d'école » en écologie évolutive. Le premier article de cette thèse est une revue sur le rôle de la médiation chimique dans ce type de mutualisme de pollinisation, où le pollinisateur utilise la plante comme site de reproduction. Du fait de la transmission horizontale de ces mutualismes, le message émis par la plante, et utilisé par l'insecte pour la localiser, est crucial pour le maintien de l'interaction. Dans ces systèmes, le message émis est donc spécifique (Dufaÿ *et al.*, 2003; Svensson *et al.*, 2005; Dötterl *et al.*, 2006; Proffit *et al.*, 2009; Ibanez *et al.*, 2010), même si le degré de spécificité de l'interaction diffère selon le cas de *nursery pollination* étudié. Ainsi, dans l'interaction *Trollius europaeus / Chiastocheta,* jusqu'à six espèces de mouches peuvent être pollinisatrices (Després *et al.*, 2002), alors que dans l'interaction *Ficus* / Agaonideae, pour la majorité des cas, la règle du « one-to-one » est très généralement respectée (Cook & Rasplus, 2003). Nous avons ainsi réalisé une étude comparative de ces différents systèmes de *nursery pollination*, en abordant différents points très discutés dans la littérature, tels que l'existence des canaux privés, la tromperie, ou encore la diversité des communautés associées à ces systèmes. Nous avons alors dégagé de grands patterns communs à ces interactions, comme le fait que les COVs « importants », c'est-à-dire ceux perçus par le pollinisateur et ainsi appartenant au signal, soient fortement sélectionnés et

constituent alors les composés majoritaires au sein du bouquet. De même, les messages chimiques dans ces interactions sont souvent relativement simples (nombre et nature des molécules produites) et abondants, facilitant la rencontre entre les deux partenaires dans un paysage olfactif complexe.

Le second article de cette thèse est un travail de réflexion autour de l'unique cas connu jusqu'à présent de canal privé intervenant dans les interactions figuier / pollinisateur. L'existence et le rôle des canaux privés comme mécanisme structurant des interactions plantes / pollinisateurs sont très débattus dans la littérature (Raguso, 2008b; Svensson *et al.*, 2010). L'existence d'un canal privé requiert : i) l'identification d'un récepteur spécifique (le pollinisateur), ii) la détection fine du signal par le receveur et iii) la faible ou non perception du signal par d'autres récepteurs (Schaefer *et al.*, 2004). Le principal problème pour démontrer l'existence d'un cas de canal privé repose sur la difficulté, notamment dans de nombreux systèmes de pollinisation généraliste, de déterminer l'identité du récepteur spécifique (c'est-à-dire le pollinisateur le plus efficace) et l'absence de détection par d'autres. De surcroît, le canal privé est souvent attribué à un seul composé spécifique, et son oxydation, sa saturation ou sa chiralité sont donc des éléments importants, et pas forcément détectés selon la méthodologie employée pour l'analyse des odeurs (par exemple, la GC-MS rend difficile la distinction entre deux énantiomères). En fait, l'hypothèse la plus répandue est que l'odeur serait un élément de « filtre floral », à même d'assurer la spécificité d'une interaction, mais en compléments d'autres éléments, comme par exemple des barrières morphologiques (Raguso, 2008b). Ainsi, chez les *Ficus*, l'odeur serait spécifique de l'espèce, mais si un autre pollinisateur la choisissait, il pourrait tout de même être bloqué physiquement, par la forme de l'ostiole, ou chimiquement, par la présence d'autres composés chimiques (non volatils, mais de surface) présents sur la paroi externe de la figue (Gibernau *et al.*, 1998). Dans le cas d'un mutualisme, comme l'interaction figuier/pollinisateur, il y aurait alors sélection d'un

message simple et abondant, ce qui le rendrait aisément détectable dans un environnement complexe, une « mosaïque d'odeurs » constituée par les bouquets volatils émis par l'ensemble des autres espèces syntopiques.

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CHEMOECOLOGY

REVIEW PAPER

# Floral scents: their roles in nursery pollination mutualisms

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**Abstract** Mutualisms are interspecies interactions in which each participant gains net benefits from interacting with its partner. In nursery pollination mutualisms, pollinators reproduce within the inflorescence they pollinate. In these systems, each partner depends directly on the other for its reproduction. Therefore, the signal responsible for partner encounter is crucial in these horizontally transmitted mutualisms, in which the association between specific partners must be renewed at each generation. As in many other interspecies interactions, chemical signals are suspected to be important in the functioning of these mutualisms. We synthesized and compared the published data available on the role of floral scents in the functioning of the 16 known independently evolved nursery pollination mutualisms. So far, attraction of pollinators to their specific hosts has been investigated in only seven of these systems, and the majority of the studies have been conducted on one of them, fig/fig wasp interactions. While such unevenness of the information limits the potential for meta-analysis, some patterns emerge from this review concerning the role of flower volatiles in maintaining the specificity of pollinator attraction, in signaling the appropriate phenological stage for pollinator visit, in attracting the pollinator toward the rewardless sex in dioecious plant species and in aiding the location and exploitation of resources by parasites and predators associated with these mutualisms. Finally, we highlight new perspectives on the evolution of signals in these diversified systems depending on the age and the degree of specificity of the interaction, and on the effect of phylogenetic inertia on the evolutionary dynamics of plant signals.

**Keywords** Chemical mediation · Specific pollination · Pollination by deceit · Community ecology · Pollinator behavior · Evolutionary ecology

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## Introduction

Mutualisms are cooperative interactions between species, in which each partner benefits from the association (Bronstein et al. 2006). In nursery pollination mutualisms, pollinators lay eggs within plant reproductive structures when pollinating flowers, using these structures of the host plant as their only breeding sites (Dufaÿ and Anstett 2003). Among these mutualisms, the most diverse and well-known examples are the interactions between figs and fig wasps (Anstett et al. 1997; Cook and Rasplus 2003; Herre et al. 2008) and those between yuccas and yucca moths (Pellmyr 2003). However, in addition to these textbook examples, a handful of other nursery pollination mutualisms have been described in greater or lesser detail (Dufaÿ and Anstett 2003; Kawakita and Kato 2009). Nursery pollination mutualisms represent high taxonomic diversity of both plants and insects, and show independent and convergent evolution (Dufaÿ and Anstett 2003). They share many properties in their ecological functioning. Moreover, they may use similar mechanisms to facilitate partner encounter. This is a crucial step in these horizontally transmitted mutualisms in which each partner depends directly on the other for its reproduction. Indeed, as in many other plant/pollinator interactions, flower scents—

often complex blends of volatile organic compounds—play a major role in mediating interactions between the two partners and offer a virtually infinite number of combinations, thanks to quantitative and qualitative variations in volatile organic compounds present in emitted scents (Raguso 2008a, b). In fact, the current revolution in techniques for odor extraction and identification has led to isolation of more than 1720 volatile organic compounds (VOCs) as flower scents in about 1000 species (Knudsen et al. 2006). Most of these compounds belong to three large chemical groups: (1) the terpenoids, synthesized by the mevalonate pathway, (2) aliphatic compounds, synthesized from fatty acids, and (3) benzenoids, synthesized by the shikimate pathway. Such "endless" variation in the composition of floral scents (Dudareva and Pichersky 2006) can be used by plants to elaborate quite complex and specific blends in order to attract pollinators (Raguso 2008a, b). The message complexity of odor blends, based on the combination of major and minor compounds (Firn and Jones 2003), makes floral scent one of the most variable aspects of plant phenotype (Knudsen and Gershenzon 2005) and a better candidate than visual signals as the basis for specific attraction (Raguso 2008a). However, synergistic effects on pollinator attraction can be expected between chemical and visual signals.

As in pollination mutualisms in general, floral scents have recently attracted some attention in studies of nursery pollination mutualisms. Diversity of floral scents has been explored in nine of the 16 nursery pollination systems (see Table 1; Fig. 1). The results of these studies indicate that chemical mediation plays a key role in partner encounter, allowing pollinators to locate host plants and find floral rewards, i.e. breeding sites (Patt et al. 1992; Gibernau et al. 1998; Grison-Pigé et al. 2002a; Dufaÿ et al. 2003; Jürgens et al. 2003; Dötterl and Jürgens 2005; Svensson et al. 2005; Okamoto et al. 2007; S. Ibanez, pers. comm.). However, fewer studies have examined in any detail how pollinators are attracted to these complex blends—in other words, which compounds in the message emitted by the host plants are used by the associated insects as a signal for their own behavioral decisions (Table 2).

Biological traits inherent to nursery pollination make these mutualisms quite specific: each partner depends exclusively, or at least partially, on the other for its reproduction, enforcing tight physiological co-adaptation. However, some variation does exist among these systems in the degree of reciprocal dependence of plant and pollinator. In some interactions, plant and pollinator have evolved extraordinary reciprocal specificity, often approaching one-to-one obligate specificity, as in most fig/fig wasp, yucca/yucca moth, or *Glochidion/Epicephala* moth mutualisms (Jousselin et al. 2008; Pellmyr 2003; Kawakita and Kato 2009). In other systems, specificity is less strict. Indeed, in some species of figs (Cook and Rasplus 2003) and cycads (Terry et al. 2004, 2008), the plant can be pollinated by two or more closely related species of nursery pollinators. Likewise, a mutualistic pollinator can in some cases reproduce in several closely related plant species. Finally, as in the case of *Lithophragma parviflorum* (Cuautle and Thompson 2010) or of some *Dianthus* and *Silene* species (Kephart et al. 2006), in addition to the main nursery pollinator, non-nursery co-pollinators belonging to different groups of insects also visit and pollinate the flowers. Nevertheless, whatever the degree of reciprocal dependence, for all these mutualistic interactions the transmission of the association is solely horizontal, i.e. the plant's specific pollinators and its seeds disperse independently. In addition, upon completing their development, pollinators find no structures at the appropriate developmental stage for pollination on their natal plant. Pollinators must thus leave their natal plant to reproduce, so that the association between specific partners must be renewed at each generation (Anstett et al. 1997). This requires tight coordination and synchronization of phenology, particularly as concerns the developmental cycles of plant reproductive structures and of pollinating insects. The scent signal emitted by the host plant must be specific, to attract its specific and obligate partner. The successful encounter of the host plant and its mutualist insect also requires a very strong signal. Selection acting on both partners should thus reinforce both the strength of the signal emitted by the plant and the capacity of the insect to detect the message (Grison-Pigé et al. 2002a; Svensson et al. 2005; Chen et al. 2009; S. Ibanez, pers. comm.) and to extract ''signal'' from ''noise'' (Raguso 2003). For these reasons, the scent emitted by the host plant and perceived by the insect should contain not only information about the specific identity of the plant, but also on its developmental stage, particularly information about whether or not the plant is receptive, i.e. ready to be pollinated (and thus has resources to offer). Transfer of information about resources opens up a large number of questions. How is specificity of the signal achieved? Once specificity is achieved, how do plant–pollinator relationships change, how do they diversify? Is the signal always "honest" [in the behavioral-ecology sense of this word, meaning reliable for mutualistic partners (Krebs and Davies 1993)] or can "cheating" occur? What are the differences between the scents emitted during receptivity and those emitted during other phenological phases? Do species other than the two mutualists use this information exchange to exploit resources?

Comparative studies remain crucial to go beyond description of proximate mechanisms of scent production to obtain a complete understanding of the evolutionary dynamics of floral scent emission in nursery pollination

**Table 1** Summary of data on floral scent emission in nursery pollination mutualism with scent extraction performed by head space techniques

| Plant/nursery pollinator interaction | | Chemical message emitted by plants (headspace extraction) | | References |
|---|---|---|---|---|
| Plant (Genus, species) | Nursery pollinator (Genus, species) | Absorbant | Classes of main compounds | |
| **Agavaceae** | **Lepidoptera: Prodoxidae** | | | |
| *Yucca filamentosa* | *Tegeticula cassandra* and *T. yuccasella* | Porapak | Homoterpenes | Svensson et al. (2005) |
| *Y. elata* | *T. elatella* | Porapak | Homoterpenes | Svensson et al. (2006) |
| **Araceae** | **Diptera: Chloropidae** | | | |
| *Peltandra virginica* | *Elachipetra formosa* | Tenax<br>TA-carbotrap | Fatty acid derivatives | Patt et al. (1992) |
| **Arecaceae** | **Coleoptera: Curculionidae** | | | |
| *Chamaerops humilis* | *Derelomus chamaeropsis* | Porapak | Monoterpenes and sequiterpenes | Dufaÿ et al. (2003, 2004) |
| **Caryophyllaceae** | **Lepidoptera: Noctuidae** | | | |
| *Dianthus barbatus* | *Hadena bicruris* and *H. compta* | Tenax<br>TA-carbotrap | Fatty acid derivatives and sesquiterpenes | Jürgens et al. (2003) |
| *D. superbus* | *H. rivularis* | Tenax<br>TA-carbotrap | Monoterpenes and sequiterpenes | Jürgens et al. (2003) |
| 3 *Silene* spp. and *Dianthus deltoïdes* | *Hadena* spp.[a] | Tenax<br>TA-carbotrap | Fatty acid derivative and benzenoids | Jürgens et al. (2002, 2003) |
| *Dianthus sylvestris*, *Saponaria officinalis* and 2 *Silene* spp. | *Hadena* spp.[a] | Tenax<br>TA-carbotrap | Benzenoids | Jürgens et al. (2002, 2003) |
| *Silena otites* and *S. vulgaris* | *Hadena* spp.[a] | Tenax<br>TA-carbotrap | Monoterpenes | Jürgens et al. (2002) |
| *S. nutans* | *Hadena* spp.[a] | Tenax<br>TA-carbotrap | Monoterpenes and benzenoids | Jürgens et al. (2002) |
| *S. latifolia* | *H. bicruris* | Tenax<br>TA-carbotrap | Monoterpenes | Dötterl et al. (2006, 2007) |
| *S. ciliata* | *H. consparcatoides* | Tenax<br>TA-carbotrap | Benzenoids | Gimenez-Benavides et al. (2007) |
| **Eupomatiaceae** | **Coleoptera: Curculionidae** | | | |
| *Eupomatia laurina*<br>*E. ennettii* | *Elleschodas sp.* | Porapak | Fatty acid derivatives | Bergström et al. (1991) |
| **Moraceae** | **Hymenoptera: Agaonidae** | | | |
| 11 *Ficus* spp. | Agaonidae[a] | Porapak | Sesquiterpenes | Grison-Pigé et al. (2002a) |
| 7 *Ficus* spp. | Agaonidae[a] | Porapak and Tenax<br>TA-carbotrap | Monoterpenes and sequiterpenes | Grison-Pigé et al. (2002b), Proffit et al. (2009) |

**Table 1** continued

| Plant/nursery pollinator interaction | | Chemical message emitted by plants (headspace extraction) | | References |
|---|---|---|---|---|
| Plant (Genus, species) | Nursery pollinator (Genus, species) | Absorbant | Classes of main compounds | |
| *F. carica and F. racemosa* | *Blastophaga psenes* and *Ceratosolen fusciceps*, respectively | Porapak | Monoterpenes | Grison-Pigé et al. (2001, 2002a), Proffit et al. (2009), Proffit, unpublished data |
| *F. burkei* and *F. natalensis natalensis* | *Elisabethiella stucken-bergii* and *E. socotrensis*, together with *E. stucken-bergii* | Tenax TA-carbotrap | Fatty acid derivatives and monoterpenes | A. Cornille, unpublished data |
| *F. hispida* | *C. solmsi marchali* | Porapak | Monoterpenes and sequiterpenes | Proffit et al. (2008, 2009), Chen and Song (2008) |
| *F. semicordata* | *C. gravelyi* | Porapak | Benzenoids | Chen et al. (2009) |
| *F. sycomorus* | *C. arabicus* | Tenax TA-carbotrap | Fatty acid derivatives and sesquiterpenes | Proffit and Johnson (2009) |
| **Phyllanthaceae** | **Lepidoptera: Gracillariidae** | | | |
| 4 *Glochidion* spp. | *Epicephala* spp.[a] | Tenax TA | Monoterpenes | Okamoto et al. (2007) |
| **Ranunculaceae** | **Diptera: Anthomyiidae** | | | |
| *Trollius europeus* | *Chiastochaeta* spp.[a] | Porapak | Monoterpenes and sequiterpenes | S. Ibanez, pers. comm. |
| **Zamiaceae** | **Coleoptera: Curculionidae** | | | |
| *Macrozamia machinii* | *Tranes* spp.[a] | Porapak | Monoterpenes | Terry et al. (2004) |
| | **Thysanoptera: Aeolothripidae** | | | |
| *M. macleayi* | *Cycadothrips* spp.[a] | Porapak | Monoterpenes | Terry et al. (2004) |
| *M. lucida* | *Cycadothrips* spp.[a] | Porapak | Monoterpenes | Terry et al. (2004, 2007) |
| *M. platyrhachis* | *Cycadothrips* spp.[a] | Porapak | Fatty acid derivative alcohol | Terry et al. (2008) |

No behavioral tests of the role of these compounds in attraction has been done so far

[a] More than two species or genera

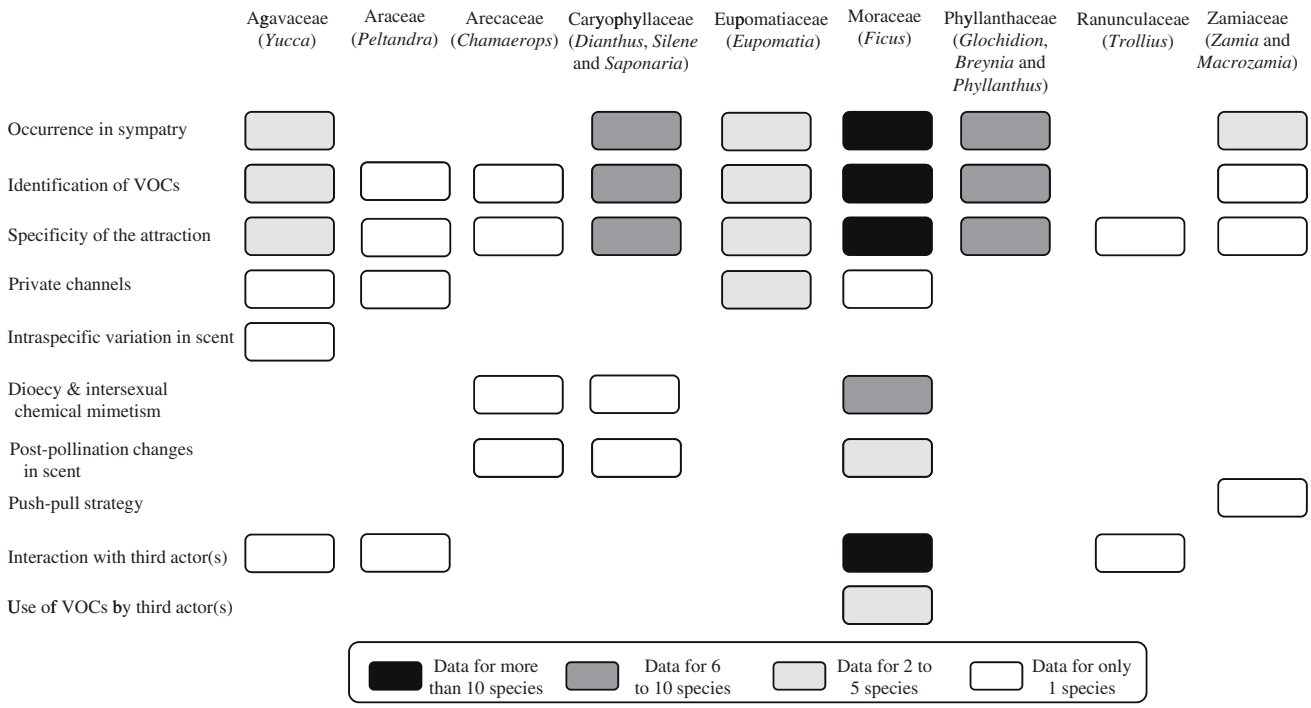


**Fig. 1** Diagram summarizing the state of our knowledge of nursery pollination interactions in which chemical mediation has been described

**Table 2** Summary table of the biological tests commonly used to investigate effects of VOCs in nursery pollination systems

| | | |
|---|---|---|
| **Physiological tests** | | |
| Electro-antennography | Insect antenna makes contact between two electrodes. If the antenna reacts when confronted with a scent an electrical signal is detected | Dötterl et al. (2006) |
| **Behavioral tests** | | |
| (Observation or measurement of behavior of the insect when confronted with a scent [synthetic or extracted in solvent]) | | |
| *Tests with free-moving insects* | | |
| Presence test | Count the number of individuals present on a test filter paper, agar–agar solution or sticky trap with a deposited scent (compared to a control) | Gibernau (1997) |
| 'Marble' test | Drop of a controlled solution (VOC+ solvent for test or solvent for control) deposited on op of a glass marble. The tested insect is deposited on the marble and the duration of its stay on the marble recorded. Interpretation is based on comparative situations with different VOCs | Grison-Pigé et al. (2002a) |
| *Tests with insects moving in an apparatus* | | |
| Wind-tunnel | Insect movements are followed in a wind-tunnel to study the attractivity of a scent (which is situated at the opposite extremity from the starting point of the tested insect) | Dötterl et al. (2006) |
| Olfactometer | Insect movement in maze displaying two branches (Y-maze), four or six branches, in which one or more branches are associated with a tested scent. Insect behavior is measured by recording the branch first entered, or the time spent by the insect in each branch | Okamoto et al. (2007), Proffit et al. (2007), Chen and Song (2008), Chen et al. (2009) |

mutualisms. As most of these interactions are specific and obligatory for both partners, there should be strong stabilizing selection to maintain the encounter of the two mutualists and the specificity of the interaction. Such selection could favor the emission, by the plant partner in each interaction, of a few major compounds, resulting in

simplification of the chemical message and thereby facilitating partner encounter. But how do these systems change? How do the constraints associated with specificity affect the partners' capacities to adapt to other associated species that exploit the resources they exchange?

Hypotheses can be generated and tested using various approaches, such as experiments, modeling, and the comparative method. However, nursery pollination mutualisms offer only restricted scope for comparative studies: the number of independently evolved systems is too small to permit a useful meta-analysis (Table 1). The purpose of this review is to synthesize published data on chemical mediation in nursery pollination mutualisms for which sufficient information is available and to try to define emerging patterns. We investigate general patterns concerning the ecological traits linked to the specificity of attraction, the evolution of the signal, the influence of dioecy on the evolution of chemical mediation, and the perception of scent signals by insect species other than the mutualistic pollinators. However, as most of the studies (about 70%) have been conducted on one group of mutualisms, those between figs and fig wasps, our review will be biased toward this well-known set of interactions.

## Specificity of the attraction

In several of these specialized nursery pollination systems, closely related species of plants and or/pollinators sometimes have overlapping distributions. Numerous cases in which closely related species co-occur are known in figs, palms, cycads and more recently *Glochidion* trees (Table 1). In most of these cases, the species-specificity of the interaction is maintained, suggesting the presence of pre-zygotic barriers limiting hybridization between co-occurring species (of plants and of pollinators). In nursery pollination mutualisms, VOCs emitted by the host plant appear to play a determining role in maintaining species-specificity. In several of these systems, the chemical message emitted by the plant to attract its pollinator has been characterized (Table 1). In fact, sympatric and co-flowering plant species involved in nursery pollination systems, and visited by different pollinators, produce qualitatively different chemical messages (Grison-Pigé et al. 2002b; Okamoto et al. 2007; Chen et al. 2009; Proffit et al. 2009; Proffit and Johnson 2009). Moreover, bioassays conducted on fig wasps and on *Epicephala* moths, pollinators of *Glochidion* trees, corroborate the role of scent as a pre-zygotic barrier between closely related plant species by showing that pollinators are able to discriminate their own host within the olfactory landscape (Raguso 2003) of compounds emitted by co-flowering plants (Grison-Pigé et al. 2002a; Okamoto et al. 2007; Chen et al. 2009; Proffit et al. 2009).

By contrast, for related plants visited by different insect species but with non-overlapping distributions, two different patterns emerge concerning inter-specific variation in VOCs emitted by the host plant. In some cases, such as fig/fig wasp interactions, the message produced by the plant seems still to be species-specific, showing significant variation among different species (Grison-Pigé et al. 2002a; Proffit et al. 2007, 2009; Proffit and Johnson 2009). In other cases, there is no apparent variation of floral scent between species pollinated by different insects in allopatry, as in the case of *Yucca elata* and *Y. filamentosa* (Svensson et al. 2006). Interestingly, in the latter yucca species floral scent is very similar among populations pollinated by different yucca moths (Svensson et al. 2005). The low intra- and inter-population variation in the fragrance blend of *Yucca* is so far quite unique among nursery pollination mutualisms (Waelti et al. 2009; S. Ibanez, pers. comm.; Soler et al. submitted). Svensson et al. (2006) hypothesized that the lack of inter-specific variation in yucca floral scent may indicate that strong selection by obligate pollinators counteracts drift or divergence in this trait. However, it is so far unknown which compounds in yucca floral scents are actually important in eliciting behavioral responses by pollinators. Direct comparison of variation in such active floral compounds and variation in non-active compounds is necessary to reach any conclusion about specificity, or lack of it, in the signal used by different species of yucca moth to find their hosts.

Specificity is often ensured by quantitative and qualitative differences in relative proportions of major and minor VOCs emitted by host plants (Raguso 2008a). However, if scent is a determinant component of host-specificity in highly specialized nursery pollination systems, one could expect that specific attraction of pollinators may be achieved through the existence of a private sensory channel: the potential for exclusive communication via unique signals and receptors (Bergström et al. 1991; Schaefer et al. 2004; Raguso 2008a). The existence or not of private channels in nursery pollination mutualisms, or indeed in any pollination system, is still debated (Raguso 2008c). In particular, rigorous experiments are needed to demonstrate one necessary condition qualifying a signal as a 'private channel', i.e. poor reception of the signal by 'non-expected' visitors (Schaefer et al. 2004) such as co-pollinators or parasites. Most of the compounds constituting floral scent in nursery pollination systems are quite common constituents of odors produced by flowers or by green parts of plants (Knudsen et al. 2006). However, in some cases, unusual compounds have been reported. In early work on the arrow arum, *Peltandra virginica,* an aquatic Araceae involved in a mutualism with its pollinator

fly (*Elachiptera formosa*), Patt et al. (1992, 1995) identified novel compounds, 4,5,7-trimethyl-6,8-dioxabicyclo[3.2.1] octane and a molecular weight 142 analog, emitted by flowers in high concentrations. Although they did not test directly for effect of these two compounds on attraction of the mutualistic fly or other co-pollinators, these novel compounds could be good candidates for 'private channels'. In the floral scent of *Y. filamentosa*, Svensson et al. (2005) detected the presence of two di-oxygenated compounds not previously reported as floral compounds. In preliminary electrophysiological recordings, one of these compounds has been shown to elicit a strong response from antennae of *Tegeticula cassandra* (G. Svensson and R. Raguso, pers. comm.). This result provides some support for the "private channel" hypothesis in yucca/yucca-moth interactions. However, structural elucidation and synthesis of this compound, as well as behavioral assays to test its function as a pollinator attractant, are still needed.

In the system for which the greatest amount of data is available on floral scent, the fig/fig wasp system, the message responsible for pollinator attraction is usually constituted by a species-specific blend of compounds, generally dominated by one or a few common terpenoid compounds, the identity of which differs among sympatric species (Grison-Pigé et al. 2002a; Chen et al. 2009; Proffit et al. 2009). Gibernau (1997) showed that a mixture of three monoterpene compounds (linalool and two linalool oxides (furanoids)) and one benzenoid compound (benzyl alcohol) in certain proportions was necessary and sufficient to attract *Blastophaga psenes*, the specific pollinator of *F. carica.* Synergistic effects between two and three compounds emitted by receptive figs have also been documented in the attraction by *F. hispida* of its specific pollinator (*Ceratosolen solmsi marchali*) (Chen and Song 2008). An exception is the case of *F. semicordata*, where the main compound emitted by receptive figs, 4-methyl-anisole, represented alone more than 90% of floral scent (Chen et al. 2009). This compound has never been reported in the scent of receptive figs of any of the other 40 fig species studied so far (Ware et al. 1993; Gibernau et al. 1998; Grison et al. 1999, Grison-Pigé et al. 2002b; Song et al. 2001; Proffit et al. 2007, 2009; Borges et al. 2008; Proffit and Johnson 2009; C. Soler, unpublished data) and has been reported very rarely, always as a minor compound, in the floral scent of other plants (Knudsen et al. 2006). This compound by itself is sufficient to attract the pollinator wasp of *F. semicordata* (Chen et al. 2009) and thus could play the role of a private channel, as no other insect, even non-pollinating fig wasps, have been reported to visit its figs at this stage. These first results on figs thus indicate that the specific attraction of fig wasps to their host is usually achieved through the emission and recognition of a specific combination of fairly common compounds, although at least one exception occurs. For the other specific nursery pollination systems, we lack information on the exact nature of the signal to which pollinators respond. Knowing the identity of the compounds that play a determining role in attraction is a pre-requisite to going any further.

In less specialized nursery pollination systems, more information is available on the identity of the compounds perceived as signals by the pollinators. For instance, in Australian *Macrozamia* cycads, in which one species is pollinated by several related nursery pollinator species, Terry et al. (2004) found differences in the volatile profiles among species pollinated by different insect groups (*Cycadothrips* vs. *Tranes* weevils). Similarly, in *Silene* species or related Caryophyllaceae, which, in addition to being pollinated by a nursery pollinator (*Hadena* or *Perizoma* moths), can sometimes also be pollinated by co-pollinators that belong to various other families and do not reproduce in the plant, volatile compounds emitted by the host plant vary between diurnally and nocturnally pollinated species (Kephart et al. 2006, Jhumur et al. 2008). In one species of each of these two last examples (*Silene latifolia* [Dötterl et al. 2006], *Macrozamia lucida* [Terry et al. 2004]), specific attraction of the pollinating insects is known to be mainly due to a blend of different isomers. What is the key to the specific recognition of the plant by its pollinators, the identity of the different isomers or their ratios? No tests have been conducted yet to answer this question. However, for the four different lilac aldehyde isomers present in *S. latifolia*, the ratios that have been shown to be most attractive to the plant's pollinating moth *Hadena bicruris* are a more conservative trait compared to the total scent, making the proportions of these isomers the more reliable cue for host-plant detection (Dötterl et al. 2007).

## Information beyond "species identification"

In most pollination mutualisms, rewards are principally food sources (Fenster et al. 2004), and their exploitation by pollinators does not involve destruction of any plant organs. In contrast, in nursery pollination mutualisms, the rewards offered to pollinators are oviposition sites in the plant's reproductive structures. Such rewards are likely to impose higher costs to the plant (on male function, female function, or both, depending on the system) than in food-reward pollination mutualisms (Dufaÿ and Anstett 2003). In systems where pollinator larvae develop in or on seeds or ovaries, for example, in monoecious figs, yucca, and some *Silene* spp., the benefits of pollination may not always be commensurate with the cost of seed predation, so that the effect on the plant could sometimes shift toward parasitism (Thompson and Pellmyr 1992; Anstett 2001;

Bronstein 2001; Gimenez-Benavides et al. 2007). The greater the number of insects developing within reproductive structures, the more seeds or ovaries will be destroyed (Anstett et al. 1996; Jaeger et al. 2001). Overexploitation by nursery pollination mutualists can also negatively affect the plant's male fitness. For instance, in figs, when a female pollinator wasp enters a fig and encounters no other female inside it, sex ratio of the offspring she produces is highly female-biased, in response to local mate competition (Herre 1985; Kjellberg et al. 2005). The greater the number of foundresses entering a single fig, the more male offspring they produce. Because only the winged female pollinator offspring disperse the fig's pollen, overexploitation results not only in higher seed predation but also in lowered male fitness.

Thus, following pollination, it may be beneficial to the plant to reduce attractiveness of the flower to pollinators (or to other potential visitors), thereby simultaneously reducing the risk of attracting enemies, avoiding the metabolic cost of continued VOC production (Grison et al. 2001) and emission, and directing pollinators to its still-receptive flowers (Arditti et al. 1973; Dudareva and Pichersky 2000; Schiestl and Ayasse 2001; Theis and Raguso 2005). In several cases of nursery pollination systems in which scent is determinant for pollinator attraction, a post-pollination decline in the total quantity of VOCs emitted by the flower has indeed been shown (Dufaÿ et al. 2004; Muhlemann et al. 2006; Proffit et al. 2008; Chen et al. 2009). Qualitative changes in the scent composition have also been demonstrated, with reduction of the proportion of the key compound(s) responsible for pollinator attraction. For example, for two fig species, *F. hispida* and *F. semicordata*, the relative proportions as well as the total amounts of the main compound emitted by receptive figs (respectively, a monoterpene and a benzenoid compound), attractive to the pollinator, decreases following pollination in both male and female figs of these dioecious species (Proffit et al. 2008; Chen et al. 2009). Similarly, in *S. latifolia*, the quantities emitted of the compounds responsible for the attraction of *H. bicruris* to the flowers, lilac aldehydes A and B as well as veratrole, decrease after pollination (Muhlemann et al. 2006).

A more unusual strategy, characterized as "push–pull" pollination strategy, has been described in the Australian cycad *Macrozamia lucida* pollinated by *Cycadothrips* (Terry et al. 2007). Like all other living cycads, *M. lucida* is dioecious. Male cones provide a nursery for pollen-feeding *Cycadothrips*, which are attracted by low levels of *β*-myrcene and a blend of three ocimene isomers dominated by (E)-*β*-ocimene. During pollination, cones are thermogenic, and they synchronize mid-day heat production with a million-fold increase in scent emissions in male cones (females reach approximately one-fifth that amount). Beta-myrcene, repellent for the thrips at high concentration, represents more than 90% of total emissions during thermogenesis, forcing the pollinating thrips to depart male cones, whence they are attracted to female megasporophylls by the same odors at lower concentrations (Terry et al. 2007).

## When selection favors dishonesty of signals about resources: the case of dioecy

Vamosi and Otto (2002) suggested that the higher rate of extinction, as well as the lower rate of speciation, in clades with a large number of dioecious species may be explained by a strong sexual dimorphism, spawning the avoidance of the non-rewarding female plants by insects. However, in nursery pollination mutualisms, where reproduction of each partner is totally dependent on that of the other, selection may favor dishonest signals, i.e. "cheating", in the female plants to optimize female fitness (Patel et al. 1995). As in most other cases of pollination by deceit (Ashman 2009), females are always the deceptive sex in *Ficus*.

In this ancient and diverse genus, which had already arisen by the late Cretaceous period (Herre et al. 2008), two different breeding systems co-occur, each characterizing about half of the 750 species: monoecy, the ancestral condition, and (gyno)dioecy (Kjellberg et al. 1987, 2005). In the latter group of species, there are functionally male trees, producing wasps and pollen but no seeds (all of which are destroyed during development of the pollinators), and female trees, in which pollinating wasps cannot oviposit in the ovule (the flowers of female fig trees, all long-styled, are inaccessible to wasp ovipositors), and which consequently produce only seeds and no rewards for the pollinator (Kjellberg et al. 1987; Cook and Rasplus 2003). While evolutionary conflicts occur in all fig/fig wasp systems, and indeed in most other mutualisms, dioecy leads to rather extreme conflicts between the host plant and its pollinator, associated with "pollination by deceit" (Thompson 1988; Grafen and Godfray 1991; Patel et al. 1995). Female trees offer no rewards to pollinators, because the latter are unable to oviposit in them. This situation raises the following question: why do pollinators not avoid female plants? Again, scents emitted by *Ficus* play a major role in answering this question. The "intersexual chemical mimicry" hypothesis proposes that vicarious selection on floral scents between sexes makes it impossible for the pollinating wasp to distinguish between male and female figs (Grafen and Godfray 1991; Patel et al. 1995; Anstett et al. 1998; Moore et al. 2003). So far, the few studies on dioecious fig species with synchronous flowering have found no significant difference in floral

scent composition between male and female figs (Borges et al. 2008; Proffit et al. 2008; Chen et al. 2009; Hossaert-McKey and Borges unpublished data), supporting the intersexual chemical mimicry hypothesis (Ashman 2009). Given the importance of scents as cues for host location, inability to discriminate between scents of the two sexes could explain why wasps continue to visit female figs, and thus how the system remains evolutionarily stable. Interestingly, there are a few exceptions, in the case of strongly seasonal species, where males and females flower largely asynchronously. In such cases, pollinators (emerging from male figs) are usually not offered the choice between male and female figs but find available only the latter. In these cases, intersexual mimicry can be relaxed (Kjellberg et al. 1987; Grison-Pigé et al. 2001).

The intersexual mimicry hypothesis has also been explored in other dioecious species involved in nursery pollination mutualisms. For example, in the dwarf palm, *Chamaerops humilis,* at the stage when plants attract pollinators, male plants (at anthesis) and female plants (at receptivity) produce similar blends of VOCs with high concentrations of E-$\beta$-ocimene and of two sesquiterpenes [E-$\beta$-farnesene and an unidentified sesquiterpene (Dufaÿ et al. 2004)] and the pollinating *Derelomus* weevil is unable to discriminate between the rewarding and the deceptive sex. Interestingly, in this system scents attracting pollinators are produced by leaves of both sexes and not by inflorescences (Dufaÿ et al. 2003). In this case, leaf volatiles act as flower scents, and they are produced only during the flowering period. Likewise, Dötterl et al. (2006) concluded that there were no overall differences between male and female flower scents of *Silene latifolia*. However, in a more recent study of the same species, Waelti et al. (2009) found some significant intersexual differences in the total amount of compounds produced, but not in the qualitative composition of the compounds emitted by the two sexes. Nevertheless, in this last study, the pollinating female moths were equally attracted by scents of both sexes, but male moths seemed to prefer male plants. However, as mentioned by the authors, these behavioral tests were performed only with virgin moths. Similar experiments using mated moths are necessary before drawing any conclusion on the attraction of pollinators to flowers of the two sexes.

All these examples provide evidence that olfactory cues play a central role in intersexual mimicry in these cases of pollination by deceit in nursery pollination mutualisms. They also show how an honest signal can be shifted to a dishonest signal by the cheating sex (females). Specialization has locked the pollinator into a specific and obligate interaction; its "options" in responding to the evolution of a deceptive sex are thus limited. Together, the constraint imposed on the pollinator by specificity and the strong selection acting on the plant to enforce intersexual mimicry, combine to allow not only the evolutionary persistence of nursery pollination mutualism in dioecious figs but also a remarkable diversification of dioecious fig species and their pollinators (Anstett et al. 1997).

## Nursery pollination mutualisms as networks of biodiversity

Pairs of species engaged in nursery pollination mutualisms do not interact in isolation, but are integrated into ecosystems. Indeed, the scent emitted by host plants is perceived not only by the pollinator but also by other insects that use this signal to locate and exploit resources exchanged by mutualists. In nursery pollination mutualisms, both parasites (Jaeger et al. 2000; Cook and Rasplus 2003), and predators (Bronstein 1988; Schatz and Hossaert-McKey 2003) have been reported to exploit plant seeds or ovaries, pollinators, associated insects, or both these kinds of insects. Exploiting the resources offered by specific mutualistic interactions requires timing of arrival with the precise developmental phase when a particular resource is present (Jaeger et al. 2000; Kerdelhué et al. 2000; Marr et al. 2001; Schatz and Hossaert-McKey 2010). This suggests that insects exploiting these mutualisms should be able to recognize not only their own host (or prey) species but also the exact time window when it is likely to be present.

For instance, several non-pollinating fig wasps (NPFWs) are known to exploit the specific and obligatory interactions between figs and their pollinators (Compton and van Noort 1992; Kerdelhué et al. 2000). Most NPFWs are assumed (as available information suggests is the case) to be strictly associated with a single *Ficus* species, and one fig species can host up to 30 NPFW species (Cook and Rasplus 2003). However, such diversity in insect communities associated with a single plant host raises the question of niche partitioning among NPFWs. Three functional groups of NPFWs have been distinguished (Cook and Rasplus 2003): gall-makers, which transform ovaries of female flower into galls (their larvae feeding on these galled ovaries); inquilines, which feed on the gall tissue induced by pollinator larvae or by non-pollinating gall-makers; and parasitoids, whose larvae feed on the larvae of other wasps (pollinators or non-pollinators). Within the fig, niche space could be partitioned via the existence of different subsets of flowers, by differences in timing of oviposition, or both (Kerdelhué et al. 2000; Proffit et al. 2008). How NPFWs detect the specific *Ficus* with which they are associated, as well as the precise time window of visit in relation to the developmental cycle of their host fig, have only recently been explored (Proffit et al. 2007, 2009).

These studies have clearly shown that chemical mediation plays the crucial role. Extraction of volatile compounds during fig development and olfactometer experiments have been combined to demonstrate (1) that both pollinators and associated NPFWs are attracted only by the specific odor of their own fig host [and not by the different odors emitted by other sympatric fig species (Proffit et al. 2009)] and (2) that each NPFW species is only able to detect the scent composition [in the case of *F. hispida*: mainly sesquiterpenes such as (E)-$\beta$-farnesene, $\beta$-caryophyllene, or $\beta$-sesquiphellandrene (Proffit et al. 2009)] reflecting the appropriate phase of fig development they use for oviposition (Proffit et al. 2007).

The diversity of parasites is poorly documented in other nursery pollination systems, with limited information on only two other cases, *Yucca filamentosa* and *Trollius europaeus*. In the latter case, several species of *Chiastochaeta* flies (Diptera: Anthomyiidae) visit the flowers successively. The earliest-arriving fly species acts as the pollinator, while later-arriving species are seed parasites only (Jaeger et al. 2000). However, the proximate mechanism explaining the succession of species is still to be discovered in this system. For *Yucca filamentosa*, two species of non-pollinating yucca moths co-occur. These appear to have evolved from a mutualist species, representing a reversion from mutualism to parasitism (Marr et al. 2001). However, for this example as well, nothing is known about the signals used by these parasites to find their host.

Nursery pollination systems are generally associated with the presence near flowers of both pollinators and other insect visitors, which constitute a potential source of prey for predators. For instance, the presence during the emergence phase of numerous fig wasps on fig surfaces often attracts various arthropod predators such as mantids, spiders, and predatory beetles (Bronstein 1988; Zachariades 1994; Schatz and Hossaert-McKey 2003; Zachariades et al. 2009). In fig/wasp systems, ants are the dominant predators of fig wasps (both pollinators and NPFWs) (Bronstein 1988; Compton and Robertson 1988; Schatz et al. 2008). Ants are particularly efficient in the capture of fig wasps, detecting the odor they emit, and exhibiting olfactory learning (Schatz et al. 2003, Ranganathan and Borges 2009; Schatz and Hossaert-McKey 2010). Moreover, as fig odors change with time (Proffit et al. 2008), some ants are also able to learn the specific odor associated with developmental phases when the probability of the presence of fig wasps is higher, and to modulate their presence and their aggressiveness using these olfactory cues (Schatz and Hossaert-McKey 2010). Armed with such abilities, ants can exert strong predation pressure. Experimental exclusion of ants leads to higher abundance of NPFWs, showing that ant presence can confer substantial protection upon pollinator larvae (Schatz et al. 2006).

Ants may also play a role as indirect mutualistic partners in yucca/yucca moth mutualisms. Indeed, Perry et al. (2004) showed that *Formica* species on *Yucca glauca* deter oviposition of the non-pollinating moth *Tegeticula corruptrix*, but not that of the pollinating moth *Tegeticula yuccasella*. Unfortunately, no studies have investigated the possible role of chemical mediation in this system.

## Discussion

The general pattern that appears from these studies is that chemical mediation is particularly determinant in the specific attraction of mutualistic partners in nursery pollination mutualisms. However, this review has also highlighted gaps in our understanding of the role of chemical mediation in the functioning of these interactions (Fig. 1). Moreover, few studies examine the potentially synergistic interplay of olfactory and visual signals in the attraction of mutualistic partners.

As is the case for most pollination interactions [with the notable exception of those involving orchids (Dodson et al. 1969)], chemical ecology has only recently been integrated into studies of nursery pollination (Raguso 2008a). However, recent work focusing on the role of scents in several nursery pollination interactions has begun to characterize the diversity of VOCs that plants produce to attract their nursery pollinators (see Fig. 1). Unfortunately, within these chemical messages, the signals (compounds singly or in combination) to which the pollinators actually respond have been identified for only a few systems, owing probably to the complexity of floral scents. The recent integration of electrophysiological recording (GC/EAD, see Table 2) into the study of behavioral aspects of plant–pollinator interactions will provide opportunities for more complete comparisons of these diversified systems. However, electrophysiological responses of insect antennae can be interpreted only when we know what behavioral response they trigger in the whole insect: attraction, repulsion, or passive behavior. Thus, an integrated view of the signals used by pollinators in these systems will only emerge when electrophysiological studies are combined with behavioral experiments (using Y-maze olfactometers, wind tunnels, and other apparatus, see Table 2).

The independent evolution of nursery pollination in different taxonomic groups of plants and insects allows attempting tentative generalizations and proposing testable hypotheses on the evolution of specificity of chemical signals. From the studies of diversified and obligate nursery pollination mutualisms, such as the interactions between figs and fig wasp and those between *Glochidion* trees and

*Epicephala* moths or between *Trollius* and *Chiastochaeta*, it seems that coevolutionary specialization has led to simplification of the signal produced by the plant, with only a few compounds facilitating partner encounter. These signals are also highly specific and only recognized by the plant's specific pollinator, corresponding to the notion of a private channel. On the contrary, where the plant is pollinated by several closely related species of the same genus, as in less specialized, perhaps more recently evolved nursery pollination interactions, the message produced by the plant should be less specific. The emergence of new results on nursery pollination interactions, particularly when they enable comparison of different systems varying in degree of specialization, will give us the opportunity to investigate such questions and to properly test the occurrence of "private channels" in these interactions.

On the other hand, extreme simplification of signals in long-coevolved interactions should have other consequences. Could it be that, the simpler the signal, the more the pair is locked into it, with few "options" for evolutionary transition to a different signal? A simple signal (combined with a fine sensitivity for this compound in the pollinator) may be an isolated adaptive peak, whereas a more complex signal offers more possibilities for change. As increasing numbers of associated species adapt to exploit the resources exchanged by mutualists, are mutualist pairs that are locked into a simple signal unable to shift, whereas those that use more complex signals can respond more easily to such pressures? Could it be that if they appear, simple-signal systems may relatively quickly disappear, rather than leaving descendant lineages? Answering such questions will require integration of the study of biosynthetic pathways in order to understand the role of such hypothesized constraints on the evolutionary dynamics of plant signals. Quantitative and qualitative changes for compound are the results of modification in the biosynthetic pathway, which occurs at multiple levels, including gene regulation, mutations, or post-translation modifications (Dudareva and Pichersky 2006). Insights into molecular mechanisms will guide our understanding of how these very specific interactions can evolve (Raguso and Pichersky 1999).

Studies on geographic variation in odor traits and maintenance of specificity in nursery pollination mutualisms over a wide geographic scale (Dötterl and Jürgens 2005; Svensson et al. 2005) are still too limited to allow tracing any general trends. Moreover, rigorous studies on this point will need to take into account not only genetic variation, but also the possibility of environmentally conditioned variation among plants from widely scattered sampling sites. Studies must, for example, include experiments using plants from different provenances grown in a common garden. Such studies would cast light on odor divergence and the role of selection in driving it.

Moreover, to understand the evolutionary dynamics of signals that ensure host specificity we also need to know to what extent phylogenetic inertia constrains evolution of the composition of signals. Do closely related plant species tend to produce similar floral scents? Alternatively, is selection so strong that floral scents can diverge very rapidly, for example, in related species that evolve association with different pollinators? Any metric of similarity would have to take into account the "biosynthetic distance" between compounds (Levin et al. 2003). The existence, or not, of such constraints could be tested in diversified nursery pollination systems in which the phylogenies of both plants and insects are well known and in which co-speciation of plant and pollinator have been demonstrated (Pellmyr et al. 2007; Jousselin et al. 2008; Kawakita and Kato 2009). Such an approach will be crucial to understanding the respective roles of phylogenetic inertia and selection pressure on the evolution of chemical signals in nursery pollination systems.

Finally, another step in our understanding of chemical mediation in nursery pollination mutualisms should be to consider the entire complex network of interacting species, as suggested recently by Raguso (2008a) in his comprehensive review of floral scents. As these resource-rich systems attract other insects, such as parasites or predators, and even frugivorous vertebrate seed-dispersing mutualists (Borges et al. 2008; C. Soler, unpublished data), especially in the case of keystone species such as figs, floral scents may be the "silk" that holds together the complex web of interactions, orchestrating the organization of an entire community focused on the pollination mutualism.

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# Private channels in plant-pollinator mutualisms

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**Volatile compounds often mediate plant-pollinator interactions, and may promote specialization in plant-pollinator relationships, notably through private channels of unusual compounds. Nevertheless, the existence of private channels, i.e., the potential for exclusive communication via unique signals and receptors, is still debated in the literature. Interactions between figs and their pollinating wasps offer opportunities for exploring this concept. Several experiments have demonstrated that chemical mediation is crucial in ensuring the encounter between figs and their species-specific pollinators. Indeed, chemical messages emitted by figs are notably species- and developmental stage-specific, making them reliable cues for the pollinator. In most cases, the species-specificity of wasp attraction is unlikely to result from the presence of a single specific compound. Nevertheless, a recent paper on the role of scents in the interaction between *Ficus semicordata* and its pollinating wasp *Ceratosolen gravelyi* showed that a single compound, 4-methylanisole, is the main signal compound in the floral scent, and is sufficient by itself to attract the obligate pollinator. Mainly focusing on these results, we propose here that a floral scent can act as a private channel, attracting only the highly specific pollinator.**

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**Key words:** chemical mediation, ficus, agaonidae, private channel, floral filter, coevolved mutualism

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Mutualisms are interspecies interactions in which each participant gains net benefits from interacting with its partner. Like many other interspecies interactions, mutualisms are usually mediated by chemical signals. For instance, floral scents act as pollinator attractants in numerous plant species.[1-3] We studied the chemical compounds that mediate a set of interactions which has become a model system for understanding the evolution of mutualisms: the interactions between Ficus (Moraceae) and their species-specific pollinating fig wasps (Chalcidoidea: Agaonidae). 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. Each pollinator species is usually associated with a single Ficus species. Fig trees mainly grow in tropical regions, and many species can co-occur in the same forest. In these regions, the density of individual species is often quite low.[4] Therefore, signals emitted by each species must be efficient at long distances and specific, to allow the attraction of the associated pollinator. In all of the Ficus species that have been studied so far (approximately 40 of a total of roughly 800 species worldwide), figs have been shown to release volatile compounds when they are receptive (i.e., at the stage when pollination can occur).[5-10] Behavioral tests have been performed with pollinators of several species, confirming for these species the role of fig scent in pollinator attraction (Soler et al. in prep).[5-7,9,11] In the floral scents of the fig species studied, at least two to five major compounds account for the majority of the total volatiles emitted by receptive figs.[8,10,12-15] These major volatiles emitted by receptive figs are generally compounds that are not rare in floral fragrances. The species-specificity of wasp attraction is thus usually not likely to result from the presence of only one single specific compound.[10,13] However,

Chen and co-workers,[16] focusing on *Ficus semicordata*, found that a single benzenoid compound, 4-methylanisole, is sufficient to attract its pollinator. Though 4-methylanisole occurs in floral fragrances, having been documented in floral scents of plants from 17 families (of the 90 families included in the review by Knudsen et al.),[17] it usually accounts for only a fraction of total volatiles, and this was the first time that this benzenoid compound has been reported in the floral scent of a Ficus species.[7,10-12,18] To our knowledge, no previous study has shown that 4-methylanisole is attractive to pollinators of any plant, or that this compound could by itself mediate the specificity of any mutualistic interaction.

Raguso (2008)[3] defined a 'private channel' as the potential for such exclusive communication via unique signals and receptors. Moreover, according to Schaefer et al.[19] private channels must fit three major criteria: (i) the identification of an intended (effective mutualist) receiver; (ii) sensitive signal detection by this receiver, and finally (iii) poor detection by unintended (less effective) receivers. Chen et al.[16] showed that a single compound, 4-methylanisole, accounted for more than 90% of the volatile compounds emitted by receptive figs of *F. semicordata*. This compound is also known not to be produced by the two other sympatric Ficus species in which floral odours have also been studied, nor by any Ficus species whose scent has been analyzed (Soler et al. in prep.).[10,13] Moreover, Chen et al.[16] found that the species-specificity of the attraction of *Ceratosolen gravelyi*, the pollinating wasp of *Ficus semicordata*, was due mainly, if not entirely, to this single major compound. Indeed, based on behavioural (olfactory) tests, they showed that the specialized pollinating wasp detects 4-methylanisole and is attracted by it, even at low concentrations (wasp response tested in concentrations ranging from $1.22 \times 10^2$ ng/100 µl to $1.22 \times 10^6$ ng/100 µl). The last criterion for a private channel proposed by Schaefer et al.[19] i.e., poor detection by unintended receivers, is the only one which has not been clearly demonstrated by Chen et al.[16] In the case of fig/fig wasp mutualisms, the unintended receivers correspond obviously to the parasites of the mutualism. Indeed, many fig species harbor numerous species of chalcidoid wasps that mature within ovaries in fig inflorescences, like the pollinator, but do not carry pollen. Each of these non-pollinating fig wasps is assumed to be associated specifically with a single Ficus species[20] and to use fig scents as cues to detect the host species, as do pollinating fig wasps.[21] However, in the case of the *F. semicordata*/*C. gravelyi* mutualism, no non-pollinating fig wasps have been observed ovipositing during the period when figs are at the receptive stage. This situation is quite unusual in Ficus species (Proffit M, unpubl. data; Rasplus J-Y, pers. comm.). The apparent absence of 'eavesdropping' parasites at the time when pollinators are attracted to the figs suggests that the last criterion of a private channel—poor detection by potential receivers other than the specific pollinator—also holds in this case, although this has not been experimentally demonstrated.

The study by Chen et al.[16] is, to our knowledge, the first that attempted to test the existence of a private channel in a fig/fig wasp interaction. Nevertheless, this is not the first case of a putative private channel in a nursery pollination mutualism. Indeed, examples have been highlighted in the interactions between *Yucca filamentosa* (Agavaceae) and its moth pollinator *Tegeticula cassandra* (Prodoxidae)[22] and in the interactions between *Peltandra virginica* (Araceae) and its pollinating fly *Elachiptera formosa* (Chloropidae).[23] Similarly, the existence of private channels has been suggested for several species in the family Eupomatiaceae, pollinated by beetles.[24] In contrast, Svensson et al.[25] showed that in the Breynia (Phyllanthaceae)/Epicephala (Gracillariidae) interaction, pollinators are attracted by common volatile compounds, supporting the hypothesis that no private channel exists in this case. In a review of the role of scents in plant-pollinator interactions, Raguso[3] highlighted the putative examples of private channels, but noted that their existence is still debated, notably in the case of non co-specialized plant-pollinator interactions. Nevertheless, some studies have suggested that private channels do exist in this class of interactions. Most examples concern the Orchidaceae. For instance, Eltz et al.[26] showed that carvone oxide and ipsdienol are volatile floral rewards emitted by Neotropical orchids pollinated by male euglossine bees, which collect volatile substances for courtship displays. However, probably the best-known examples are Ophrys spp., temperate-zone terrestrial orchids whose flowers mimic the female pheromone of the pollinator to attract males, which pollinate flowers by pseudo-copulation.[27-29] In his review of the role of scents in plant pollinator interactions, Raguso[3] also proposed that floral filters do not need to rely upon exclusive olfactory signals or receptors. Indeed, few studies have determined whether pollinator-attractive compounds could alone assure species-specificity (private channel), or whether specificity is mediated by more complex 'floral filters', of which scent is only one component. These latter may integrate mechanical or other kinds of barriers, as seems to be the case in the interaction between the dwarf palm *Chamaerops humilis* and the weevil *Derelomus chamaeropsis*.[30]

In the literature about private channels in chemical mediation of mutualisms, two points still seem unclear. One is a semantic point: do cases in which specificity is ensured by specific ratios of several more common compounds constitute a private channel, or is this concept restricted to cases in which specificity is ensured by a single rare compound? The literature on private channels emphasizes cases of the latter type. A related question is whether specificity is sometimes ensured not by a single rare compound, but by a combination of rare compounds. While all the putative cases of private channels that have attracted attention concern emission of and attraction to a single rare compound,[3,16,18] the limited number of studied cases does not allow drawing firm general conclusions. Nevertheless, we might think that a hypothetical private channel constituted by several rare compounds might be more difficult to evolve (if it requires the acquisition of several biosynthetic pathways by the plant, and of specific receptors by the insect) or counter-selected (owing to greater costs).

We propose here that in the mutualism between *F. semicordata* and *C. gravelyi*, despite the existence of mechanical barriers to flower visitation (as in all Ficus species), available information on the

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chemical communication between plant and pollinator constitutes a strong case for the mediation of a highly specific interaction through a private channel, which acts largely alone as a floral filter that prevents 'cheaters' from finding and exploiting a potential resource. An interesting long-term consequence of such an adaptation in a highly co-specialized plant-pollinator interaction is that it might reduce evolutionary flexibility, preventing host shifts, and perhaps making it difficult for the mutualists to evolve counter-adaptations to a new parasite that 'decodes' the private channel. Private channels may be isolated adaptive peaks, even more difficult to escape than they are to reach. This could explain their apparent infrequent occurrence in nature.


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# CHAPITRE 2 :
# Travaux Expérimentaux

# CHAPITRE 2 :
# Travaux Expérimentaux

## PARTIE A :

## PARFUM DE FIGUES :

## CONTRAINTES & MÉDIATION CHIMIQUE

**Articles associés :**

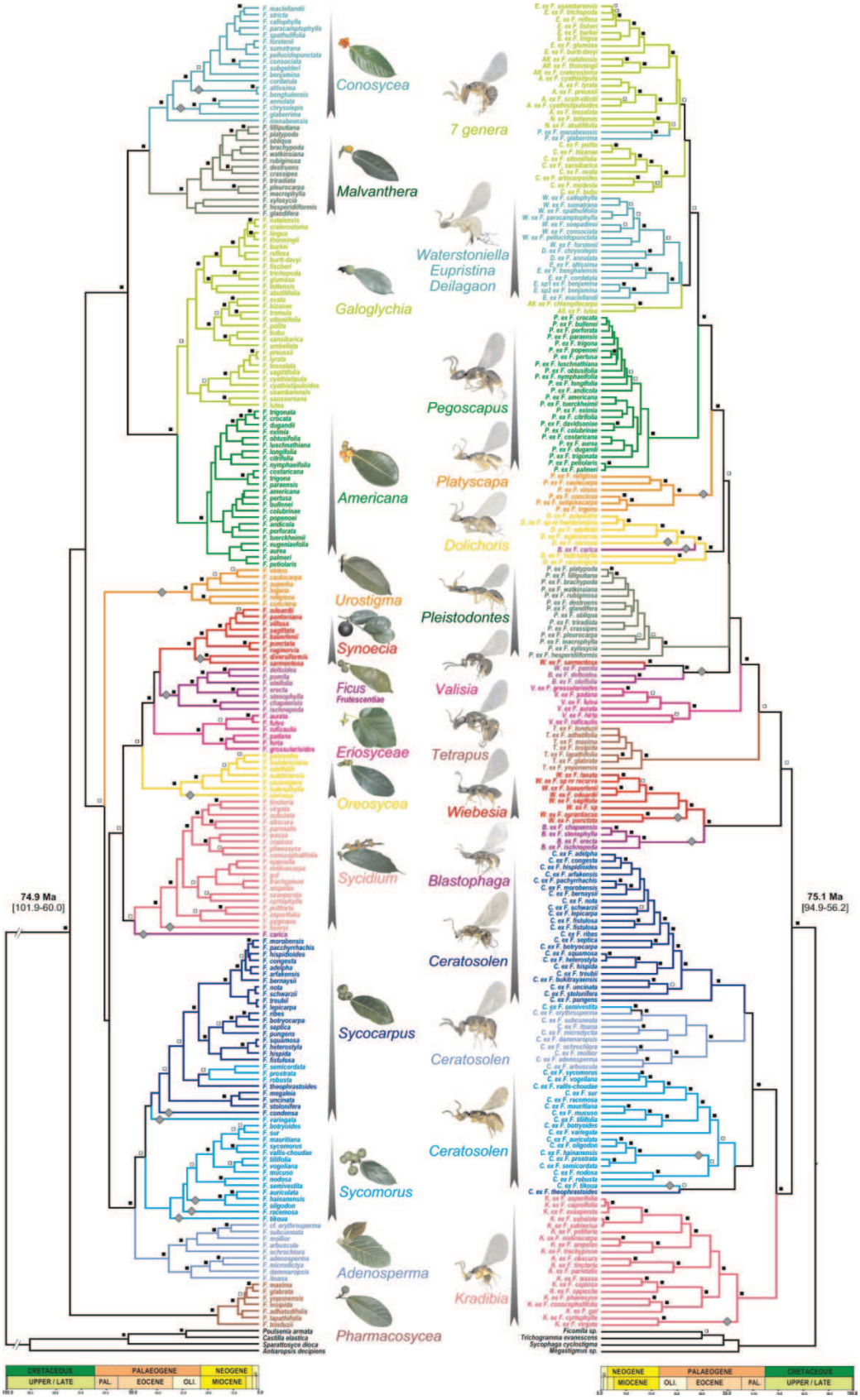
Manuscrit 3 : **Soler C.**, Alvarez N., Bastelica F. , Proffit M., Bessière J.-M., Schatz B. and Hossaert-McKey M. No phylogenetic conservatism of fig odours**.** *In prep.*

Manuscrit 4 : **Soler C.**, Hossaert-McKey M., Buatois B., Bessière J.-M., Schatz B., Proffit M. 2010. Geographic variation of floral scent in a highly specialized pollination mutualism . *Phytochemistry* (in press).

Communiquer engendre des coûts : il existe des contraintes liées à toute forme de communication, y compris la médiation chimique. Ces contraintes peuvent être générales, c'est-à-dire applicables quel que soit le mode de communication utilisé (par exemple, être repéré par un prédateur), ou spécifiques du mode de communication utilisé (par exemple, diffusion efficace d'un signal électrique uniquement en milieu aqueux). De même, les traits d'histoire de vie des partenaires impliqués, entre lesquels la communication est utilisée, représente une contrainte. Ainsi, dans le cas d'une interaction mutualiste spécifique et obligatoire à transmission horizontale, comme les *nursery pollination*, les pressions évolutives sur le message/signal sont fortes, car il est déterminant dans la rencontre entre les partenaires. Etonnamment, il n'existe que peu d'études sur les contraintes liées à la médiation chimique. Chez les figuiers, deux points ont déjà été analysés : le coût en carbone de la production du bouquet volatil (Grison-Pigé *et al.*, 2001b) et la contrainte phylogénétique (Grison-Pigé, 2001; Soler, 2007), mais ces derniers travaux n'étaient que très préliminaires. Grison-Pigé *et al.* (2001b) ont montré que les coûts estimés en carbone investis dans la production des COVs sont très faibles (environ 0,1%), comparés à ceux investis dans le reste de la reproduction des figues (notamment dans la formation d'organes reproducteurs charnus). En ce qui concerne les contraintes phylogénétiques, les deux études précédemment citées suggèrent l'existence d'une certaine inertie phylogénétique sur l'émission du bouquet. Cependant, les auteurs restent très prudents vis-à-vis de leurs résultats, notamment en raison des méthodes utilisées et d'un échantillonnage relativement faible.

Dans quelle mesure production et émission des COVs, chez chacune des espèces, dépendent de leur histoire évolutive ? Existe-t-il des contraintes phylogénétiques sur l'émission de bouquets de COVs ? Les voies de biosynthèse des COVs nécessitant la mise en place de toute une machinerie enzymatique associée à l'activation du métabolisme secondaire

**Figure 9 :** Cocladogénèse figuiers/ pollinisateurs *(d'après Cruaud et al., soumis)*.



(comme étudié pour la voie de l'acide shikimique ou l'isopentényl pyrophosphate par Dudareva & Pichersky, 2000), une certaine inertie phylogénétique pourrait contraindre la production des bouquets volatils. Le modèle figuier est très pertinent pour examiner cette question, car il constitue un exemple de coévolution forte (Cf. Fig. 9), où les phylogénies des agaonides pollinisateurs et des *Ficus* sont quasiment des miroirs l'une de l'autre (Machado *et al.*, 2001; Cruaud et al., soumis), et où la spéciation peut se faire par capture latérale (Jousselin *et al.*, 2008). De plus, le fait que la rencontre de ces partenaires se réalise grâce à la détection par l'insecte des bouquets de COVs émis par les figuiers, fournit une situation où l'inertie phylogénétique sur les bouquets volatils pourrait être forte. L'objectif de l'article N°3 était de tester cette hypothèse. Cette étude n'est pas encore achevée, et nous affinons actuellement différentes façons de traiter les informations, tout en les complétant avec des espèces d'autres taxa phylogénétiques (sections non encore représentées dans notre jeu de données). Cependant, nous obtenons des résultats différents de ceux des deux études précitées. Nous avons réalisé un regroupement des espèces en utilisant un dendrogramme basé sur les similarités chimiques des COVs, afin de le comparer avec la nouvelle phylogénie moléculaire des figuiers (Cruaud *et al.*, soumis). Deux «chémogrammes », obtenus selon des méthodes phylogénétiques différentes, sont présentés dans notre article. Le premier chémogramme est basé sur les distances de Sorensen, et donc sur la présence/absence des COVs, le second sur les quantités émises de COVs (*hierarchical clustering* de Ward). Dans le premier cas, les espèces sont bien séparées, et ce contrairement au second cas. Il y a donc discrimination des espèces possibles en fonction de la présence/absence des COVs émis, et non de leurs proportions relatives (%). Cela signifie que les analyses basées sur les quantités, même relatives, ne permettent pas l'utilisation des odeurs comme marqueurs phylogénétiques, notamment du fait de la variation interindividuelle très forte chez les figuiers. *A contrario*, les qualités (présence/absence) des COVs sont informatives du point de vue de la

phylogénie. Ce résultat concorde avec des études précédentes, telles celle de Levin et al. (2003) sur les Nyctaginacées, où les topologies exprimées en présence / absence suivent mieux la phylogénie des espèces que lorsque les COVs sont exprimés en proportions relatives. Ce chémogramme de Sorensen ne révèle donc aucun conservatisme phylogénétique du message chimique émis à réceptivité, même dans le cas d'espèces sympatriques. Cependant, certaines espèces, voisines phylogénétiquement, émettent des messages chimiques proches. C'est notamment le cas de *F. natalensis natalensis* et de *F. burkei*, de *F. oligodon* et de *F. auriculata*, ou encore de *F. asperifolia* et de *F. pachyclada pachyclada*. Il est alors très intéressant de constater que les deux premiers exemples correspondent à des cas tout à fait exceptionnels de *Ficus* sympatriques pollinisés par les mêmes insectes, alors que le troisième exemple est un cas où l'identité des pollinisateurs est encore débattue, tout comme la position de ces deux espèces de *Ficus* dans la phylogénie moléculaire (F. Kjellberg, com. pers.). En fait, en combinant différentes approches, nous avons pu mettre en évidence des variations interspécifiques des bouquets de COVs émis à réceptivité par les figuiers, tant qualitatives que quantitatives, ce qui rejoint les différentes modalités de variation de la médiation chimique proposées par Raguso (2008). Il existe d'importantes différences dans la qualité du bouquet par perte ou gain de certains COVs. À chaque espèce de figuier est associée une odeur spécifique, résultant de la mise en place, au cours des évènements de spéciation, d'un système 'on-off' dans les voies de biosynthèse des COVs. Ces messages émis par les figuiers pour attirer leurs pollinisateurs sont soumis à de fortes pressions de sélection biotiques, de part la spécificité de l'interaction *Ficus*/ Agaonidae, et de part l'importance des *Ficus* au sein des écosystèmes en tant qu'espèces clefs de voûte. Ces fortes pressions biotiques pourraient ainsi avoir effacé l'empreinte de contraintes phylogénétiques préalablement existantes sur ces messages.

Une seconde contrainte exercée sur le message chimique émis par les figuiers à réceptivité correspond aux variations dues aux facteurs

environnementaux, ce qui constitue l'objectif de notre article n°4. En effet, certaines espèces du genre *Ficus* présentent des aires de distribution très larges, et les populations sont ainsi soumises à des conditions abiotiques différentes. Dans cette étude, nous avons analysé les bouquets de COVs émis à réceptivité par deux espèces sympatriques de figuiers à large aire de distribution, *F. racemosa* et *F. hispida*, et ce pour trois populations différentes par espèce. Nous avons ainsi échantillonné *F. racemosa* en Inde, en Chine et en Thaïlande, alors que *F. hispida* a été échantillonné en Chine, et dans 2 populations indiennes distinctes, dans les Ghâts. Plus d'une centaine de COVs différents ont été identifiés dans ces bouquets. La spécificité du bouquet a été trouvée également pour ces espèces : en effet, les individus de *F. racemosa* émettent à réceptivité un bouquet d'odeurs significativement différent de celui des individus de *F. hispida*. Etonnamment, nous avons également mis en évidence une variation intraspécifique du bouquet : pour les deux espèces, les individus indiens émettent à réceptivité des bouquets différents de ceux des individus est-asiatiques. Ces différences consistent surtout en des variations de proportions relatives de certains terpènes au sein des bouquets, qui sont souvent des COVs principaux dans l'interaction figuier/pollinisateur (Hossaert-McKey *et al.*, 1994; Gibernau *et al.*, 1998; Grison *et al.*, 1999; Song *et al.*, 2001; Proffit *et al.*, 2009). Par ailleurs, des résultats récents sur la génétique des populations de ces espèces de figuiers (Alvarez *et al.*, 2010; Dev *et al.*, 2010, Bain et al., in prep) et des pollinisateurs (Kobmoo *et al.*, 2010, Cruaud et al., in prep), ainsi que des données morphologiques sur ces derniers (J-Y Rasplus, comm. pers.) tendent à montrer qu'un processus de spéciation est en cours chez ces espèces. En effet, les populations de figuiers indiens ont pu être isolées des autres populations asiatiques, notamment par la présence de la chaine de montagnes des Eastern Ghâts. Nous avons ainsi mis en évidence que la spécificité du bouquet émis à réceptivité par les figuiers est conservée sur de longues distances, malgré des milieux différents, et que la variation inter-populations significativement détectée correspondrait plutôt à un début de variation entre nouvelles espèces, ce

qu'il faudrait cependant vérifier, notamment par des tests comportementaux sur le signal utilisé par les insectes récepteurs.

Nos études de deux contraintes sur la médiation chimique de l'interaction figuier / pollinisateur aboutissent ainsi à des résultats similaires : ces deux contraintes ne semblent pas exercer de fortes pressions de sélection sur les messages émis alors que « l'élément important » reste le respect de la spécificité du message. La variation interspécifique du message émis semble donc soumise à une forte sélection diversifiante, probablement liée à des contraintes biotiques, comme celles exercées par le pollinisateur.

# No phylogenetic conservatism of fig odours

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This is just a draft of our paper. Before publication, we have to complete our dataset (*Ficus* species from other sub-genus have been sampled but do not still appear here). We also planned to work on the biosynthesis pathways. Moreover, we want to try different phylogenetic methods to build the chemograms (as using the phylogenetic software PAUP for example) and perhaps some independent contrasts methods. We planned to submit this work for publication to *Systematic Biology* or *Evolution*.

## Abstract

Mutualisms are interspecies interactions in which each participant gains net benefits from interaction with the other partner. Like many other interspecies interactions, mutualisms are mediated by chemical signals. Understanding how mutualisms function and evolve depends on deciphering the chemical information transmitted between mutualists, for example, those that guide one partner to the resources offered by the other in exchange for services. Most studies on chemical mediation between plants and pollinators stress the direct impact of selection by pollinators on the composition of the message. Nevertheless, constraints on the message emission might also occur in addition to pollinator effects. We studied whether phylogenetic history constrains the composition of plant chemical signals that mediate interactions with pollinators, i.e., we tested whether the evolution of floral scents exhibits some phylogenetic conservatism. We studied the composition of chemical bouquets that mediate a group of interactions that becomes a model system in our understanding of the evolution of mutualisms: the interactions between figs and their species-specific pollinating fig wasps. 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. Encounter of the pollinator and the receptive fig is mediated by specific chemical signals. We used headspace adsorption/desorption methods to collect scents of several fig species in tropical forests from different tropical regions, and analyzed the composition of their volatile compounds using gas chromatography/mass spectrometry (GC-MS). We then built different chemograms, using both qualitative and quantitative traits of the bouquets of compounds, and compared them with phylogenies. Our results show that phylogenetic constraints on scents are negligible, and that scent divergence may be essentially driven by selection by pollinating fig wasps.

## Key-words :

Mutualism, scent, phylogeny, *Ficus*, chemograms, interspecific variation

## INTRODUCTION

Olfactory signals are often key component of the interaction between communicating organisms in an ecosystem. In plants, fragrances are typically blends of Volatile Organic Compounds (VOCs), produced by several biosynthetic pathways, and belonging to three different classes (fatty acid derivatives, benzenoid or terpenes) (Knudsen et al., 2006). Emitted by reproductive as well as vegetative structures (Croteau and Karp, 1991; Dudareva and Pichersky, 2006; Dufaÿ et al., 2003), these fragrances have many functions, as pollinator attraction or herbivores repulsion (Dudareva and Pichersky, 2006; Raguso, 2008; Schiestl, 2010). Pellmyr and Thien (1986) even proposed that the role of VOCs as pollinator attractants evolved from repellent odors against herbivorous animals. However, before being repellent, fragrance may have initially been not functional, being merely a byproduct of necessary metabolic processes. Moreover, the adaptive context within which fragrance has evolved may have changed (Levin et al., 2003).

Floral bouquets can vary in their composition (identity and quantity of the VOCs), and such variation can be used by plants to elaborate specific blends in order to attract specific pollinators (Raguso, 2008). Moreover, variation in VOC production by plants may reflect factors other than pollinator attraction or anti-herbivorous defense, including current environmental conditions and evolutionary history (Majetic et al., 2009; Raguso, 2008). Thus, in addition to the role that fragrance plays in plant–animal interactions, fragrance traits may also reflect phylogenetic history. To date, few researchers have rigorously examined the evolution of floral fragrance in a phylogenetic context (but see Azuma et al., 1997; Barkman, 2001; Barkman et al., 1997; Dobson et al., 1997; Knudsen et al., 2006; Levin et al., 2003; Raguso et al., 2006; Williams and Whitten, 1999), in part because such study requires scent data

from a quite large number of species, as well as a robust phylogeny of the studied group.

Study the evolution of scent in a phylogenetic context requires character coding of fragrance (Levin et al., 2003). The individual constituents of a fragrance are the VOCs, which among plants vary in quality (presence/absence) and quantity (amounts or relative proportions) (Raguso, 2008). Many previous researchers have ignored quantitative differences in volatiles and simply scored presence/absence of compounds or biosynthetic pathways (as Barkman, 2001; Dobson et al., 1997). However, a few of them have including quantitative information when coding scent data (as Azuma et al., 1997; Levin et al., 2003; Williams and Whitten, 1999). Moreover, individual compounds are not all independent (Dudareva and Pichersky, 2006; Pichersky and Gershenzon, 2002), since several of them are produced by the same biosynthetic pathway among the different existing ones (Barkman, 2001). As for VOCs, information about pathways could be incorporated by coding either quantitatively or qualitatively.

Models with a long history of specific interactions should be ideals to examine the relationship between fragrance and phylogenetic history. We decided to study these relationships in the well-known example of the genus *Ficus*. Fig/fig wasp interactions are a set of highly specialized and diversified mutualisms, with roughly 800 replications of a species-specific interaction (Janzen, 1979). In this system, each partner is totally dependent of the other for its reproduction, and it has been shown in several cases that fig pollinating wasps are attracted by olfactory signals (Chen et al., 2009; Gibernau et al., 1998; Grison-Pigé et al., 2002; Hossaert-McKey et al., 1994; Proffit et al., 2009; Vannoort et al., 1989; Ware and Compton, 1994; Ware et al., 1993). The fig/fig wasp mutualism is among the best examples of co-evolution

(Jousselin et al., 2003), and the phylogeny of figs and figs wasps are an example of co-cladogenesis (Herre et al., 1996; Cruaud et al, subm.). In this system, the existence of phylogenetic inertia on scents has never been really investigated, even if one study, looking on interspecific variation of scents, discussed about its importance in fragrance composition (Grison et al., 1999). In most studies on pollination ecology, pollinators are considered as the major selection pressure on the evolution of chemical signals (Majetic et al., 2009). However, in order to understand the role of pollinators as agents of selection on fragrance, it requires first to take into account the role that plant phylogeny plays in constraining or enhancing plants responses.

In this study, we examined the role of plant phylogeny by raising the following questions. Do differences in fragrance profiles between species exceed differences among individuals of the same species? How do the topologies based on fragrance data compared with phylogenetic trees resulting from DNA sequence data? Do the associations with pollinators better explain the observed variations in the bouquets of emitted VOCs?

## MATERIAL & METHOD

### Material

We studied the VOC emissions of receptive figs (i.e. those at the developmental stage ready to be pollinated) of 195 individuals, from 25 different species of fig, from different phylogenetic sections (see Fig. 1 for details). Fig floral stages were estimated by their physical characteristics: an open ostiole and brightness are for example both signals showing that a fig is receptive. At least 3

individuals per species, but more generally at least 5 individuals per species have been sampled.

**Collection of Volatiles**

For collection of odor samples, we used the Chromatoprobe head-space technique. Receptive figs were cut off from plants and were enclosed in a polyethylene terephtalate (Nalophan®) bag for 30 min. Then, the air was pulled out of the bag (flow rate: 200 mL min–1), over a filter (filled with 1.5mg of Carbotrap 20-40 and 1.5mg of Tenax 60-80), in which the compounds were trapped. Each collection lasted 5 min, and was done at any time between 11:00 and 15:00. One microlitre of a solution of internal standards (nonane and dodecane) was added to each filter, before scent extraction, to allow quantification. The samples were then stored at -20°C until chemical analyses.

**Chemical Analyses**

The odor samples were then analyzed by GC-MS, using a CP-3800 chromatograph (Varian) coupled with a Saturn 2000 spectrometer (Varian). The characteristics of the columns are the following ones: Varian, CP-Sil 8CB Low Bleed MS 30mx0.25mmI Dx0.25µm film Thickness. The injection is made with the injector 1079PTV (Programmed temperature Vaporizator) with Chromatoprobe equipment (Varian, Walnut Creek, USA). The injection is made at 40°C for 1 min, and then warm up for 3 min, until reaching 250°C, at the rate 200°C/min. The temperature program for the analysis was: 40°C for 3 min, ramped from 40°C to 100°C at 3.3°Cmin–1 to 250°C, from 100°C to 140°C at 2.9°Cmin–1, then 140°C to 180°C at 2.7 °Cmin–1,

and finally from 180°C to 2500°C at 10°Cmin–1. Temperature is still 250°C for the 8 following minutes.

Compound identification was based on matching of the mass spectra with NIST 98 MS and Adams 2007, and on confirmation by comparison of RI with libraries and published data (Adams, 2007). Identification of some compounds was confirmed by comparison of both mass spectrum and GC retention indices with those of standards. These analyses were performed at the “Plate-Forme d’Analyses Chimiques en Ecologie” (platform for chemical analyses in ecology) of “IFR 119 Montpellier Environnement Biodiversité”.

**Data Analysis**

We first grouped all the emitted COV in a general matrix. This gave us two datasets: one on the presence/absence of the VOCs in the emitted bouquets, and a second one on their relative amounts. Thus, to test if some phylogenetic inertia exists on scents emitted by receptive figs, we built two chemograms, from two different methods. Using R software (2.7.2 version), we first performed a Ward hierarchical clustering on the relative amounts dataset, and then we build a tree on Sorensen distances from the presence/absence dataset. If by-species clustering was respected, we then compared the chemogram with fig tree phylogeny (Ronsted et al., 2008), and fig wasps phylogeny (Cruaud et al, subm). Indeed, fig and fig wasps phylogenies being roughly congruent, it’s interesting to compare the chemogram with both of them.

## RESULTS & DISCUSSION

In this study, we highlight the large scent diversity of *Ficus*: figs of 25 species emitted ca. 300 compounds. The two chemograms we built gave us different results. Indeed, the Ward hierarchical clustering, based on quantitative data, did not clustered individuals by species, except for individuals of four species (*F. stuhlmanii, F. semicordata, F. fulva* and *F. subulata*; see Fig. 1). On the contrary, the Sorensen method, based on presence/absence of the compounds, clustered the individuals according to the species they belong. Indeed, only 7 individuals (on the 195 studied here) were not properly clustered (see Fig. 2). The species-specificity of the message emitted by receptive figs, found on previous studies (Grison et al., 1999; Proffit and Johnson, 2009; Soler et al., 2010), is thus confirmed by the second method. Therefore, we decided to consider only the presence/absence matrix, rather than the one of relative amounts of volatiles compounds in the bouquet, as suggested by similar study on Nyctaginaceae (Levin et al., 2003). We consequently used the Sorensen chemogram to compare it with phylogenies. Thus, the presence/absence fragrance dataset had significant hierarchical signal, but this signal was not congruent with signal from DNA sequence data from figs trees (Fig. 3), neither DNA sequence data from fig wasps (Fig. 4).

These results highlighted the absence of phylogenetic inertia on scents emitted by receptive figs. In general, fragrance characters may be too evolutionarily labile to be useful in phylogenetic inference (Barkman, 2001; Williams and Whitten, 1999). However, the general lack of congruence of our chemograms with the phylogenies does not mean that we should exclude the possibility of phylogenetic constraints on scents, since phylogenetic inertia might be only visible when working at biosynthetic pathways level, as suggested by Levin et al. (2003). Moreover, many

of the pathways involved in the biosynthesis of insect-attractive compounds also synthesize compounds that play roles in herbivore defense. Besides, a same compound may be both attractant for pollinators and repellant for herbivores (Dobson, 1994; Euler and Baldwin, 1996). Indeed, floral odor has been shown to act as a feeding and/or oviposition cue for herbivores, thus suggesting a high indirect cost of scent production (Dobson, 1994; Hossaert-McKey et al., 2010). This has been shown for *Ficus*, for which a large community of parasites, using scent to localize their host plants, is associated with (Proffit et al., 2009). Such results suggest the existence of another selection pressure than pollinators, which may have a stronger impact on scent than phylogenetic history. Consequently, certain compounds may be more homoplastic than others because they are more constrained by the selection pressure exerted by herbivores. Moreover, some compounds may be simply by-products of the synthesis of other ones.

More studies combining investigations of the function, cost, and physiology of production of VOCs are necessary to understand fully the role that phylogenetic history may have play in the evolution of scents. Notably, a clear identification of the physiologically active VOCs for pollinators, and how they react to them, should shed light on evolutionary processes mediating the massive radiation of a group such the genus *Ficus*.

**Table1:** Taxonomic and phylogenetic subdivisions of the *Ficus* species used in this study [classified according to (Berg and Corner, 2005) and (Ronsted et al., 2008) and Kjellberg, unpub. data.]. The thick lines correspond to the phylogenetic sections.

| Sub-genus | Section | Sub-section | Species |
|---|---|---|---|
| Ficus | Ficus | Ficus | *F. carica* |
| Ficus | Eriosycea | Eriosycea | *F. esquiroliana*<br>*F. fulva* |
| Sycidium | Sycidium | / | *F. exasperata*<br>*F. asperifolia*<br>*F. montana*<br>*F. pachyclada pachyclada*<br>*F. cyrtophylla* |
| | Paleomorphae | / | *F. subulata*<br>*F. tinctoria gibbosa* |
| Urostigma | Urostigma | / | *F. tsjahela* |
| Sycomorus | Sycomorus | Sycomorus | *F. sycomorus*<br>*F. racemosa*<br>*F. botryoïdes* |
| | | Neomorphae | *F. auriculata*<br>*F. oligodon* |
| | Sycocarpus | / | *F. fistulosa* |
| | | Sycocarpus | *F. hispida* |
| | Hemicardia | / | *F. semicordata* |
| Urostigma | Galoglychia | Galoglychia | *F. lutea* |
| | | Chlamidodorae | *F. burkeï*<br>*F. natalensis natalensis* |
| | | Platyphyllae | *F. stuhlmannii* |
| | | Platyphyllae | *F. trichopoda*<br>*F. glumosa* |

**Figure 1:** Chemogram obtained by Ward hierarchical clustering method (on relative amounts of VOCs).

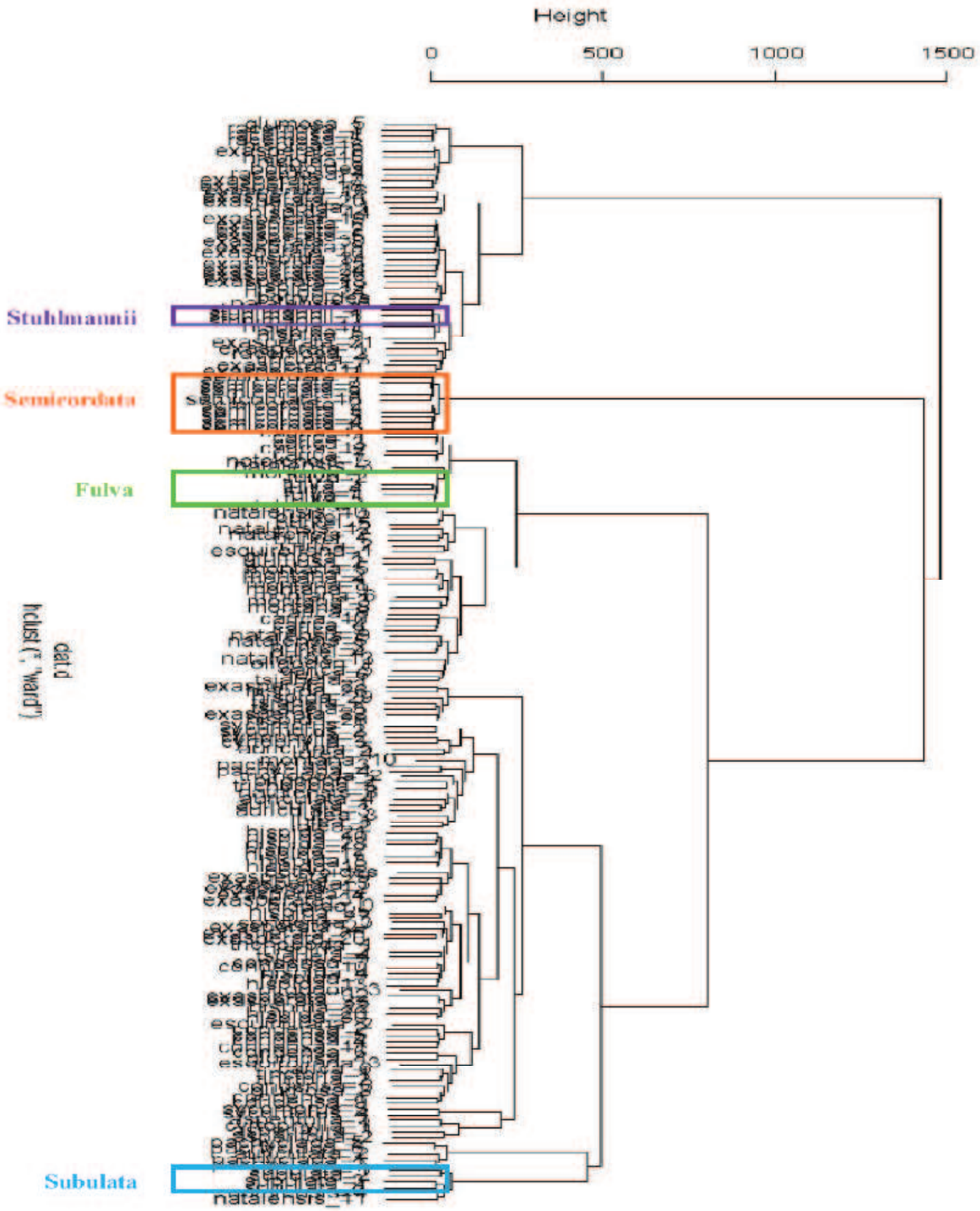


**Figure 2:** Chemogram obtained by Sorensen clustering method (from presence/absence of VOCs dataset).

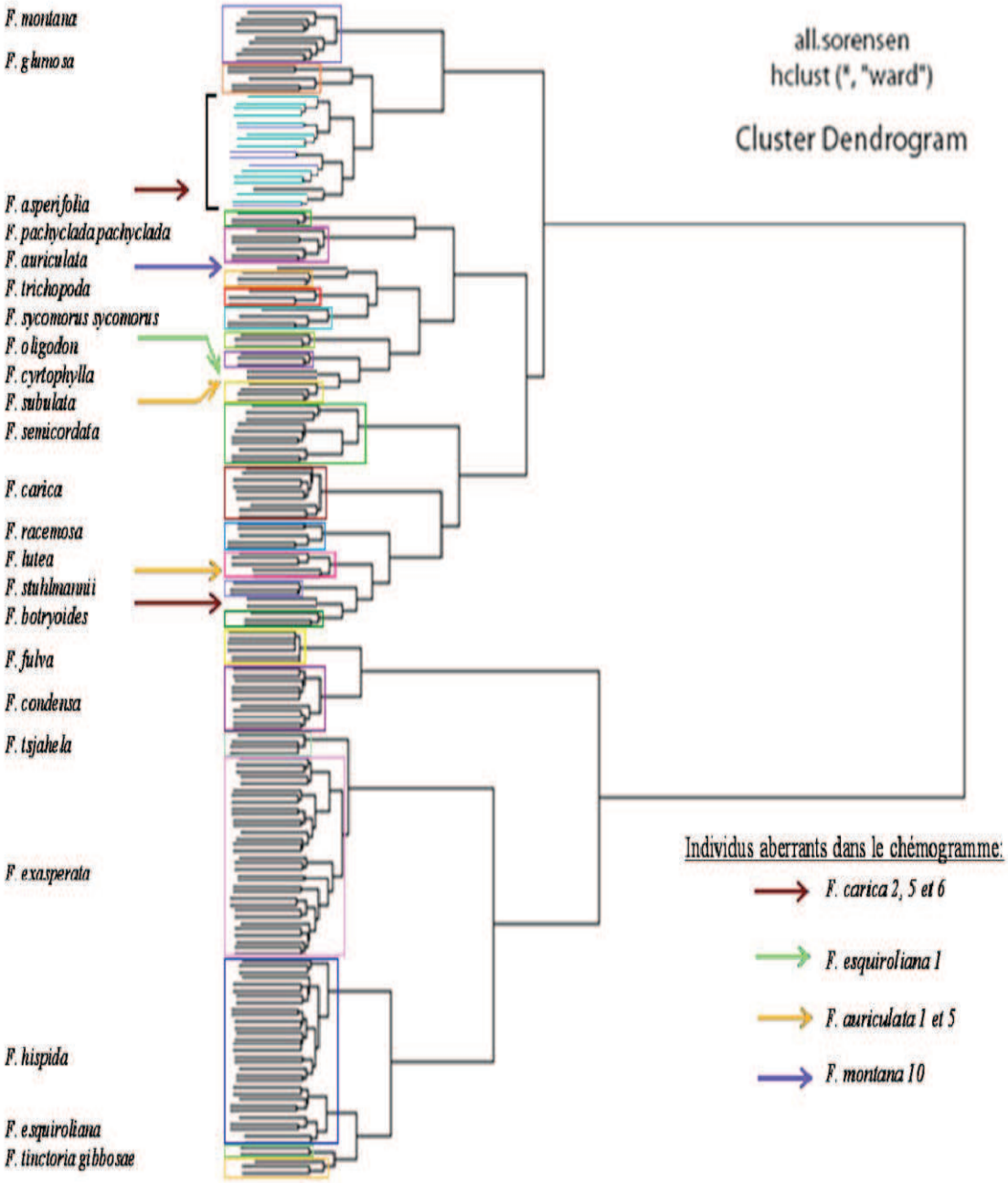


**Figure 3:** Comparison between Sorensen chemogram (left side) and *Ficus* phylogeny (right side)

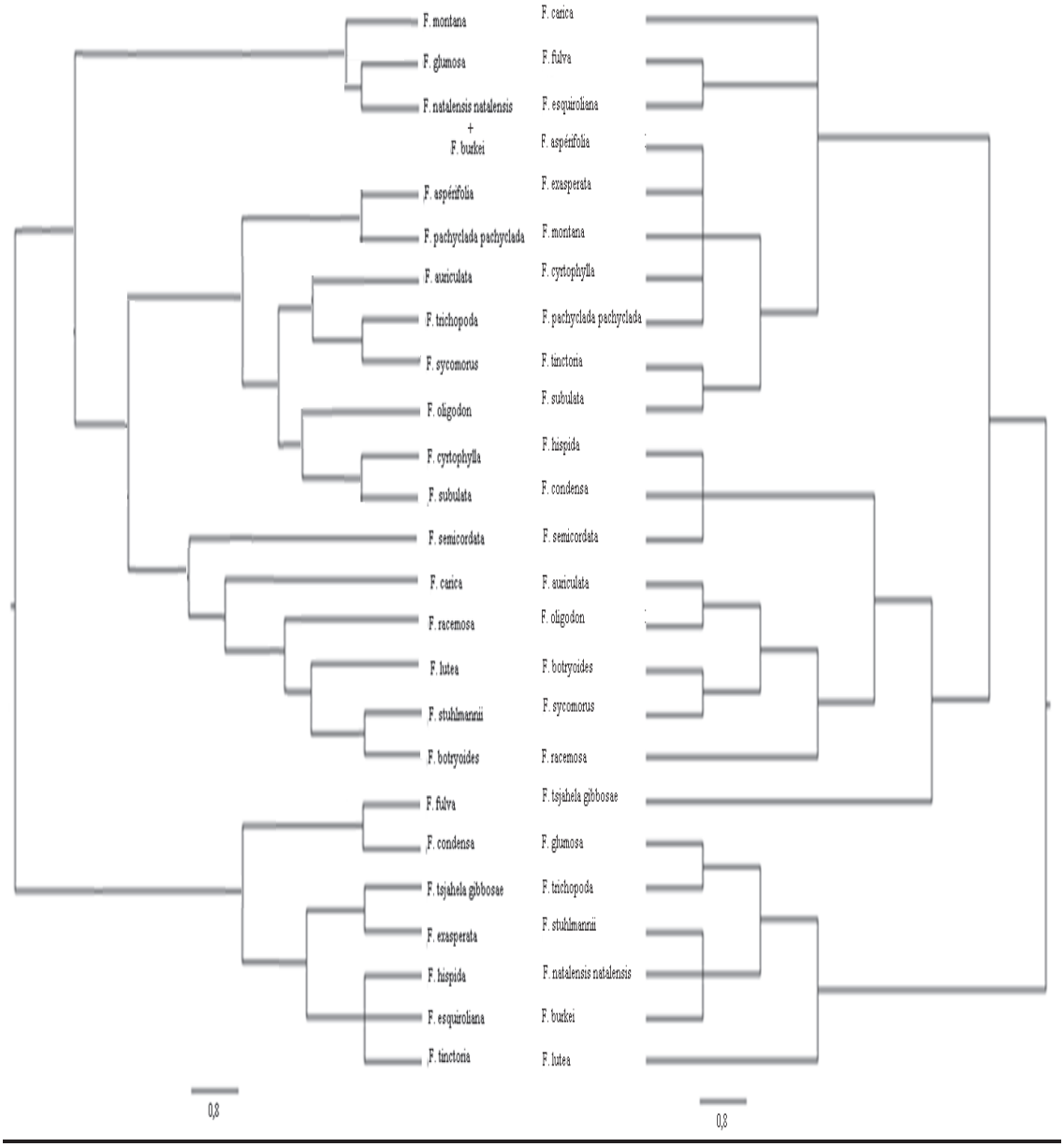
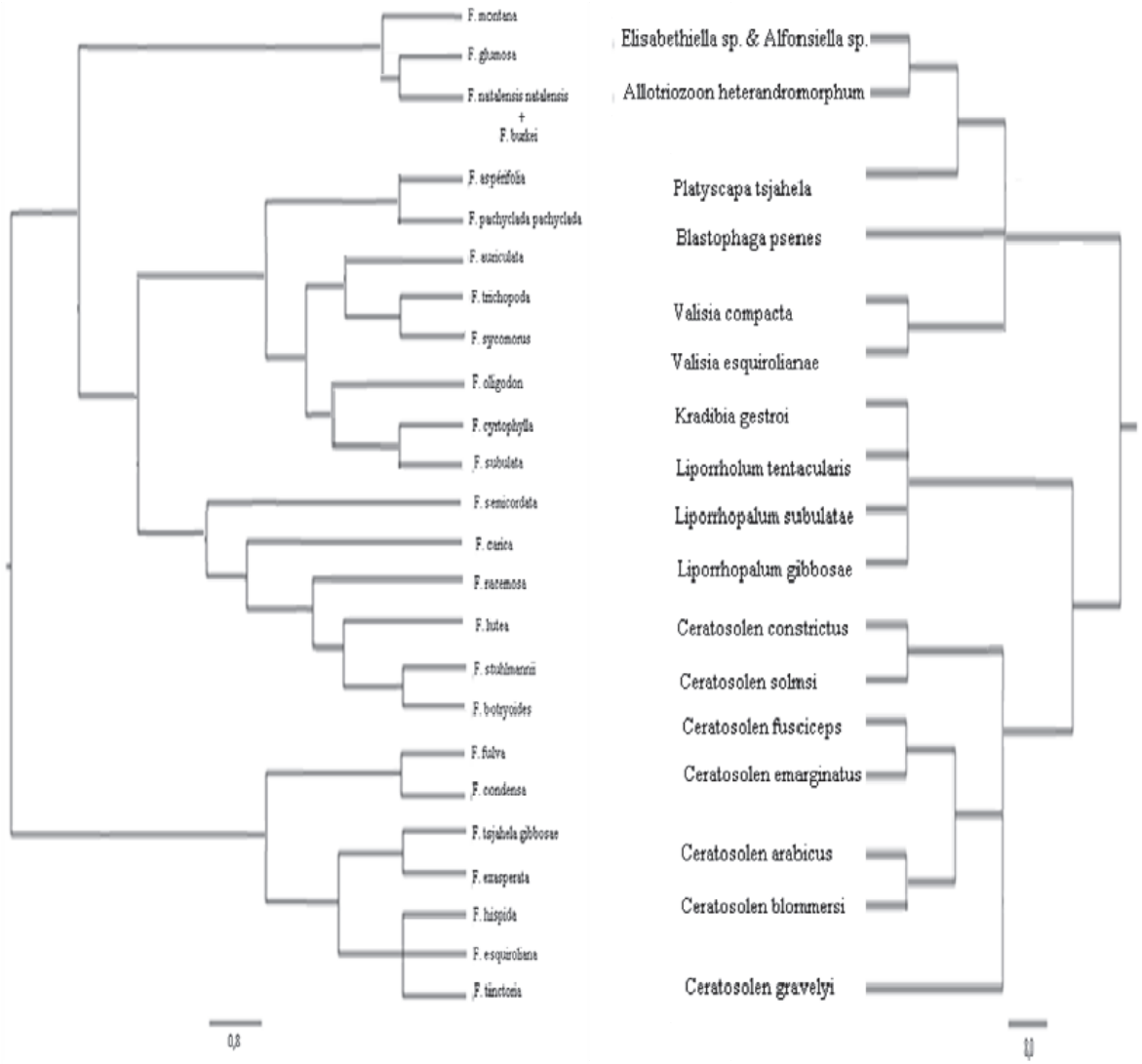


**Figure 4:** Comparison between Sorensen chemogram (left side) and fig wasps phylogeny (right side)



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# Geographic variation of floral scent in a highly specialized pollination mutualism

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ABSTRACT

Floral scents are important signals for communication between plants and pollinators. Several studies have focused on interspecific variation of these signals, but little is known about intraspecific variation in flower scent, particularly for species with wide geographic distributions. In the highly specific mutualism between *Ficus* species and their pollinating wasps, chemical mediation is crucial for partner encounter. Several studies show that scents, i.e. blends of volatiles, are species-specific, but no studies address interpopulation variation of scents in fig pollination mutualisms, which often have broad geographic distributions. In this study, using absorption/desorption headspace techniques, we analyzed variation in floral scent composition among three populations of each of two widely distributed Asian *Ficus* species. We identified more than 100 different volatile organic compounds, predominantly terpenes. In both species, significant differences were found between scent bouquets of East Asian and Indian populations. These differences are discussed in relation to geographical barriers that could disrupt gene exchange between these two areas, thereby isolating Indian populations from those of Eastern Asia.
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## 1. Introduction

Volatile organic compounds (VOCs) emitted by plants often mediate interactions between plants and animals, as for example in many pollination systems (reviewed by Raguso (2008)) and other mutualistic interactions, such as animal-mediated seed dispersal (Borges et al., 2008; Hodgkison et al., 2007) and ant/plant protection mutualisms (Schatz et al., 2009). Among the different classes of compounds-fatty acid derivatives, benzenoids and terpenes-the last-named group is the largest, with the widest assortment of structural types (Degenhardt et al., 2009). Floral bouquets are usually mixtures of numerous components, varying among species in their composition, blend ratio, and overall concentrations (Raguso, 2008). In specific pollination mutualisms, such differences in floral scents, and in animal responses to them, contribute to reproductive isolation between closely related sympatric species (Levin et al., 2001).

Contrary to interspecific variation, variation among populations of a single species in production of floral scent has been studied only very recently and in a limited number of examples (Dötterl et al., 2005b; Füssel et al., 2007; Hossaert-McKey et al., 2010; Ibanez et al., 2010; Knudsen, 2002; Majetic et al., 2009; Schlumpberger and Raguso, 2008; Svensson et al., 2005). In a few cases, no interpopulation differences in floral scents were found (Dormont et al., 2010; Knudsen, 2002), or only quantitative differences could be observed (Svensson et al., 2005). However, in most studies, consistent interpopulation differences in floral scent have been reported (Azuma et al., 2001; Schlumpberger and Raguso, 2008). Why floral volatiles vary among different populations of the same plant species remains poorly understood. Intraspecific variation has been notably explained by hypotheses such as relaxed selective pressure, genetic drift, introgression of scent traits through hybridization, pleiotropic effects of plant defense on scent biosynthesis, or phenotypic plasticity resulting from edaphic or climatic differences (Raguso, 2008). Another explanation is that not all compounds contribute to the signal used by the pollinator, and only compounds without a signalling function are variable (Dötterl et al., 2006; Mant et al., 2005). In the literature, the two most frequently proposed explanations are (i) relaxed selection pressure on floral scents as visual cues assume an important role in pollinator attraction (Azuma et al., 2001; Ellis and Johnson, 2009), and (ii) the occurrence of different pollinators in different geographic areas, as in the case of species visited by a spatially variable set of generalist pollinators (Schlumpberger and Raguso, 2008). The extent to which selection on specificity of the plant-insect interaction leads to selection pressure on scent production is unclear. For example, different populations of *Echinopsis*

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*ancistrophora* (Cactaceae), pollinated by a broad spectrum of insects, were shown to emit different floral scents that attracted different pollinators (Schlumpberger and Raguso, 2008). But surprisingly, in the case of the interaction between *Yucca* and their pollinating moths (*Tegeticula yuccasella* and *Tegeticula cassandra*), in which specificity is high (but not complete), no difference of scent appeared among populations of *Yucca filamentosa* pollinated by different yucca moths (Svensson et al., 2005).

However, none of the cases mentioned above are entirely species-specific. Interpopulation variation of floral scents has never been investigated in obligatory, strictly species-specific pollination mutualisms (Hossaert-McKey et al., 2010). We can expect that in highly specific interactions, in which scents promote partner encounter, stabilizing selection should tightly constrain interpopulation variation of scent. Indeed, selection pressure could lead to strong conservatism of scents over a wide geographic scale, allowing the specific pollinator to recognize its host throughout its range, which can be quite large if the pollinator is a good disperser and often encounters host individuals a long distance from where it was born (Ahmed et al., 2009; Zavodna et al., 2005). The objective of the present study was to test whether intraspecific variation of scent exists in a highly specific plant-insect pollination interaction, the fig/fig wasp mutualism.

The genus *Ficus* (Moraceae) comprises about 800 species, most of them living in the intertropical region. The pollination of *Ficus* inflorescences (the fig or syconium) is carried out solely by agaonid wasps (Hymenoptera:Chalcidoidea:Agaonidae), which reproduce inside the figs. The relationship between *Ficus* and agaonid wasps is obligate for both partners and generally species-specific [one fig species is pollinated by one species of wasp [(Janzen, 1979; Wiebes, 1979); but see (Cook and Rasplus, 2003)]. In such a close-knit nursery pollination mutualism, the encounter between partners is a crucial step for the continuity of the life cycle of each partner. In tropical forests, where several *Ficus* species can live in sympatry, partner encounter is particularly problematic and requires specific chemical signals for each species pair. Several studies have shown that different species of *Ficus* emit clearly distinct chemical messages to attract only their specific pollinating wasp (Grison-Pigé et al., 2002b; Grison et al., 1999; Hossaert-McKey et al., 2010; Song et al., 2001). Behavioral evidence was also found for specific fig pollinator attraction to host volatiles (Chen et al., 2009; Grison-Pigé et al., 2002a; Hossaert-McKey et al., 1994; Proffit and Johnson, 2009; Song et al., 2001).

In general, variation in plant traits involved in pollinator attraction would be expected only when the most effective pollinators in each population exhibit divergent sensory preferences (Ellis and Johnson, 2009), even if some scent variation can be in part conditioned by phenotypic plasticity, in response to environmental variation on a large geographic scale, as shown in the family Brassicaceae (Majetic et al., 2009). In fig/fig wasp interactions, where (i) the plant is generally pollinated by only one pollinator species throughout its distribution, and (ii) scent is known to be responsible for the attraction of the obligate mutualistic partner, the interpopulation variation of the olfactory message might be limited (Hossaert-McKey et al., 2010). Indeed, a change in floral scents could disrupt partner recognition, leading to a drastic reduction in fitness of both partners. In this study, we examined the variation of floral scents emitted at receptivity in two *Ficus* species, one monoecious and one dioecious, and investigated variation among three Asian populations of each species. Our aim here is to test the hypothesis that, even on large geographical scales, the scent of both studied species is species-specific and invariant among populations. To our knowledge, this study is one of the rare ones (Svensson et al., 2005) to examine interpopulation variation in chemical signals in a pollination mutualism that is both obligatory and specific.

## 2. Results and discussion

A total of 114 different VOCs, including six unidentified compounds, were found in the bouquets emitted by receptive figs (i.e. those at the developmental stage ready to be pollinated) of these two species. The 108 identified VOCs belong to four different classes of compounds, including 25 monoterpenes, 59 sesquiterpenes, 10 benzenoids and 14 fatty acid derivatives. In the bouquets of *Ficus racemosa* individuals, we found 57 different VOCs, and in those of *Ficus hispida* 94 compounds (37 VOCs were common to both species; see major compounds for both species in Table 1). Most of these volatile compounds are quite common in floral scents (Knudsen et al., 2006). Only 51 VOCs had a mean relative proportion in the bouquet higher than 1% (respectively 10 VOCs for *F. racemosa* and 45 for *F. hispida*).

### 2.1. Interspecific variation in floral scents

To test if each species had a particular blend of VOCs allowing reproductive isolation of sympatric species in our highly specific system, we performed multivariate analyses on our dataset of relative abundance of each compound for each individual of the two species. The PCA (Principal Component Analysis) showed a separation of the scents emitted by receptive figs of the two species (Fig. 1a). This difference between the two species was confirmed by the MANOVA performed on the coordinates (two first components) of the PCA ($F_{(15;91)} = 11.42$; $p < 0.0001$. Furthermore, the species effect was highly significant in the MANOVA performed on the relative abundance dataset (Wilks's Lambda ["species effect"]: $F_{(19;17)} = 7.95$; $p < 0.0001$). Eleven different VOCs are responsible for this interspecific variation of scents (see Table 1). These results confirm previous studies showing that the chemical signals emitted by different *Ficus* species are sufficiently distinct to allow specific attraction of their own pollinating wasps (Chen et al., 2009; Grison-Pigé et al., 2002b; Grison et al., 1999; Proffit and Johnson, 2009). Indeed, in the case of sympatric species, the differences between species bouquets may be reinforced by selection pressure reducing the frequency of mistakes by the pollinating wasps and scents may act as a barrier to host shifts (Chen et al., 2009; Proffit et al., 2009). Moreover, our results are in agreement with previous studies on odour extracts of receptive figs of *F. hispida* (Proffit et al., 2008, 2009) where similar VOCs have been identified. However, our results differ from those presented in Song et al. (2001). That study used solvent extraction, a technique that recovers not only volatile compounds emitted by figs, but in addition chemicals present in the superficial cell layers of the plant. Our study, as well as those of Proffit et al. (2008, 2009) based on dynamic headspace extraction, reported only volatile compounds, which are potentially detectable by pollinators and other animals at distance.

### 2.2. Intraspecific variation in floral scents

In this very specialized pollination system, we examined whether the blends of VOCs emitted by receptive figs of these two *Ficus* species vary over a large geographic scale. We first performed MANOVAs on our global dataset on relative abundance of each compound in different populations of each species (VOCs > 1% for each species), testing for a population effect. There was significant variation among populations of a single species in scents emitted by receptive figs (Wilks' Lambda ["population within species effect"]: $F_{(76;69)} = 5.62$; $p < 0.0001$). Most of the compounds contributing to interpopulation differentiation are monoterpenes and sesquiterpenes (Tables 2 and 3). In fact, previous studies on fig/fig wasp interactions showed that volatiles from these two

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**Table 1**
Relative abundances (mean ± standard deviation) of main VOCs (>1%) found in bouquets of scents emitted by *Ficus racemosa* and *Ficus hispida* at receptivity for each studied population.

| Compound | S_inj | R.I. | Occ | *Ficus racemosa* India | | *Ficus racemosa* China | | *Ficus racemosa* Thailand | | *Ficus hispida* China | | *Ficus hispida* India A | | *Ficus hispida* India B | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Occ | (*N* = 6) | Occ | (*N* = 5) | Occ | (*N* = 11) | Occ | (*N* = 6) | Occ | (*N* = 6) | Occ | (*N* = 7) |
| *Monoterpenes* | | | | | | | | | | | | | | | |
| α-Pinene | X | 932 | 36 | 5 | 3.05 ± 2.76 | 4 | 0.67 ± 0.81 | 11 | 2.71 ± 1.58 | 4 | 1.99 ± 2.11 | 5 | 5.41 ± 2.52 | 7 | 2.52 ± 1.28 |
| Sabinene | X | 965 | 29 | 0 | | 5 | 2.77 ± 3.24 | 9 | 1.13 ± 0.89 | 6 | 5.69 ± 4.39 | 3 | 2.8 ± 4.1 | 6 | 0.56 ± 0.97 |
| β-Pinene* | X | 977 | 24 | 0 | | 2 | 1.21 ± 2.14 | 6 | 0.14 ± 0.18 | 4 | 2.63 ± 3.53 | 5 | 3.13 ± 1.18 | 7 | 0.7 ± 0.62 |
| p-Cymene | X | 1026 | 19 | 0 | | 0 | | 9 | 0.37 ± 0.25 | 2 | 0.35 ± 0.66 | 4 | 2.38 ± 1.86 | 4 | 0.92 ± 1.99 |
| 1,8-Cineole* | X | 1030 | 18 | 0 | | 0 | | 8 | 0.49 ± 0.56 | 4 | 2.55 ± 3.09 | 3 | 3.17 ± 5.06 | 3 | 0.55 ± 1.20 |
| Limonene | X | 1031 | 31 | 5 | 2.83 ± 0.02 | 0 | | 11 | 6.04 ± 4.01 | 5 | 3.18 ± 3.67 | 5 | 11.57 ± 9.77 | 5 | 0.48 ± 0.48 |
| *cis*-β-Ocimene* | X | 1036 | 31 | 1 | 0.71 ± 0.03 | 5 | 7.21 ± 3.98 | 11 | 5.99 ± 1.74 | 2 | 0.33 ± 0.53 | 5 | 1.12 ± 1.1 | 7 | 1.18 ± 1.18 |
| *trans*-β-Ocimene* | X | 1047 | 39 | 6 | 34.59 ± 0.04 | 5 | 72.54 ± 15.5 | 11 | 59.24 ± 11.6 | 5 | 3.33 ± 2.9 | 5 | 19.36 ± 14.9 | 7 | 31.57 ± 24.44 |
| *cis*-Linalool oxide furan | X | 1074 | 13 | 2 | 0.08 ± 0.05 | 0 | | 4 | 0.12 ± 0.19 | 1 | 0.18 ± 0.44 | 1 | 0.03 ± 0.07 | 5 | 1.18 ± 1.11 |
| Linalool | X | 1099 | 37 | 4 | 1.66 ± 0.06 | 5 | 7.13 ± 4.89 | 10 | 3.96 ± 3.51 | 6 | 13.33 ± 10.3 | 5 | 1.27 ± 1.16 | 7 | 4.22 ± 5.40 |
| Perillene | | 1103 | 23 | 4 | 0.64 ± 0.69 | 2 | 1.2 ± 2.41 | 5 | 0.25 ± 0.34 | 5 | 2.48 ± 2.93 | 3 | 0.45 ± 0.49 | 4 | 0.12 ± 0.20 |
| Unknown a | | 1114 | 9 | 0 | | 0 | | 2 | 0.04 ± 0.09 | 0 | | 0 | | 7 | 3.51 ± 3.89 |
| Sum | | | | | 43.6 | | 92.7 | | 80.5 | | 36 | | 50.69 | | 47.52 |
| *Sesquiterpenes* | | | | | | | | | | | | | | | |
| α-Ylangene | | 1375 | 15 | 0 | | 0 | | 4 | 0.36 ± 0.67 | 0 | | 4 | 0.17 ± 0.12 | 7 | 3.64 ± 4.64 |
| α-Copaene* | X | 1377 | 30 | 5 | 2.94 ± 3.87 | 0 | | 11 | 4.07 ± 3.26 | 2 | 0.68 ± 1.23 | 5 | 6.94 ± 2.91 | 7 | 10.31 ± 7.28 |
| Daucene | | 1381 | 10 | 0 | | 0 | | 0 | | 2 | 0.93 ± 1.53 | 1 | 0.30 ± 0.68 | 7 | 1.13 ± 1.07 |
| β-Elemene | | 1392 | 19 | 0 | | 0 | | 6 | 0.16 ± 0.37 | 4 | 2.28 ± 2.14 | 3 | 0.39 ± 0.37 | 6 | 0.65 ± 0.49 |
| Longifolene | | 1407 | 7 | 2 | 0.54 ± 1.05 | 0 | | 0 | | 0 | | 3 | 2.53 ± 2.38 | 2 | 0.20 ± 0.46 |
| *cis*-α-Bergamotene | | 1414 | 14 | 0 | | 3 | 0.18 ± 0.20 | 4 | 0.08 ± 0.13 | 3 | 1.35 ± 1.64 | 4 | 1.43 ± 1.57 | 0 | |
| β-Caryophyllene* | X | 1421 | 24 | 2 | 2.20 ± 4.17 | 0 | | 4 | 0.63 ± 1.29 | 6 | 4.79 ± 3.90 | 5 | 4.05 ± 1.83 | 7 | 7.50 ± 4.18 |
| *trans*-α-Bergamotene* | | 1440 | 26 | 5 | 2.98 ± 5.02 | 0 | | 5 | 0.26 ± 0.50 | 4 | 3.02 ± 3.03 | 5 | 5.77 ± 6.82 | 7 | 3.71 ± 2.50 |
| Zizaene | | 1453 | 7 | 3 | 0.79 ± 1.53 | 4 | 1.94 ± 1.66 | 0 | | 0 | | 0 | | 0 | |
| *trans*-β-Farnesene* | X | 1458 | 15 | 4 | 0.44 ± 0.55 | 0 | | 0 | | 6 | 16.12 ± 8.49 | 0 | | 5 | 0.38 ± 0.50 |
| α-Humulene* | X | 1462 | 20 | 1 | 0.07 ± 0.17 | 0 | | 3 | 0.10 ± 0.20 | 4 | 1.81 ± 1.88 | 5 | 1.42 ± 0.93 | 7 | 2.57 ± 1.83 |
| Selina-4,11-diene | | 1471 | 13 | 0 | | 0 | | 0 | | 2 | 0.59 ± 1.04 | 4 | 3.45 ± 4.60 | 7 | 2.19 ± 1.77 |
| γ-Muurolene | | 1478 | 15 | 0 | | 0 | | 0 | | 3 | 0.63 ± 0.73 | 5 | 1.51 ± 1.15 | 7 | 2.55 ± 2.45 |
| Germacrene D | X | 1484 | 22 | 4 | 1.03 ± 1.62 | 0 | | 5 | 0.51 ± 1.02 | 2 | 0.38 ± 0.60 | 4 | 0.59 ± 0.55 | 7 | 3.49 ± 4.77 |
| β-Selinene | | 1492 | 9 | 0 | | 0 | | 0 | | 2 | 1.51 ± 3.40 | 0 | | 7 | 0.48 ± 0.30 |
| α-Muurolene | | 1497 | 8 | 0 | | 0 | | 0 | | 0 | | 1 | 0.39 ± 0.88 | 7 | 1.53 ± 1.95 |
| Bicyclogermacrene | | 1499 | 20 | 0 | | 0 | | 8 | 4.86 ± 4.70 | 2 | 0.37 ± 0.57 | 3 | 0.75 ± 0.87 | 7 | 0.98 ± 0.81 |
| (*cis*,*cis*)-α-Farnesene | X | 1506 | 20 | 4 | 9.89 ± 18.99 | 3 | 0.81 ± 0.93 | 5 | 1.09 ± 1.82 | 6 | 2.67 ± 0.86 | 0 | | 2 | 0.17 ± 0.44 |
| Germacrene A | | 1509 | 21 | 0 | | 0 | | 10 | 1.29 ± 1.58 | 5 | 4.02 ± 3.14 | 1 | 0.11 ± 0.24 | 5 | 0.41 ± 0.55 |
| 7-Epi-α-cedrene | | 1516 | 11 | 0 | | 0 | | 0 | | 1 | 1.06 ± 2.59 | 5 | | 5 | 1.11 ± 1.76 |
| β-Sesquiphellandrene | | 1518 | 7 | 0 | | 0 | | 0 | | 6 | 3.11 ± 1.36 | 1 | 0.12 ± 0.28 | 0 | |
| δ-Cadinene* | | 1521 | 24 | 4 | 0.82 ± 1.13 | 0 | | 6 | 0.51 ± 0.67 | 2 | 0.26 ± 0.43 | 5 | 2.41 ± 1.33 | 7 | 2.63 ± 1.68 |
| Unknown b | | 1434 | 4 | 0 | | 0 | | 0 | | 0 | | 4 | 0.52 ± 0.31 | 0 | |
| Sum | | | | | 21.69 | | 2.94 | | 13.92 | | 45.57 | | 32.86 | | 45.6 |
| *Fatty acid derivatives* | | | | | | | | | | | | | | | |
| *cis*-3-Hexenyl acetate | X | 1006 | 14 | 4 | 14.99 ± 17.09 | 0 | | 5 | 0.55 ± 0.79 | 3 | 1.75 ± 2.62 | 2 | 0.85 ± 1.35 | 0 | |
| Tridecane | X | 1300 | 9 | 0 | | 3 | 0.39 ± 0.37 | 0 | | 3 | 1.47 ± 2.28 | 0 | | 3 | 0.10 ± 0.18 |
| Tetradecane | X | 1400 | 11 | 0 | | 0 | | 0 | | 3 | 1.01 ± 1.71 | 0 | | 8 | 0.65 ± 0.61 |
| Sum | | | | | 14.99 | | 0.39 | | 0.55 | | 4.23 | | 0.85 | | 0.75 |
| *Shikimic compounds* | | | | | | | | | | | | | | | |
| Benzylnitrile | | 1142 | 5 | 0 | | 5 | 1.08 ± 0.63 | 0 | | 0 | | 0 | | 0 | |
| Methyl salicylate | X | 1195 | 10 | 4 | 2.21 ± 2.01 | 3 | 0.48 ± 0.62 | 3 | 0.11 ± 0.20 | 0 | | 0 | | 0 | |
| Indole* | X | 1295 | 14 | 0 | | 0 | | 0 | | 3 | 1.65 ± 3.16 | 5 | 6.13 ± 6.60 | 6 | 2.73 ± 5.98 |
| Sum | | | | | 2.21 | | 1.57 | | 0.11 | | 1.65 | | 6.13 | | 2.73 |

RI = Retention Index; Occ = occurrence of each VOC, total and by population; *N* = number of samples; S_inj = VOCs for which identification has been confirmed by standards injection.
* VOCs with significantly different relative proportions between the two species.

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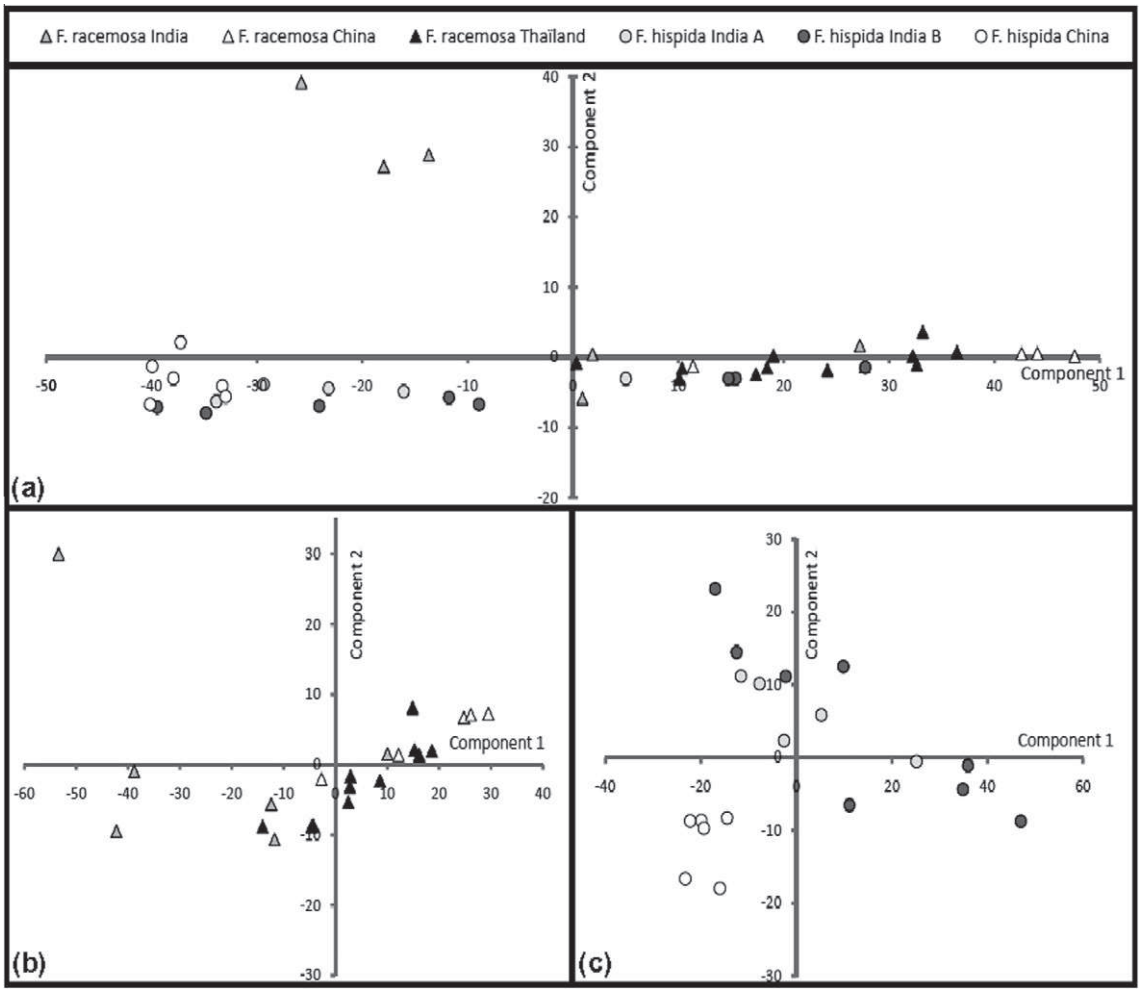


**Fig. 1.** PCA analysis on relative abundance of VOCs produced by receptive figs of: (a) *Ficus racemosa* and *F. hispida* together (first component explains 68% and second component 28% of the total variation observed). (b) *Ficus racemosa* from three populations (first component explains 68%, and second component 11% of the total variation observed). (c) *Ficus hispida* from three populations (first component explains 55% and second component 15% of the total variation observed).

**Table 2**
Relative abundances of VOCs from the bouquet of *Ficus racemosa* receptive figs for which interpopulation variation was significant (LS means comparisons).

| | trans-β-Ocimene | cis-3-Hexenyl acetate | cis-β-Ocimene | Bicyclogermacrene |
|---|---|---|---|---|
| China | 72.54 ± 15.51 (a) | 0.00 ± 0.00 (a) | 7.21 ± 3.98 (a) | 0.00 ± 0.00 (a) |
| Thailand | 59.24 ± 11.57 (a) | 0.55 ± 0.79 (a) | 5.99 ± 1.74 (a) | 4.86 ± 4.70 (b) |
| India | 34.59 ± 18.40 (b) | 14.99 ± 17.09 (b) | 0.71 ± 1.74 (b) | 0.00 ± 0.00 (a) |

Different letters in parantheses within a column indicate significant differences between populations at $p < 0.05$.

**Table 3**
Relative abundances of VOCs from the bouquet of *Ficus hispida* receptive figs for which interpopulation variation was significant (LS means comparisons).

| | trans-β-Farnesene | β-Sesquiphellandrene | δ-Cadinene | Germacrene A |
|---|---|---|---|---|
| China | 16.12 ± 8.49 (a) | 3.11 ± 1.36 (a) | 0.26 ± 0.43 (a) | 4.02 ± 3.14 (a) |
| India A | 0.00 ± 0.00 (b) | 0.12 ± 0.28 (b) | 2.41 ± 1.33 (b) | 0.11 ± 0.24 (b) |
| India B | 0.38 ± 0.5 (b) | 0.00 ± 0.00 (b) | 2.63 ± 1.68 (b) | 0.41 ± 0.55 (b) |
| | α-Pinene | γ-Terpinene | Limonene | |
| China | 1.99 ± 2.11 (a) | 0.00 ± 0.00 (a) | 3.18 ± 3.67 (a) | |
| India A | 5.41 ± 2.52 (b) | 4.60 ± 3.85 (b) | 11.57 ± 9.77 (b) | |
| India B | 2.52 ± 1.28 (a) | 0.18 ± 0.36 (a) | 0.48 ± 0.48 (a) | |

Different letters in parantheses within a column indicate significant differences between populations at $p < 0.05$.

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chemical classes are the major compounds in scents of most *Ficus* species (Gibernau et al., 1998; Grison-Pigé et al., 2001; Proffit et al., 2008), even if some systems are mediated by benzenoids (Chen et al., 2009). In Fig. 2, which illustrates variation of different classes of volatile compounds among populations of each species, some interpopulation differences appear clearly. The most striking is the significant variation of mean proportions of monoterpenes among *F. racemosa* populations. Indeed, for differences between mean proportions of monoterpenes in Indian and Chinese populations, Indian and Thai populations and Chinese and Thai populations, the results of Wilcoxon tests were respectively ($S = 45.00$; $p = 0.0043$), ($S = 27.00$; $p = 0.0048$) and ($S = 66.00$; $p = 0.0055$).

To study interpopulation variation in greater detail, the two *Ficus* species were then examined independently. The PCA performed on relative abundance of VOCs emitted only by *F. racemosa* (Fig. 1b) did not show clear differentiation among populations. Nevertheless, the scents produced by individuals from southern India were relatively distinct compared to those produced by the two other populations (northern Thailand and southern China). A MANOVA performed on the same *F. racemosa* VOCs dataset (but including only those with mean proportion >1%) showed that the population effect was significant (Wilks' Lambda: $F_{(18;22)} = 3.95$; $p = 0.0014$). Four VOCs contributed to this interpopulation difference: bicyclogermacrene, for which relative proportions were significantly different in bouquets of the Thai population compared to those of both Chinese and Indian populations, and *trans*-β-ocimene, *cis*-β-ocimene and *cis*-3-hexenyl acetate, for all of which relative proportions were significantly different between scents of Indian figs and those of both Thai and Chinese populations (see Table 2). The last of these compounds was totally absent, or present in very low quantity, in bouquets from Thai and Chinese populations but present in scents of most *F. racemosa* individuals from Indian populations. This is quite surprising since this compound, a typical green leaf volatile, is known in other plants to be induced by damage from herbivory (Pichersky and Gershenzon, 2002), and to act as a repellent against herbivores or as a parasitoid attractant. For *F. racemosa*, the significant differences between scents of receptive figs from Indian populations and those of figs from populations in the two other Asian regions sampled also resulted from the variation of *trans*-β-ocimene. This volatile compound is present in lower proportion in the bouquets from the Indian population than in bouquets from populations of the two other regions. Interestingly, quantities of the isomer *cis*-β-ocimene varied in a similar manner. Consequently, selection on one of these compounds as part of the signal also acts on the presence (and quantity) of the second one. The much higher proportion of *trans*-β-ocimene in the bouquets might mean that selection is acting principally on this compound, or alternatively might simply reflect the chemical instability of *cis*-β-ocimene. In fact, most documented cases suggest a 10:1 ratio or more of *trans*:*cis* isomers in floral scents (Arimura et al., 2004; Flamini et al., 2004). Bicyclogermacrene is the only VOC showing significant differences in proportion between the fig bouquets from the Thai population and those of populations from the two other Asian regions. Despite this last difference, globally, scents of receptive figs from Chinese and Thai populations of *F. racemosa* were not significantly different (Wilks' Lambda: $F_{(9;6)} = 3.49$; $p = 0.0709$).

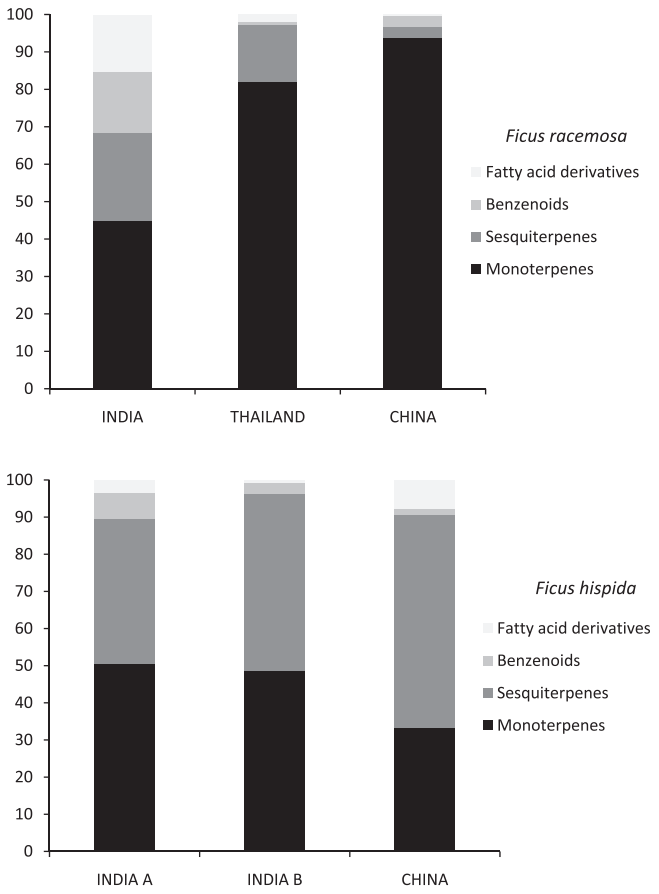


**Fig. 2.** Mean relative abundances by species and populations of chemical classes of volatile compounds emitted by *Ficus racemosa* and *F. hispida*.

In the case of the second species, *F. hispida*, the PCA performed on the relative contribution of the different compounds in scents emitted by receptive figs (Fig. 1c) shows that the VOCs emitted by figs from both Indian populations were relatively similar, and could be separated from the VOCS emitted by figs of the population from South China. A MANOVA performed on the same VOCs dataset (but including only compounds with mean proportion >1%) showed that the population effect was significant (Wilks' Lambda: $F_{(22;12)} = 11.72$; $p < 0.0001$). This result is explained by the significantly different proportions of four compounds [*trans*-β-farnesene, β-sesquiphellandrene, δ-cadinene and germacrene A] between scents of Chinese *F. hispida* and those of both Indian populations (see Table 3). Surprisingly, three compounds (α-pinene, γ-terpinene and limonene) showed significantly different proportions between fig scents of one Indian population (India A, Agumbe) and those of both Indian B (Kudremukh) and Chinese populations, although scents from the Indian A population are graphically not distinct from those of the Indian B population (Fig. 1c). An interesting point here is the contribution of *trans*-β-farnesene to interpopulation differences. This compound is one of the major compounds of the bouquet (about 25% for some individuals), although at the same time, quantities of *cis*-β-farnesene and (*trans*,*trans*)-α-farnesene are very low. The biosynthesis of these three compounds is linked (Schnee et al., 2002), and we can thus hypothesize that the presence of *trans*-β-farnesene may be highly selected, or, as explained above for *cis*-β-ocimene, the quantity of *trans*-β-farnesene might simply reflect the chemical instability of *cis*-β-farnesene. Nevertheless, the presence of both δ-cadinene and germacrene A suggests that two different pathways (corresponding to two types of initial cyclization reactions of farnesyl cation) are engaged here for the biosynthesis of *trans*-β-farnesene (Degenhardt et al., 2009), further supporting the hypothesis that its presence may be highly selected.

### 2.3. Geographical variation in scents of receptive figs: incipient allopatric speciation?

These results show that even in an obligate specific mutualism, where the emitted scent is species-specific, interpopulation variation can occur at large geographic scales. The mutualistic pollinator

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should be able to adapt to such variation in the species-specific scent. Consequently, divergence in scent and in pollinator response to it could lead to allopatric speciation. Indeed, recent studies on spatial genetic structure of *F. racemosa* (using microsatellite markers), are consistent with such a scenario. Indian populations are genetically distinct from populations of the rest of continental Asia, whereas within India and within continental Asia, genetic differentiation was very limited (Alvarez et al., 2010). For the wasp pollinator of this species, morphological observations suggest that Indian wasps are somewhat different from all the others, in contrast to wasps from the rest of continental Asia which form one genetic entity (Kobmoo et al., 2010). Fewer genetic data are available for dioecious *Ficus*. However, recent results showed strong isolation by distance in several dioecious fig species, including *F. hispida* (Dev et al., in press), as well as the existence of different species or subspecies of the pollinating wasp of this species in India and East Asia (A. Cruaud, unpublished data). Over the wide range of our study, the Eastern Ghats, for example, might have acted as a physical barrier that initiated a breakdown in gene flow, and consequently a process of incipient allopatric speciation (Rajagopalan et al., 1997). In our case, for both studied species, the major differentiation we observed is between scents in Indian fig populations and those elsewhere in Asia. The results of genetic and phylogeographic studies suggest that geographic barriers (present or past) may have led as well to the observed discontinuities in scent variation.

Interestingly, our results are very different from those found in another nursery pollination mutualism where interpopulation variation of scent has been investigated, the *Yucca*/yucca moth interaction (Svensson et al., 2005). These authors demonstrated that the scents were similar among populations even when pollinators were different. In another study on scent variation in a nursery pollination system, the case of *Trollius* and its pollinating flies, Ibanez et al. (2010) also found some geographical variation of scent, but with large overlap in scent samples among the populations. However, in both of these studies, the geographic distances covered are much lower than in our fig study. Moreover, specificity in these other nursery pollination systems is less strict than in fig/fig wasp interactions. Consequently, in both of these studies, pressure for scent constancy and probability of occurrence of geographical barriers are both lower than in our case.

Alternatively, geographic variation in scent could concern only compounds not used by the pollinator to localize its host, as has been shown in *Silene latifolia* and *Salix* species (Dötterl et al., 2005a,b, 2006; Füssel et al., 2007). While genetic data thus provide strong arguments in favor of incipient speciation in our fig/fig wasp example, further experiments are necessary to draw conclusions. GC-EAD tests and coupled behavioral experiments with fig wasps are necessary to determine whether global fig scent and compounds used as cues by the wasps show a similar pattern of interpopulation variation.

## 3. Concluding remarks

Our work shows that even in a highly specific obligate mutualism, where the odour emitted by the plant is necessary and preponderant in its specific partner attraction, the scent can be constant over large geographical scales, but may vary if geographical barriers occur. Indeed, these latter may disrupt gene flow, which could lead to an incipient speciation process. However, geographic variation in scent within each of the species studied here remains less than that between species, so that in each population, specificity with respect to other sympatric fig/pollinator pairs is preserved. Coupled physiological and behavioral experiments would enable determining whether the VOCs principally contributing to geographical differentiation are compounds that actually contribute to the olfactory signal that pollinators detect and find attractive, or whether such variation is possible due to plasticity in the olfactory response of the pollinator.

## 4. Experimental

### 4.1. Plant material

We studied the scents of two broadly sympatric Asian *Ficus* species: *F. hispida* Linnaeus (section Sycocarpus) and *F. racemosa* Linnaeus (section Sycomorus). *F. racemosa* is pollinated by *Ceratosolen fusciceps* Mayr (Chalcidoidea, Agaonidae), and *F. hispida* by *Ceratosolen solmsi marchali* Mayr (Chalcidoidea, Agaonidae), (http://www.figweb.org). *F. hispida*, a (gyno)dioecious species, is a short free-standing fig usually found along streams. *F. racemosa*, a monoecious species, is a large tree up to 30 m high that grows mainly in moist valleys or along rivers. All individuals used were wild, i.e. non-planted trees. For *F. racemosa*, Indian samples came from the campus of the Indian Institute of Science (12°58′ N, 77°35′ E), Bangalore, Karnataka State, and Thailand samples from the Khao Yai region (14°24′ N, 101°22′ E). Both species were sampled from wild populations in Xishuangbanna Tropical Botanical Garden (XTBG) in Yunnan, China (21°55′ N, 101°15′ E). For Indian samples of *F. hispida*, half were collected in Agumbe, Shimoga District (13°30′ N, 75°5′ E), and the other half in Kudremukh, Chikmagalur District of Karnataka state in the Western Ghats of India (13°15′ N, 75°7′ E). These two Indian sites are separated by 75 km. In each study site, the availability of trees with figs at the right stage was often quite low due to variable phenology and low density of fig trees. This sometimes limited the number of trees we could sample. We were able to sample the scents from 22 individuals of *F. racemosa* (6 from India, 5 from China and 11 from Thaïland) and 19 individuals (males only) of *F. hispida* (6 from India A (Agumbe), 7 from India B (Kudremukh) and 6 from China).

### 4.2. Collection of volatiles

To collect the scent of receptive figs, pre-receptive figs were isolated from pollinators using mesh bags. Fig floral stages were estimated by their physical characteristics. For example, an open ostiole and attraction of pollinators to the outside of the bag are both signals showing that a fig is receptive.

The scent of receptive figs of both *F. hispida* and *F. racemosa* was collected using the same dynamic headspace technique (Proffit et al., 2009; Raguso and Pellmyr, 1998). Sampling was conducted for 3 h, from 12:00 to 15:00, during the peak of odour emission. Receptive figs were enclosed in a polyethylene terephtalate (Nalophan®) bag. A glass cartridge (1/4″ OD Borosilicate tubing) filled with 30 mg of Porapak® Q (or Alltech super Q) adsorbent was connected to the bag. During odour collection, air was passed through the filter at a rate of 300 ml/min. Air purified with charcoal cartridges was also blown into the bag (entrance flux: 400 ml/min). Controls with empty bags were also performed. After collection, the filter was eluted with 150 µl of dichloromethane and the extracts were stored at −18 °C.

### 4.3. Chemical and data analysis

The extracts were analyzed in a CP-3800 (Varian Inc., Palo Alto, CA) gas chromatograph with an FID detector coupled with a Saturn 2000 mass spectrometer (Varian). For each of both gas chromatography (GC) and mass spectrometry (MS), a CP-SIL 8CB low bleed MS Varian column (30 m, ID 0.25 mm, film thickness 0.25 µm), helium carrier gas (at 1 ml $min^{-1}$), and injection in a split mode at 1:4 ratio were used. The temperature of the two injectors was 200 °C.

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The oven temperature programme for the analysis was: injection at 50 °C and maintained at that temperature for 3 min, then ramped by 3 °C $min^{-1}$ to 100 °C, by 2.7 °C $min^{-1}$ to 140 °C, by 2.4 °C $min^{-1}$ to 180 °C and by 6 °C $min^{-1}$ to 250 °C. The temperatures of the different parts of the mass spectrometer (transferliner, manifold and trap) were 250, 80 and 170 °C. These analyses were performed at the "Plate-Forme d'Analyses Chimiques en Ecologie" (platform for chemical analyses in ecology) of "IFR 119 Montpellier Environnement Biodiversité".

Compound identification was based on matching of the mass spectra with NIST 98 MS and Adams (2007) and on confirmation by comparison of RI with libraries and published data (Adams, 2007). Identification of some compounds was confirmed by comparison of both mass spectrum and GC retention indices with those of standards (see Table 1). We used the MS data to identify the VOCs, and the FID data for their quantification.

For the statistical analysis of the chemical blend, we ran Principal Component Analysis (PCA, covariance matrix, Stat Box v6.3) to compare patterns of scent composition of receptive figs between the two species and among populations. For these multivariate analyses, we used the relative amounts (percentages) of the compounds which represented at least 1% of the total compound emission. This restriction is appropriate here, because in mutualisms such as fig/fig wasp interactions that have undergone long periods of coevolution, selection pressures should have led to high levels of signal compounds to facilitate encounter as suggested by Hossaert-McKey et al. (2010). To confirm or invalidate the results of the PCAs, we then performed on the same dataset a multivariate analysis of variance followed by a multiple comparison of means (SAS v9: manova, proc glm, and LSMEANS with Tukey–Kramer multiple comparison tests; predictor "population within species"). The MANOVA also included univariate (i.e. sequential) analyses on each dependent variable (relative amount of each VOC) to test which compounds contributed to the overall significance of the statistical analysis (Stevens, 1992). We also analyzed the variation among populations of mean proportions of compound classes, comparing populations two by two, by using the nonparametric Wilcoxon test.

For this study, we worked on the relative amounts of VOCs only. As previously reported (Proffit et al., 2008), even a single species in a single site may emit a different global amount at different periods of the year. Variable abiotic constraints among geographical scales, and at different periods of the year, mean that comparisons between global amounts of VOCs produced by figs in different sites and at different seasons would not have been relevant for this study.

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# CHAPITRE 2 :
# Travaux Expérimentaux

## PARTIE B :

## *PARFUM DE FIGUES :*

## MÉDIATION CHIMIQUE & POLLINISATION

**Articles associés :**

Manuscrit 5 : **Soler C.**, Bard Y., Bessière J.-M., Hossaert-McKey M. and Schatz B. Scent and hybridization: the case of the highly specific figs / fig wasps interactions**.** *In prep.*

Manuscrit 6 : Hossaert-McKey M., **Soler C.**, Proffit M., Chen C., Bessiere J.-M., Schatz B. and Borges R. How to be a dioecious fig ? Case of chemical mimicry and pollination by deceit in a nursery pollination mutualism**.** *In prep.*

Manuscrit 7 : **Soler C.**, Bessière J.-M., Proffit M., Hossaert-McKey M. and Schatz B.. When males change their scents in presence of females, the case of the plant *Ficus carica***.** *In prep.*

Manuscrit 8 : Proffit M., Schatz B., Bessiere J.-M., Chen C., **Soler C.**, Hossaert-McKey M. 2008. Signalling receptivity: Comparison of the emission of volatile compounds by figs of *Ficus hispida* before, during and after the phase of receptivity to pollinators. *Symbiosis* 45: 15-24.

Dans le cas d'une interaction mutualiste spécifique et obligatoire à transmission horizontale, les pressions sur le message / signal sont d'autant plus fortes que la survie des deux partenaires dépend directement du succès de la communication permettant leur rencontre à chaque génération. L'interaction *Ficus* / Agaonidés, notre modèle d'étude, est une interaction interspécifique où des pressions évolutives très fortes pour maintenir l'association ont conduit à la coévolution des espèces du genre *Ficus* (Moraceae) et des agaonides pollinisateurs (Hymenoptera, Chalcidoidae). Cette coévolution, associée à une cospéciation, a été démontrée pour plusieurs traits chez les deux partenaires (Jousselin *et al.*, 2003), comme par exemple : les poches à pollen et peignes coxaux chez les pollinisateurs de figuiers permettant une pollinisation active, la forme des stigmates (Jousselin & Kjellberg, 2001), la forme de l'ostiole et la forme de la tête du pollinisateur (Verkerke, 1987). Le cycle phénologique d'un figuier est caractérisé par cinq phases successives (détaillées en introduction), où les phases de réceptivité et de dispersion sont des phases très sensibles du cycle, puisqu'elles correspondent aux deux étapes de la dispersion des gènes du figuier. Le succès de ces deux phases dépend particulièrement du succès de la communication entre le figuier et ses partenaires mutualistes. Ainsi, les Agaonidés assurent la pollinisation, car ils sont capables de détecter et de s'orienter vers des figues réceptives, grâce notamment à la reconnaissance des signaux olfactifs. De nombreuses études démontrent que le pollinisateur est attiré par les bouquets de COVs émis par le figuier, bouquets qui sont spécifiques de l'espèce (Vannoort *et al.*, 1989; Ware *et al.*, 1993; Hossaert-McKey *et al.*, 1994; Gibernau *et al.*, 1998; Grison *et al.*, 1999; Grison-Pigé *et al.*, 2002; Chen & Song, 2008; Proffit & Johnson, 2009). L'interaction figuier/pollinisateur est, parmi les interactions figuier / partenaire (mutualiste ou non), celle pour laquelle la médiation chimique a été la plus étudiée (Hossaert-McKey et al., 2010). Ainsi, le rôle prépondérant, voire même indispensable des bouquets volatils pour la rencontre entre ces deux partenaires a pu être démontré. Cependant, de part sa forte spécificité, sa

diversité, et son ancienneté, cette interaction laisse encore de nombreuses questions sans réponses. Nous avons vu dans le chapitre précédent que des contraintes existant sur l'émission du bouquet volatil, a priori importantes, n'avaient pas été identifiées comme fortes, et que la « nécessité de spécificité » du bouquet représentait une pression de sélection majeure sur ce dernier.

La variation interspécifique du bouquet est donc un élément important dans le maintien de la spécificité de l'interaction figuier / pollinisateur, et peut par conséquent constituer une barrière pré-zygotique s'opposant au changement d'hôte (Proffit *et al.*, 2009), et donc à l'hybridation. Cependant, même s'ils sont très rares, quelques cas d'hybridation entre espèces de *Ficus* existent en conditions naturelles particulières (Bronstein & McKey, 1989; Parrish *et al.*, 2003). Nous avons alors cherché à comprendre précisément le rôle que pouvait jouer les bouquets volatils émis à réceptivité dans un cas exceptionnel d'hybridation contrôlée, décrit dans l'article n°5. Dans cette étude, nous avons analysé, quantitativement et qualitativement, les bouquets d'odeurs émis à réceptivité par deux espèces de figuiers, naturellement allopatriques mais réunis en serres, ainsi que par leurs hybrides et par des individus issus de rétrocroisements (back-cross). Nous avons ainsi pu mettre en évidence que la composition chimique du bouquet de COVs émis par les hybrides et les rétrocroisements est une combinaison de celle des deux parents, sans qu'elle ne se rapproche clairement plus de celle de l'un ou de l'autre. Dans cette étude, l'odeur apparait comme un élément de filtre floral, qui empêcherait l'hybridation uniquement en complément d'autres éléments, pré- ou post-zygotiques.

La très forte spécificité d'interaction entre figuiers et pollinisateurs impose une différenciation interspécifique forte sur le bouquet volatil émis par les figues réceptives. Comme suggéré dans le chapitre précédent, dans l'article n°3, même s'il existe de fortes pressions pour la stabilité intraspécifique du message, les analyses des bouquets volatils de différents espèces

montrent une forte variabilité interindividuelle des bouquets de COVs (Hossaert-McKey *et al.*, 2010). Certaines variations sont possibles, sans risques pour le maintien du mutualisme, tant qu'elles restent moins fortes que la variation interspécifique. Ce problème des variations intraspécifiques potentielles soulève la question de la variation intersexuelle du bouquet émis à réceptivité. Comme expliqué en introduction, près de la moitié des espèces de figuiers sont des espèces (gyno)dioïques, c'est-à-dire des espèces à sexes séparés. Dans ce cas, les figuiers mâles assurent la survie des populations d'hyménoptères, alors que les individus femelles assurent la survie des figuiers. En effet, pour des raisons morphologiques, les agaonides ne peuvent pondre dans les ovaires de ces figues femelles. Ils peuvent toutefois rentrer à l'intérieur de ces figues et les polliniser : il s'agit donc d'une pollinisation par tromperie. Une question se pose alors : pourquoi les pollinisateurs continuent-ils à visiter les figues femelles, alors qu'ils n'en retirent aucun bénéfice? Grafen et Godfray (1991) ont émis l'hypothèse d'un mimétisme chimique, obtenu par sélection vicariante : un message identique émis par les figues des deux sexes rend toute discrimination impossible par le pollinisateur. Tester cette hypothèse a été l'objet de l'article n°6. Nous avons analysé l'odeur émise à réceptivité par des figuiers mâles et femelles appartenant à sept espèces dioïques différentes, et vérifié ainsi l'hypothèse de mimétisme chimique. En effet, si une variation intersexuelle existe, elle se confond avec la variation interindividuelle, et reste donc inférieure à la variation interspécifique : le message émis est spécifique de l'espèce, mais pas du sexe de l'individu. La sélection vicariante, codant pour le mimétisme chimique inter-sexes, est donc une explication forte du non-évitement des figuiers femelles par les pollinisateurs (Grafen & Godfray, 1991). Cependant, elle n'est pas la seule. Patel et al. (1995) ont ainsi émis trois autres hypothèses, non exclusives. Premièrement, du fait de leur courte durée de vie (seulement quelques heures), les pollinisateurs sont soumis à la « selection to rush » : dans le paysage olfactif complexe des milieux tropicaux, ils n'ont en pratique « pas le temps » de détecter une possible variation intersexuelle. La deuxième

hypothèse proposée est que les pollinisateurs auraient, pour certaines espèces, appris à distinguer les mâles des femelles, et ainsi à éviter ces dernières. Mais dans un tel système, la reproduction des figuiers n'existe plus, puisqu'il n'y a plus de production de graines. Le système serait alors évolutivement instable, et l'interaction n'aurait pas survécu au cours des temps. La dernière hypothèse proposée est celle du non-choix : dans les milieux plus saisonniers, les phénologies des arbres mâles et femelles sont asynchrones, et donc le pollinisateur ne se retrouve jamais face à un choix ! Dans une première étude, cette dernière hypothèse a été testée et confirmée par Grison-Pigé et al. (2001a) chez *F. carica*, le figuier méditerranéen. Il reste donc à démontrer si les résultats obtenus sur le figuier méditerranéen sont généralisables ou non à d'autres figuiers, notamment car *F. carica* représente un cas à part et plus complexe que les autres *Ficus*…

Notre article n°7 avait, lui, pour objectif de tester l'hypothèse de mimétisme chimique pour l'exemple particulier de *F. carica*, où le pollinisateur se retrouve parfois en situation de choix, et parfois non (Anstett *et al.*, 1997). Présent dans un milieu saisonnier (le bassin méditerranéen), *F. carica* présente une phénologie dite saisonnière. Les arbres femelles portent des figues réceptives seulement une fois par an, en début - milieu d'été. Les arbres mâles portent, eux, des figues réceptives deux fois par an. La première production a lieu au printemps, et les figues sont appelés *profichi*, alors que la seconde a lieu en milieu - fin d'été. Ces figues, appelés *mammes*, permettent de « boucler le cycle », et, contrairement aux *profichi,* apparaissent donc partiellement simultanément aux figues femelles (Kjellberg *et al.*, 1987). Dans la mesure où les *Ficus* ont évolué en milieu tropical, nous pouvons considérer que la situation « classique » est celle de la présence en synchronie des figues réceptives mâles et femelles, et donc de l'existence d'une pression pour le mimétisme chimique. En d'autres termes, les *profichi* sont une particularité de *F. carica*. Nous avons pu mettre en évidence qu'il y a bien mimétisme chimique entre les bouquets émis à réceptivité par les *mammes* et les

femelles, et que la pression de sélection pour le mimétisme étant relâchée lors de la réceptivité des *profichi*, ces derniers émettent un bouquet différent et simplifié. Nous confirmons les résultats obtenus par Grison-Pigé et al (2001a), et nous les complétons puisque cette première étude ne s'était pas intéressée au cas des *mammes*. Nous avons donc mis en évidence qu'un même individu, pour attirer le même partenaire, dont il est totalement dépendant pour sa reproduction, peut émettre un message différent en fonction des pressions biotiques environnantes.

La possibilité qu'un pollinisateur puisse être exposé à un « choix », entre plusieurs espèces et plusieurs sexes de figuiers, représente donc une pression forte sur l'évolution du message émis par le figuier à réceptivité. Ce point suggère que le message émis par le figuier à réceptivité doit être spécifique de ce stade, et qu'il doit donc être différent avant et après réceptivité. C'est pourquoi, dans l'article n°8, nous avons mis en évidence chez *F. hispida* les variations du message émis par les mêmes figues, avant, pendant, et après réceptivité ; les figues n'étant pas attractives en dehors de la période de réceptivité. Ces changements selon le stade constituent une nouvelle pression sur le message émis à réceptivité, correspondant à ces changements pré- et post-réceptivité. De même, nous avons mis en évidence que la quantité totale de COVs émis est plus importante à réceptivité qu'avant ou après. Les *Ficus* étant des arbres à densité généralement assez faible, le pollinisateur peut être amené à parcourir de longues distances (Zavodna *et al.*, 2005) : il a ainsi été démontré que le pollinisateur de *F. sycomorus* pouvait parcourir plus de 160 km (Ahmed *et al.*, 2009) ! L'augmentation de la quantité totale d'odeur émise à réceptivité a ainsi pu être sélectionnée, renforçant la possibilité de détection du message. De surcroît, diminuer cette quantité totale après réceptivité peut minimiser le risque de détection du figuier par des partenaires non mutualistes (telle la large communauté de parasites associés au système figuier/pollinisateur) (annexe1 ; Proffit *et al.*, 2007).

La médiation chimique est donc un élément central de l'interaction figuier/pollinisateur, et le message responsable de la rencontre entre ces deux partenaires est façonné par différentes pressions de sélection d'origine biotique, telles que la présence d'espèces sympatriques de *Ficus*, la présence d'arbres des deux sexes, la nécessité de détection à longue distance, ou encore la présence de parasites/prédateurs. Nous allons aborder maintenant le rôle que peut jouer la médiation chimique dans l'interaction entre les figuiers et d'autres partenaires mutualistes, les disperseurs de graines.

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# □he case o□the h□□hly spec□□c □□s □□□ □asps □nteract□ons

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This paper is still 'in prep'. Our dataset is already complete; however we still have some experiments to perform with the insects (mainly electrophysiology). Moreover, thanks to previous experiments where scents have been extracted by figs washing in solvent, we may be able to distinguish between emitted VOCS and surface compounds, which can be an important element of our discussion. We planned to submit this work to journals as *American Journal of Botany* or *Journal of Chemical Ecology*.

## A□stract

Fig/fig wasp interactions are obligate and specific mutualisms in which each partner depends on the other one for its reproduction. The encounter between the two partners is assured by the production of a specific chemical message by the receptive figs, i.e. the figs ready to be pollinated. This chemical message is used as the signal by the pollinating wasps to find their host species. Using “head-space” sampling and GC-MS analyses, we studied the bouquet of volatile compounds emitted by two dioecious species*,* *Ficus montana* and *F. asperifolia,* as well as those of their hybrids and back-crosses, thanks to culture in greenhouse. We thus highlighted the significant difference between the chemical messages emitted at receptivity by both parent species, as well as between hybrids, back-crosses and the corresponding parent species. To our knowledge, this study is the first focusing on the role of the chemical mediation in hybridization process in the case of a both highly specific and obligatory mutualism. Our study confirms the central role of chemical mediation in the interactions between *Ficus* and their pollinators, and we thus discuss the function of these chemical messages as floral filters to maintain the specificity of such interactions.



## □ey□or□s□

Nursery pollination mutualism, chemical mediation, pollinator specificity, hybridization, pre-zygotic barrier, floral filters, *Ficus*, fig wasps.

## Introduction

Floral odours are important signals for communication between flowering plants and their pollinators (Raguso, 2008b). Floral scents are mostly made up of complex blends of volatile compounds, which often belong to several distinct chemical classes (Knudsen et al., 2006; Raguso, 2008a). The complexity of floral scent in terms of the number, identity, and relative amounts of constituent volatile compounds allows plants to produce a large range of signals which vary among and within species (Knudsen et al., 2006). In some specific plant–pollinator interactions, the emission by the plant of a chemical signal and its specific recognition by the exclusive pollinator is crucial for plant reproduction (Ayasse, 2006; Schatz et al., 2010). In several highly specialized nursery pollination mutualisms, where pollinators reproduced within the inflorescences they pollinate, chemical signals mediate encounter between mutualists and are thus a critical determinant of reproduction of plants and pollinators (reviewed by Hossaert-McKey et al., 2010), notably in the fig / fig wasp interactions (Vannoort, Ware, and Compton, 1989; Ware et al., 1993; Hossaert-McKey, Gibernau, and Frey, 1994; Gibernau et al., 1998; Grison-Pigé, Bessière, and Hossaert-McKey, 2002; Proffit et al., 2009).

All *Ficus* species (roughly 800 species worldwide) are, in general, pollinated by one mutualistic fig wasp species *(Hymenoptera, Chalcidoidea,* Agaonidae) (Cook and Rasplus, 2003). They lay eggs in ovaries of a fraction of the flowers they pollinate, thereby initiating the formation of galls, each corresponding to the development site of one pollinator larva (Galil and Eisikowitch, 1971). Female pollinating wasps transport fig pollen (most often actively) from their natal fig to a receptive one (usually on a different individual tree). Female wasps use chemical signals emitted by receptive figs (VOCs) to locate their host, sometimes over long

distances (Ahmed et al., 2009). Following pollination, the composition of fig bouquet changes (Proffit et al., 2008), and pollinating wasps are no longer attracted to them (Chen and Song, 2008). Knowing that *Ficus* species often coexisted in the same area in tropical habitats, the message emitted by receptive figs must specifically attract the associated pollinator species, especially since pollinating fig wasps live only for a few hours. Volatiles from headspace collection of receptive figs have now been identified for more than 30 species of *Ficus* (Hossaert-McKey et al., 2010; Soler et al., in prep). Several studies have shown that species-specificity of the chemical message emitted by receptive figs and recognition of this message by pollinating fig wasps can promote the specificity of the interaction (Grison, Edwards, and Hossaert-McKey, 1999; Grison-Pigé et al., 2002; Proffit and Johnson, 2009).

However, hybridization and the production of viable hybrids are known to be possible within *Ficus*. Indeed, some artificial interspecific hybrids of *Ficus* (in some cases from taxonomically diverse groups) have been formed and, and in few cases even viable offspring have been produced (Parrish et al., 2003). Natural hybrids in *Ficus*, however, are rare (Corner 1958, Ramirez 1970) and hybridization is considered of little importance in *Ficus* evolution (Ramirez 1986). Nevertheless, recognition of hybrid individuals may be difficult in the field due to the high similarity between parental species. Thus, hybrid events have been recorded more frequently in species outside of their natural range (Parrish et al., 2003). To date, the presence of natural hybrids has been rarely genetically ascertained (Grison, Edwards, and Hossaert-McKey, 1999; Parrish et al., 2003; Proffit and Johnson, 2009), and nothing is known about the role that scents can play in hybridization in *Ficus*.

More than 25% of the current plants species may be issued from hybridization (Mallet, 2005), making it a major mechanism in plant evolution (Waser, 2001;

Hegarty and Hiscock, 2005). Hybridization is of common used in agrosciences in cultivated trees as lemon, or plants as roses or mint (Gancel et al., 2005), but as well occurs *in natura*, and has notably be studied in the case of the European oaks (Schnitzler et al., 2004) or of several species of orchids (Cozzolino and Widmer, 2005). Certain species even seem to be more easily hybridizable in nature, such the rhododendron group (Milne et al., 1999). Interestingly, the possible role of scent as a pre-zygotic barrier against hybridization remains poorly studied, even if several studies raised this question in European orchids (Schatz et al., 2010). In the few existing cases, hybrids generally appear to emit bouquets of VOCs intermediate to those of their parent, and new compounds appear very rarely (Schnitzler et al., 2004; Gancel et al., 2005; Salzmann, Cozzolin, and Schiestl, 2007). For example, in the case of crosses between *Quercus robur* and *Q. ilex*, hybrids will emit both categories of main VOCs of the parents, respectively isoterpenes and monoterpenes (Schnitzler et al., 2004). In fact, most differences highlighted between hybrids and parents bouquets are quantitative. Thus, Gancel et al. (2005) showed that global amount of sesquiterpenes emitted by hybrids of citrus fruits was much lower than parents ones (from 65 to 90% less).

For insect-pollinated plants, hybridization mainly correspond to a cross between two differentiated pollination syndromes, and the hybrids have been investigated for several vegetative traits, but more rarely on traits (like scents) implied in the interaction with their partners (Schatz et al., 2010). Cases of hybridization was never performed in species involved in a nursery pollination mutualism (Hossaert-McKey et al., 2010), certainly because of the very constraining conditions requested for testing this situation, which are very difficult to recreate in laboratory. However, we reported here a first case study of hybridization between two fig species

performed in greenhouse, possible only under certain conditions. The first condition is to use small fig species compatible with greenhouse dimensions, and to find occasion to perform successful transfer from fields to greenhouse. The second one is the use of dioecious species in order to better control the manual displacement of pollinators, and then control the artificial hybridization. The third one is the opportunity to cultivate the hybrids throughout several years during which pollinator populations should be maintained. All these points explained our choice for the following species: *Ficus montana* and *F. asperifolia.* However, we realized *a posteriori* that this couple of parental species permit hybridization only in one direction, i.e. a pollen transfer from *F. montana* males towards *F. asperifolia* females. Indeed, pollinator of *F. asperifolia* cannot reproduce in *F. montana* figs for morphological reasons (length of the flowers styles). Nevertheless, this exceptional case study allowed us to investigate the scents emitted at receptivity by two parental species and by their artificial hybrids and back-crosses, in order to better understand the role of fig scent as a pre-zygotic barrier against hybridization.

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*Ficus montana* Burman (Sycidium, Sycidium) and *Ficus asperifolia* Miquel (Sycidium, Sycidium) are (gyno)dioecious. Dioecy in figs occurs in half of all roughly 800 *Ficus* species, and is present only in the Old Word. The hybrids are issued from males *F. montana* * females *F. asperifolia*, and the back-crosses from males *F. montana* * females hybrids. The trees grow up in a greenhouse of Leeds University, in England. *Ficus montana* is pollinated exclusively by *Liporrhopalum*

*tentacularis grandi* (Agaonidae), which was the only pollinating wasp introduced in the greenhouse, and which was able to perform 'natural' pollination of its host species under these conditions. In Nature, *F. asperifolia* is pollinated by *Kradibia gestroi afrum* (Wiebes). *Ficus montana* trees are originated from Indonesia (Krakatau islands) and *F. asperifolia* trees from Tanzania.

**Collection of volatiles**

We studied the volatile emissions of 48 individuals with 12 individuals (6 males and 6 females in each case) for the two parents, the hybrids and the back-crosses, all growing on the greenhouses of Leeds University, Yorkshire, England. Receptivity stage was estimated by physical characteristics, as open ostiole, softness and brightness.

For collection of odor samples, receptive figs were cut off from plants and were enclosed in a polyethylene terephtalate (Nalophan®) bag for 30 min. Then, the air was pulled out of the bag (flow rate: 200 mL min–1), over a filter (filled with 1.5mg of Carbotrap 20-40 and 1.5mg of Tenax 60-80), in which the compounds were trapped. Each collection lasted 5 min, as was done at any time between 11:00 and 15:00, when the emission of scent is the highest. One microlitre of a solution of internal standards (nonane and dodecane) was added to each filter, before scent extraction, to allow quantification. The samples were then stored at -20°C until chemical analyses.

## □he□ □cal Analyses

The sampled were then analyzed by GC-MS, using a CP-3800 chromatograph (Varian) coupled with a Saturn 2000 spectrometer (Varian). The characteristics of the columns are the following ones: Varian, CP-Sil 8CB Low Bleed MS 30mx0.25mmI Dx0.25µm film Thickness. The injection is made with the injector 1079PTV (Programmed temperature Vaporizator) with Chromatoprobe equipment (Varian, Walnut Creek, USA). The injection is made at 40°C for 1 min, and then warm up for 3 min, until reaching 250°C, at the rate 200°C/min. The temperature program for the analysis was: 40°C for 3 min, ramped from 40°C to 100°C at 3.3°Cmin–1 to 250°C, from 100°C to 140°C at 2.9°Cmin–1, then 140°C to 180°C at 2.7 °Cmin–1, and finally from 180°C to 2500°C at 10°Cmin–1. Temperature is still 250°C for the 8 following minutes.

Compound identification was based on matching of the mass spectra with NIST 98 MS and Adams 2007, and on confirmation by comparison of RI with libraries and published data (Adams, 2007). Identification of some compounds was confirmed by comparison of both mass spectrum and GC retention indices with those of standards. These analyses were performed at the “Plate-Forme d’Analyses Chimiques en Ecologie” (platform for chemical analyses in ecology) of “IFR 119 Montpellier Environnement Biodiversité”, such as the electrophysiological ones.

## □lectrophys□olo□y

***(NOT DONE YET! PLANNED FOR EARLY 2011!)***

To test the sensory capacities of wasps, we recorded antennal electrical response to compounds or solutions with a Syntech electroantennograph device

(Hilversum, Netherlands). Each stimulus was being tested by depositing 1 µl of pure on a rectangle of filter paper (2 cm²) placed in a glass Pasteur pipette. The stimulus was directed to the antenna by blowing an air pulse through the pipette into a tube with continuous air flow. Stimuli were released at least one-minute intervals to avoid receptor saturation. We recorded antennal responses of *L. tentacularis* (X naïve individuals, probably 10) to pure synthetic versions of (NAMES OF VOCs, main compounds of the bouquets which are commercially available). Each individual was tested for all compounds in a random order. In each series of stimuli, before each compound injection, control stimuli were performed, consisting of an air flow through a glass Pasteur pipette containing a filter paper.

**Data Analysis**

For the statistical analysis of the chemical blend, we ran Principal Component Analysis (PCA, covariance matrix, Stat Box v6•3) to compare patterns of scent composition of the four species-case of receptive figs (*F. montana, F. asperifolia*, hybrids and back-cross). We then performed a MANOVA (SAS v9: Manova, proc glm, and LSMEANS with Tukey–Kramer multiple comparison tests) on the coordinates of the PCA with the species-case of receptive figs as effect. Then, we performed MANOVA on our relative amounts dataset (for compounds with occurrence in at least 80% of individuals) and Kruskall-Wallis tests on our global amount dataset. We then performed the same analyses on the effect of the type of receptive figs, but with a data set restricted only to the active compounds (determined thanks to EAG experiments).

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In order to test if each species-case had a particular blend of VOCs, we performed multivariate analyses on our dataset of relative abundance of each compound for each individual of the four species-cases. The PCA (Principal Component Analysis) do not show a separation of the scents emitted by receptive figs of the four species-cases (Fig. 1). However, according to the MANOVA performed on the same dataset (relative amounts of VOCs), the species effect is globally significant ($F_{(114;15)}$ = 176; $p$ < 0.0001). To study interspecies variation in greater detail, the four cases were then compared two by two (Fig. 2). *Ficus asperifolia* and *F. montana* emit scents which are significantly different (PCA Fig. 2 and confirmed by a MANOVA: $F_{(9;12)}$ = 4 ; $p$ = 0.01). On the contrary, both hybrids and back-crosses bouquets are not different of parent bouquets (PCAs Fig. 2; all MANOVAs have $p$ > 0.05), and hybrids and back-crosses bouquets are not different between each other other (PCA Fig. 2; MANOVA: $F_{(5;18)}$= 4 ; $p$ = 0. 44). Moreover, no significant differences at all have been detected between our four categories of receptive figs regarding to the global quantities of emitted scents. (Kruskall-Wallis tests, all p > 0.05). Interestingly, four VOCs have been found, at low concentration, in hybrids and/or back-crosses bouquets and not observed in parent species bouquets (a-himachalene, bergamotene, dodecene and one unidentified acetate).

## □□□□□□□□

We tried here to understand which role can play scent in hybridization processes, using the highly specific figs / fig wasps mutualism as a model. Our results show i) the receptive figs of both parent species produced different chemical

messages and ii) hybrids and back-crosses produce intermediary (and non different) scents to the parent species. These results confirm other studies showing the specificity of the chemical message emitted by receptive figs of different species (Grison, Edwards, and Hossaert-McKey, 1999; Proffit et al., 2009; Soler et al., 2010; Hossaert-McKey et al, in prep; Soler et al, in prep). However, hybridization was still possible in the conditions of our greenhouse.

According to wasps observations in the greenhouse and to some additional experiments (S. Ghana, unpub. data), we know that spontaneous pollination of *F. asperifolia,* hybrids and back-crosses, occured in the greenhouse. Indeed, even if pollinators does not seem to be attracted by *F. asperifolia* figs*,* if they land on one of these figs (which must happen quite often in the greenhouse), they will try to enter through the ostiole. The same behavior is observed with hybrids and back-crosses figs. Consequently, *F. asperifolia*, hybrids and back-crosses may produce some key components of the volatile blend of *F. montana*. However, especially for *F. asperifolia* and back-crosses, wasps do not seem to enter in figs as often as for *F. montana* figs. Even if EAG experiments are necessary to clarify this point, however, a realistic explanation is that *F. montana* bouquet permits long-distance attraction, and cannot promote strict specificity by its own. Thus, the compounds present on the fig surface may also play a role in species discrimination, since they may also initiate the behavior of entering within the fig. Moreover these surface compounds may be common for both species, as well as for hybrids and back-crosses. Another possible explanation of the spontaneous pollinations is that, due to the greenhouse conditions of culture, the wasp antennae might be saturated by *F. montana* VOCs, which make the wasps unable to discriminate among fig species, and thus fail in their choice.

According to these results, hybridization can occur even if the scent, responsible of the pollinator attraction, is different between parent species. Consequently, scents have to be completed by other barriers to prevent hybridization. For example, hybrids do not support wasp larvae development, which corresponded to a strong post-zygotic barrier. Indeed, even if hybrids may support female functions (production of viable seeds), since they cannot support male functions, the mutualistic interaction is not viable. However, we also demonstrated here that hybrids and back-crosses produced and emitted particular VOCs, which were absent in both parent species bouquets. These VOCs may attract other pollinators (from other species) which may, in their turn, succeed to reproduce within theses figs. However, since the parent species are naturally allopatric, most of the selection pressures on scent discrimination by figs wasps should be released.

Consequently, species specificity of the chemical messages emitted by figs and recognition of this message by fig wasps can be not sufficient by itself, in some cases, to promote strict specificity in the relationship between figs and their pollinating wasps. However, such results need to be confirmed, notably by disproving the “antennae saturation” hypothesis (by coupled EAG and behaviour experiments on antennae-saturated individuals). We thus propose here that scents can act as a barrier against host shift in the highly specific fig / fig wasp mutualism in complement with other elements of floral filters, such as surface chemicals, length of flower styles or geographic distribution of the species.

**Figure 1:** Principal Component Analysis on the global dataset of the relative amounts emitted by the four fig categories (the first component explains 21% of the variance, the second 14%).

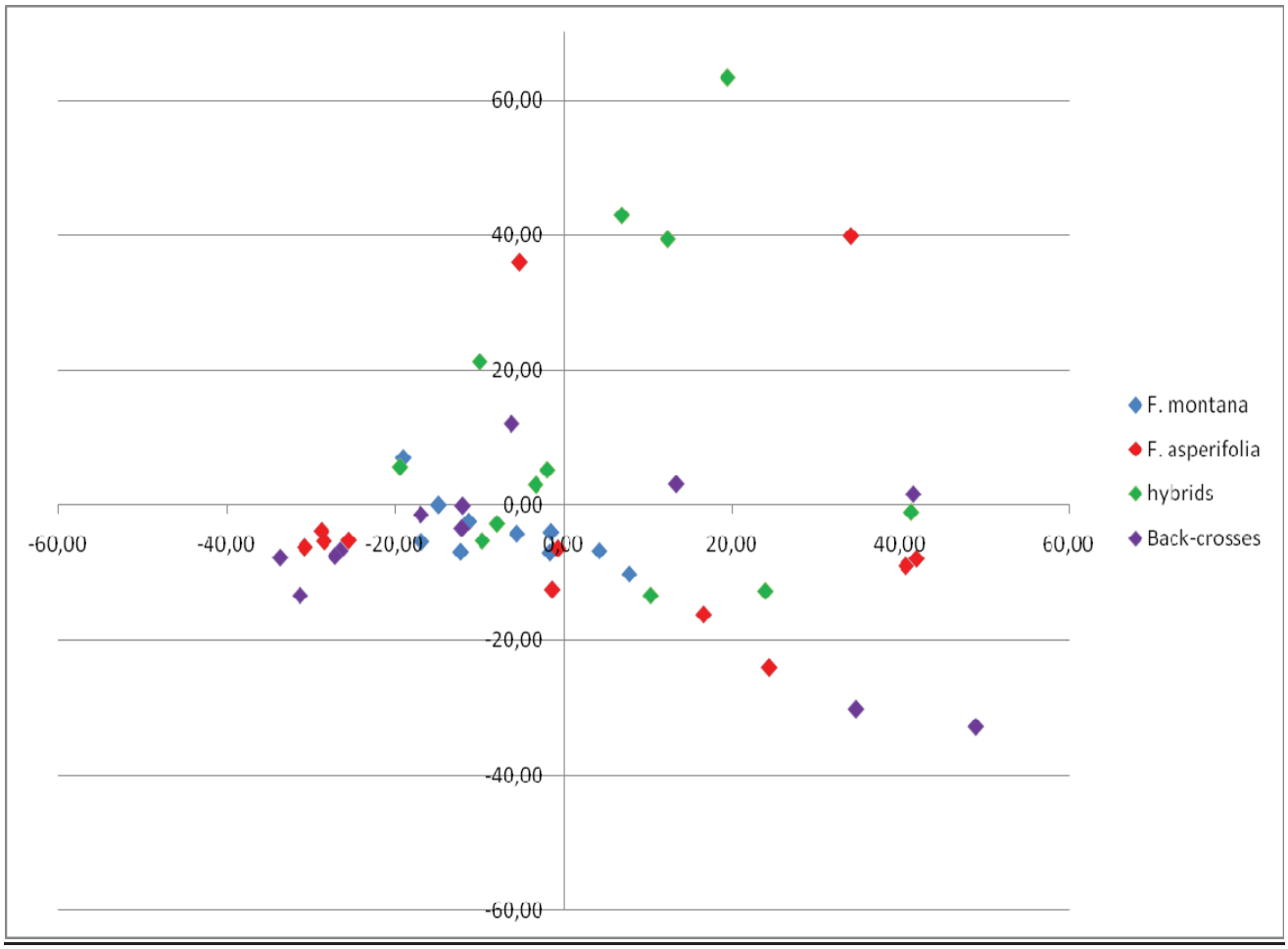
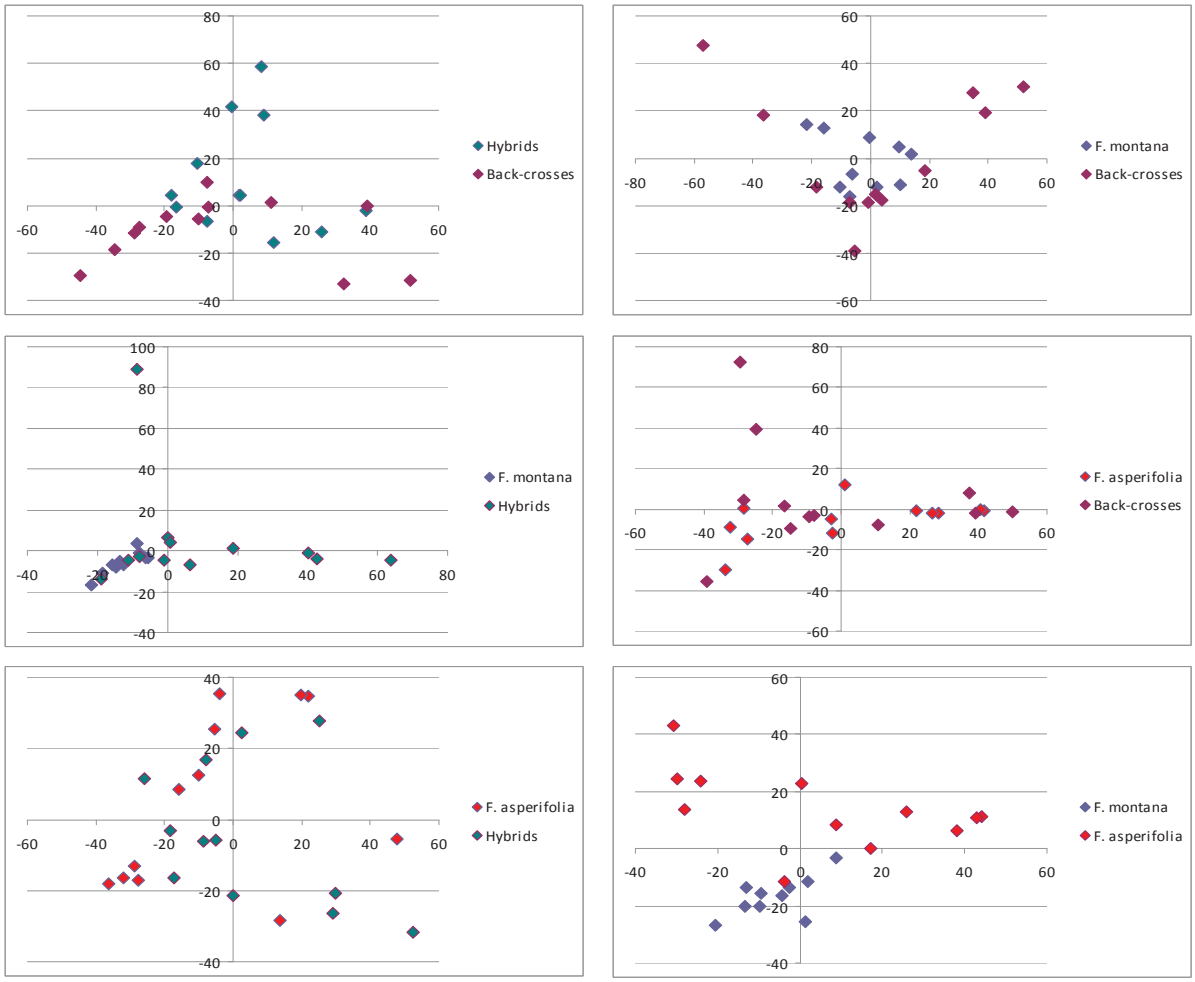


**Figure 2:** Principal Component Analysis on relatives amounts on compared couples of fig categories. Variations were explained by the component 1 and the component 2 at 22 and 18% for the comparison between back-crosses & hybrids, 25 and 18% for back-crosses & *F. montana*, 21 and 19% for *F. montana* & hybrids, 34 and 18% for back-crosses & *F. asperifolia*, 25 and 20% for *F. asperifolia* & hybrids and 23 and 17% for *F. montana* & *F. asperifolia*.



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## A□□□□A□□

Chemical mediation—olfactory attraction of pollinators to volatile compounds released by receptive figs—is important in ensuring the encounter between figs and their species-specific pollinating wasps. However, pollinator attraction in dioecious figs presents particular problems, and general traits of the chemical ecology of wasp/fig interactions in dioecious figs—half of all fig species—are still poorly understood. Pollination in dioecious figs requires that pollinating wasps enter female figs in which they die without laying eggs. Mechanisms for the evolutionary stability of dioecious fig/pollinator mutualisms are still unclear. One very appealing hypothesis holds that in dioecious figs, individuals of each sex are selected to produce odours that are difficult for wasps to distinguish from those produced by the other sex. In this paper we compared 7 dioecious species in order to explore the potential of chemical mediation in intersexual mimicry in dioecious fig species. We used headspace adsorption/desorption methods to collect odours of several worldwide dioecious fig species from different subgenus. We then analyzed the composition of their volatile compounds using gas chromatography/mass spectrometry (GC-MS). Our results showed that in fig species in which male and female receptive figs are present simultaneously, variation in odour composition among individuals of the same sex was much greater than variation between sexes, explaining the failure of wasps, in choice experiments, to discriminate between figs of the two sexes. In contrast, odours of male and female figs were more distinct for fig species in which male and female receptive figs are rarely found simultaneously (low-intensity flowering by males during the female season permits closure of the cycle).



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Dioecy, chemical ecology, nursery pollination mutualism, mimicry.

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The evolutionary transition from hermaphroditism (combined sexes) to dioecy (separate sexes) has occurred numerous times in plant evolutionary history (Charlesworth, 1999), leading researchers to conclude that the evolution of dioecy represents one of the major evolutionary transitions in flowering plants (Barrett, 2008). Thus, understanding the selective forces favoring dimorphism (Ashman, 2009) remains crucial to approach the patterns of sexual dimorphism in plants. One of the consequences of sexual specialization is the difference of reward levels in male and female flowers, as a result of sexual selection. Vamosi and Otto (2002) suggested that the higher rate of extinction, as well as the lower rate of speciation, in clades with a large number of dioecious species may be explained by a strong sexual dimorphism, spawning the avoidance of the non-rewarding female plants by insects. In nursery pollination mutualisms, where reproduction of each partner is totally dependent on that of the other, selection may favor dishonest signals, i.e. “cheating”, in the female plants to optimize female fitness (Patel et al. 1995).

Although mutualisms were first described as nice stories about cooperation, numerous authors have now recognized them as reciprocal exploitations, in which each partner is selected to increase its own benefits and reduce the costs of the interaction (Thompson, 1988; Dufaÿ & Anstett, 2003). In this view, there is a gradient from mutualistic to antagonistic interspecific associations, depending on the net result of the conflicts of interests between partners (Bronstein, 1994). Mutualisms are thus generally considered to be frequently derived from ancestral antagonistic interactions, which have evolved towards commensalism and then mutualism (Thompson, 1988). Some mutualisms appear highly coevolved and co-adapted, such as the fig/fig wasp interaction where pollinators lay eggs when pollinating and larvae

develop at the expense of the plant (Wiebes, 1979; Anstett *et al.*, 1997; Dufaÿ & Anstett, 2003). Nearly every species of fig tree (*Ficus*, Moraceae) is obligatory pollinated by a single species of fig wasp (Chalcidoidea: Agaonidae), which can only reproduce within the fig (Janzen, 1979). The female pollinator wasps locate figs of the host by a species-specific volatile attractant produced by the fig at receptivity, i.e. developmental stage during which stigmates are ready for pollination (Ware *et al.*, 1993; Hossaert-McKey *et al.*, 1994; Grison-Pigé *et al.*, 2002). In dioecious fig species, half the trees are female and half are functionally male (Janzen, 1979; Kjellberg *et al.*, 1987). Figs of “male” trees are structurally hermaphrodite, but their female flowers function only to breed pollinators. Female fig wasps enter male figs to lay eggs in the ovaries and then die. Some weeks later, the mature offspring emerge and mate within the fig; female wasps then collect pollen from male flowers before exiting their natal fig in search of other receptive figs in which to lay eggs. The male figs therefore produce pollen and pollinator wasps, but no or very few seeds. The flowers of female figs have a different structure, which enables the female wasp to pollinate, but not to lay eggs (Verkerke, 1989). A pollinator wasp that enters a female fig dies without leaving any offspring; hence female figs produce only seeds. In other words, in the dioecious fig/pollinator wasp mutualism, the wasps that ensure pollination in female figs pay the very high price of dying without reproduction. Thus, selection should favor wasps that choose to avoid female figs and only enter male figs (where they reproduce). This “simple” behavioral shift in turn would lead to the end of fig seed production and to a breakdown of the system. As in most other cases of pollination by deceit (Ashman 2009), females are always the deceptive sex in *Ficus*.

Why this conflict has not caused the mutualism between wasp and tree to dissolve has been explained by different hypothesis (Patel *et al.*, 1995). One explanation for why wasps enter female figs is that in some dioecious fig species, wasps have virtually no choice. In *F. carica* (section Ficus) and *F. exasperata* (section Sycidium), male trees are receptive more or less simultaneously, well before or after the grouped receptivity of the female trees. Wasps that exit male figs during the time of grouped female receptivity have virtually no choice, and must enter female figs or die. The wasp population in these species survives by the production of a few wasps that emerge after the period of female flowering and enter some of the few male figs that are receptive out of season (Kjellberg *et al.*, 1987).

However, there are other dioecious fig species in which male and female trees are frequently receptive simultaneously, making the “no available choice” hypothesis unavailable in these cases. A possible explanation then is the “limited partial preference” hypothesis, where wasps may be able to develop evolutionarily stable partial preferences for male figs (Patel *et al.*, 1995). In such case, wasps must somehow discriminate between male and female figs when there is variability in appearance within sex and overlap in traits (e.g., diameter) between sexes. When wasps are faced with limited discrimination between sexes, they should adjust their choosiness somewhere between a maximum (avoiding female figs but also avoiding many male figs, i.e., decreasing the chances of entering male figs) or minimum (increasing chances of entering male figs, but also increasing chances of entering female figs). The degree of selectivity exerted by the wasps will therefore depend on a trade-off between the risk of never entering a male fig and the risk of entering a female fig.

Another way in which limited partial preferences may be maintained is "selection to rush": differences between sexes of figs exist that may be detectable by the wasps, but due to their short life they cannot afford the time to choose, because time spent in choosing increases the risks of reproductive failure (Patel *et al.*, 1995). Those risks include the lack of remaining receptive figs due to termination of receptivity after a short period of time (few days), the absence of other receptive figs during the wasp's short lifespan (only few hours for most of the fig wasp species), scramble competition among a large number of wasps for egg-laying sites in male figs, and mortality of wasps during flight. Hence, the best evolutionary strategy for wasps would be to prefer male figs if they encounter receptive male and female figs close together, but otherwise to enter the first receptive fig they detect. Selection may thus, at extreme, favor lineages that enter figs rapidly, whether they are male or female. In effect, rapid entry into male figs and into female figs is pleiotropically correlated traits.

Another possible explanation of female fig pollination is the "complete preference" hypothesis (Patel *et al.*, 1995). In this case, wasps do systematically choose male trees in a fig population, but such a system is evolutionarily unstable, and rapidly goes extinct. This type of system would be hard to spot because of the rapidity of extinction of that population.

The last possible explanation is the "no preference" hypothesis, in which male and female trees are receptive simultaneously, but wasps cannot distinguish between sexes. Grafen and Godfray (1991), discussing vicarious selection in the dioecious fig/pollinator system, suggested that pollinators cannot choose between sexes because there are no detectable differences between male and female trees. Female trees need to attract pollinators to be fertilized and male trees need to attract

pollinators whose offspring will eventually disperse their pollen, i.e. be attracted by female trees. Thus, trees of both sexes are strongly selected to mimic each other. Since wasp attraction is due to chemical components, it is a case of pollination deceit thanks to chemical mimicry, as it has been shown for example in orchids (Ayasse, 2006).

Supports for this last hypothesis exist in some species, but only few species have been studied so far. Indeed, the few studies on dioecious fig species have found no significant difference in floral scent composition between male and female figs (Borges *et al.*, 2008; Chen *et al.*, 2009), supporting the intersexual chemical mimicry hypothesis. However, on a more seasonal species, the Mediterranean fig (*F. carica*) where male and female figs are not flowering in synchrony, Grison-Pigé *et al.* (2001) found a more limited inter-sexual mimicry between both sexes. Here, using a larger data set (7 species, combining seasonal and aseasonal species) we present a comparative study to better explore the potential of chemical mediation in intersexual mimicry in this case of pollination by deceit in nursery pollination mutualisms.

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### □tu□e□ spec□es

In figs species, dioecy evolved at least three times from monoecy and is found in *Ficus, Sycomorus* and *Sycicidium* subgenus, among the six subgenus of figs. Reversion towards monoecy also occurred once in the *Sycomorus* section. Dioecious fig species are only present in the Old Word. In order to have a good phylogenetical and geographical representation for this comparative study, we chose species from

different sections, among these three subgenus, and from different geographic regions distributed in the Old World (Table 1).

Our selected species represent also the diversity found in the genus *Ficus.* Fig can be found in typical tropical aseasonal environment such as *F. fistulosa and F. hispida,* but they can be also found in more seasonal environment such as *F. carica*, the Mediterranean edible fig, and *F. exasperata* in India. Figs are very diverse in their growth forms and in our selected species, we included 'big' trees (as *F. exasperate* and *F. fulva*) versus small trees (as *F. carica*), cauliflorous species (as *F. hispida* and *F. fistulosa*) versus trees bearing figs on stolons (*F. semicordata* and *F. auriculata*), as well as big figs (as *F. auriculata* and *F. carica*) versus small figs (as *F. exasperata* and *F. fulva*). From published data or our personal observations we can divide these species in two classes regarding their flowering phenology. The first class with species where both sexes flower in synchrony: *F. carica* (here we analyzed only receptive male figs produced in summer, the *mammes*, Kjellberg *et al.* 1987), *F. hispida, F. fistulosa, F. fulva* (Patel *et al.,* 1995 & unpublished data) and the second one where receptive figs of male and female trees co-occur very rarely: *F. exasperata* and *F. semicordata*. The phenology of *F. auriculata* is intermediate (C. Soler, unpublished data) with very few male trees flowering at the same time as female trees.

Floral scents were collected only when the figs have reach receptivity, i.e. when they attract mutualistic pollinators. This stage is easily recognizable in figs: the ostiole is soft and bracts not tightly closed, and many pollinators are flying around the receptive figs.

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The scents were collected by adsorption-desorption (dynamic headspace technique) (Grison-Pigé *et al.*, 2002; Dufaÿ *et al.*, 2003). For odor collection, receptive figs were enclosed in polyethylene terephtalate (Nalophan®) bags, which have been shown not to release volatiles. For these extractions we used two different techniques, depending of the type of traps we used:

i) Traps containing 30 mg of Alltech Super Q absorbent (ARS Inc., Gainesville, FL) with Airflow was maintained through the bags by two pumps. Entrance and exit flow rates (controlled by flowmeters) were 400 and 300 ml/min. The extraction last for 3 hours. Then the filters were eluted by Trapped volatiles were eluted with 150 µl of dichloromethane; two internal standards (IS, nonane and dodecane, 200 ng µl) were added to each sample for gas chromatography. Volatile compounds were analyzed by injection in a CP-3800 (Varian Inc., Palo Alto, USA) gas chromatograph with FID detector coupled with a Saturn 2000 mass spectrometer (Varian Inc., Palo Alto, USA).

ii) Traps filled with 100 mg of a 1:1 mixture of Tenax-TA 60–80 and Carbotrap 20–40). Air was sucked from the bags and over the trap by a pump with a controlled airflow (200 ml/min). Extractions lasted for 5 min after letting the figs and traps in the bags for 30 min. The samples were analyzed on the same CP-3800 Varian GC/MS but system using a 1079 injector that had been fitted with the ChromatoProbe kit. This kit allows the thermal desorption of small amounts of solids or liquids contained in the quartz traps. Some comparisons of the 2 methods (Hossaert-McKey, unpub data) allowed us to use both for this study.

**Analysis of volatile compounds**

Traps were stored at dark in a freezer until analyzes. For both sampling techniques a Varian column CP-SIL low blend MS (30 m, ID 0.25 mm, film thickness 0.25 µm, carrier with helium (at 1 ml/min) was used. The injector split vent was opened (1/4). Oven temperature was programmed at 50°C during 3 min; then increasing by 3°C min–1 to 100°C, by 2.7°C min– to 140°C, by 2.4°C min–1 to 180°C and by 6°C min–1 to 250°C. For Chromatoprobe samples, the injection was made at 40°C for 1 min, and then warm up for 3 min, until reaching 250°C, at the rate 200°C/min. The temperature program for the analysis was: 40°C for 3 min, ramped from 40°C to 100°C at 3.3°Cmin–1 to 250°C, from 100°C to 140°C at 2.9°Cmin–1, then 140°C to 180°C at 2.7 °Cmin–1, and finally from 180°C to 2500°C at 10°Cmin–1. Temperature is still 250°C for the 8 following minutes.

Component identification was based on computer matching of the mass spectra with NIST 98 MS library and on retention indices reported in the literature (Adams, 2007) and, when the products were available, by injection of reference compounds. By comparing spectra of each sample with the respective blank (empty bag, same day and condition of collection), supposedly contaminant compounds were subtracted. By comparing spectra of each sample with the respective blank (empty bag, same day of collection), supposedly contaminant compounds were subtracted.

**Data analysis**

All statistical analyses were performed using R version 2. 7. 0 (The R Foundation for Statistical Computing 2008-04-22). In order to compare scent composition both among different species and between sexes within each species,

we used the function metaMDS (Vegan package; Oksanen *et al.*, 2008). Data were first square-root transformed and then standardized using a Wisconsin double standardization to normalize the data. Then a data matrix of pairwise comparisons among samples was calculated using the Bray-Curtis distance index (Bray & Curtis, 1957), ranging between 0 and 1. A non-metric multidimensional scaling (NMDS) was used to find the best low-dimensional representation of the distance matrix. NMDS is a nonparametric dimension reduction technique that works without assumption of linear or unimodal response and escapes many of the distortions of eigenvector techniques (Legendre & Legendre, 1998). To evaluate how well (or poorly) the particular configuration produces the observed distance matrix, the stress value is given. The smaller the stress value, the better the fit of the reproduced distance matrix to the observed distance matrix (Clarke, 1993). In this analyze, several iterative processes were run until obtaining two similar configurations with minimum stress values. Finally, the solution was scaled by rotating the solution, so the largest variance of samples was on the first axis. The null hypothesis of no difference in patterns of scent composition (between sexes at receptive stage) was tested with a multiple response permutation procedure (MRPP) on the matrix of dissimilarity with 10000 permutations. MRPP is a non-parametric multivariate test appropriate for community data sets which fail to meet assumptions of normality or homogeneity of variances (McCune *et al.*, 2002). MRPP calculates a within-group agreement A that is based on the fraction of permuted pairwise dissimilarities that are less than observed pairwise dissimilarities between the sampling plots (McCune *et al.*, 2002). $A_{max}$ = 1 when all items are identical within groups, A = 0 when heterogeneity within groups equals expectation by chance, and A < 0 with there is more heterogeneity within groups than expected by chance.

## Results

### Specificity of the signal

Our data on volatile compounds emitted by 7 different species of figs allowed us to reexamine the determining role of chemical mediation in maintaining species-specificity (see Hossaert-McKey *et al.*, 2010, for review). The floral scent emitted by figs at receptivity is mainly composed of monoterpenes and sesquiterpenes. The blend of compounds is generally dominated by one or a few common terpenoid compounds, the identity of which differs among the studied species (Fig. 1).

Ordination (NMDS) of the different odor samples from the different species, based on Bray-Curtis distance, is presented in Fig. 2. On this ordination graph, based on dissimilarity distances, each species is clearly separated on the plan made by the two first dimensions. The volatile chemical profiles of receptive figs of both sexes varied significantly among species (NPMANOVA, $P < 0.001$, Table 2).

Our findings corroborate the role of scent as a pre-zygotic barrier between closely related plant species in nursery pollination systems. Behavioral experiments conducted on the pollinator of some of our studied species (*F. hispida, F. semicordata, F. montana* and *F.carica*) have already shown that this specific chemical message emitted by figs are in fact used as olfactory signals by the pollinating fig wasp (Chen *et al.*, 2009; Proffit *et al.*, 2009; C.Soler unpub. data).

### Chemical mimicry and sexes

We then used each separated dataset to test the hypothesis on chemical mimicry between sexes of dioecious fig species. Ordination (NMDS) of the different odour samples between sexes for each species and based on Bray-Curtis distance is

presented in Fig. 3. Two cases can be distinguished in these analyses. First, the species with both sexes flowering in synchrony, where volatile compounds emitted by figs cannot be separated on the plan made by the two first dimensions. It is the case for *F. carica, F. fistulosa, F. fulva*, and *F. hispida.* These results are confirmed by the Non Parametric MANOVA (Table 2) where the factor sex is not significant (respectively P = 0.63; 0.69; 0.83; and 0.15). For these species, where both male and female figs are available at the same time within populations, we thus confirmed the chemical mimicry hypothesis.

When figs of both sexes are not flowering synchronously, our results are different. On the ordination graphs based on Bray-Curtis distances, the volatile compounds emitted by receptive figs are not really overlapping, and are even clearly separated such as the case of *F. semicordata* and *F. exasperata*. The effect of sex on the composition of the floral blends emitted by receptive figs is confirmed by the NPMANOVA (*F. semicordata* P = 0.03; *F. exasperata* P= 0.08. The situation of *F. auriculata* can be considered as intermediate (P= 0.09).

For the two species, where the difference between sexes was significant, we conducted Indicator species analysis. The indicator value (fidelity and relative abundance) refers to the compounds that are the most different between the two sexes. These indicators pointed out that linalool and $\alpha$-terpinene are more characteristic of male figs of *F. exasperata,* and 4-methylanisole of male figs of *F. semicordata*.

From these results there is evidence that there is two different ways of ‘how to be a dioecious fig species’. In fig species in which male and female receptive figs are present simultaneously, variation in odor composition among individuals of the same sex was much greater than variation between sexes, explaining the failure of wasps,

in choice experiments, to discriminate between figs of the two sexes. For these species, vicarious selection on both sexes results in the production of chemical messages that are difficult for wasps to distinguish. Thus our results provide strong support to the intersexual chemical mimicry hypothesis (Grafen & Godfray, 1991).

In contrast, in fig species where male and female receptive figs are rarely found simultaneously (low-intensity flowering by males during the female season permits closure of the cycle), odors of male and female figs were more distinct. However, in this situation, chemical mimicry is not obligate for the cycle to be maintained because fig wasps are rarely faced to the choice between male and female figs.

Whatever the way to be a dioecious figs, in all cases, the deceptive sex will be pollinated, and the interactions between pollinating fig wasps and dioecious fig species will be maintained.

**Table 1** Presentation of the studied species and samples sizes

| Species | Subgenus | Section | Sites | Geographical coordinates | Sample size (number of trees) | |
|---|---|---|---|---|---|---|
| | | | | | Male | Female |
| *F. carica* | Ficus | Ficus | Montpellier (France) | 43°61N 3°88'E | 6 | 6 |
| *F. fulva* | Ficus | Eriosycea | Brunei | 4°94N 114.95'E | 4 | 4 |
| *F. auriculata* | Ficus | Sycocarpus | Yunnan (China) | 21°41'N 101°25'E | 4 | 3 |
| *F. exasperata* | Sycidium | Sycidium | Karnataka (India) | 13°51'N 75°09'E | 10 | 13 |
| *F. fistulosa* | Sycomorus | Sycocarpus | Brunei | 4°94N 114.95'E | 6 | 5 |
| *F. hispida* | Sycomorus | Sycocarpus | Karnataka (India) | 13°51'N 75°09'E | 8 | 10 |
| *F. semicordata* | Sycomorus | Hemicardia | Yunnan (China) | 21°41'N 101°25'E | 5 | 5 |

**Table 1** Results on the Non Parametric MANOVA performed on the proportion of volatile compounds emitted by figs at receptivity. All species correspond to the NP MANOVA performed to test the interspecies variations, and the other analyses are the comparison between sexes within each species.

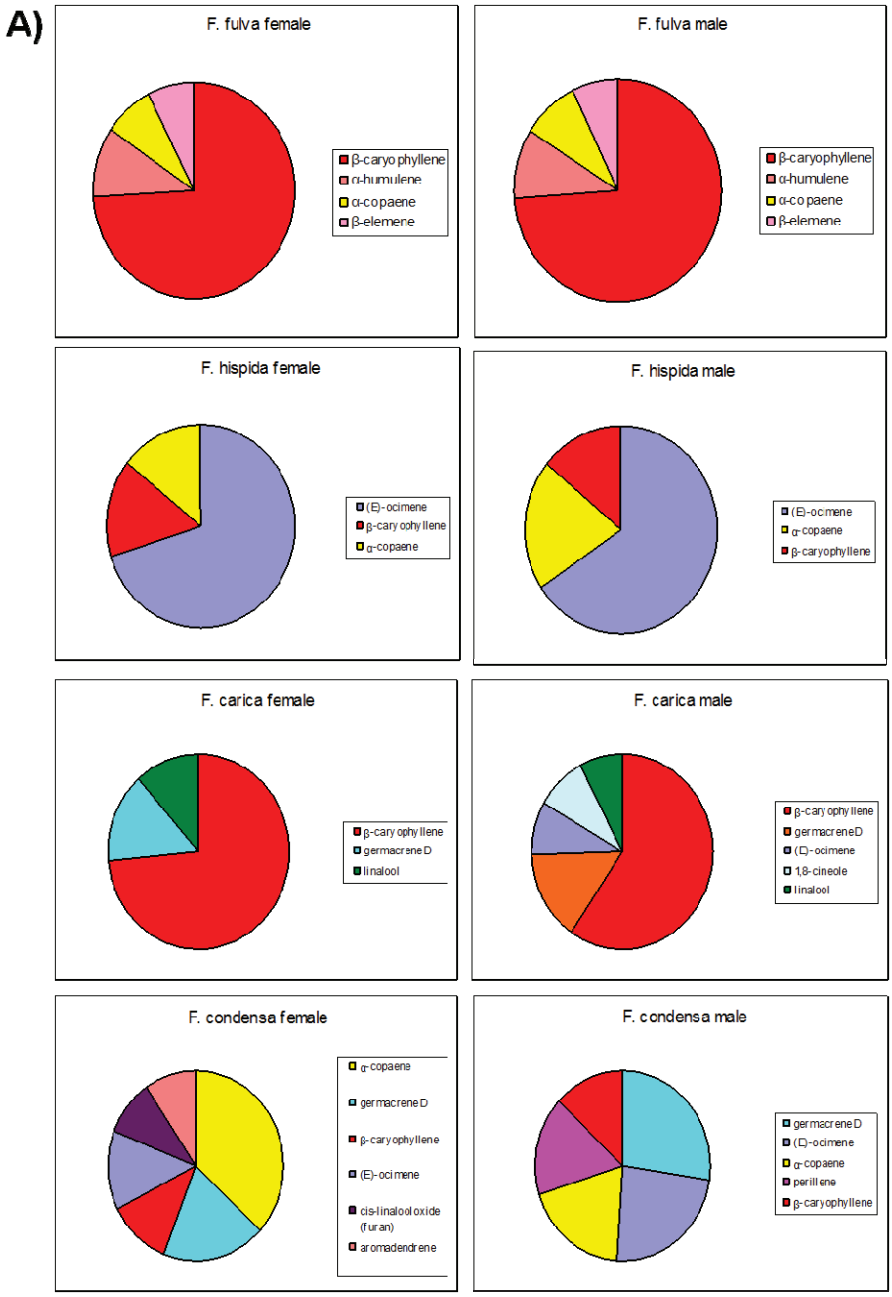
| | **stress** | MRPP | | ANOSIM | | NPMANOVA (ADONIS) | | |
|---|---|---|---|---|---|---|---|---|
| | **n** | **A** | **p** | | **p** | | | **p** |
| All species | 18,369 | [a] | [a] | [a] | [a] | 1; 106 | 1,147 | 0,001 |
| *F. exasperata* | 17,463 | 0,034 | 0,002 | 0,203 | 0,001 | 1; 25 | 2,535 | 0,008 |
| *F. hispida* | 15,893 | 0,007 | 0,161 | 0,149 | 0,035 | 1; 24 | 1,392 | 0,145 |
| *F. carica* | 9,503 | 0,004 | 0,549 | 0,067 | 0,781 | 1; 10 | 0,870 | 0,633 |
| *F. fistulosa* | 11,874 | 0,017 | 0,713 | 0,067 | 0,658 | 1; 9 | 0,716 | 0,686 |
| *F. fulva* | 0,881 | 0,013 | 0,666 | 0,185 | 0,858 | 1; 5 | 0,691 | 0,828 |
| *F. semicordata* | 5,843 | 0,069 | 0,081 | 0,268 | 0,048 | 1; 8 | 2,363 | 0,032 |
| *F. auriculata* | 10,024 | 0,047 | 0,032 | 0,231 | 0,099 | 1; 7 | 1,588 | 0,098 |

[a]: not done

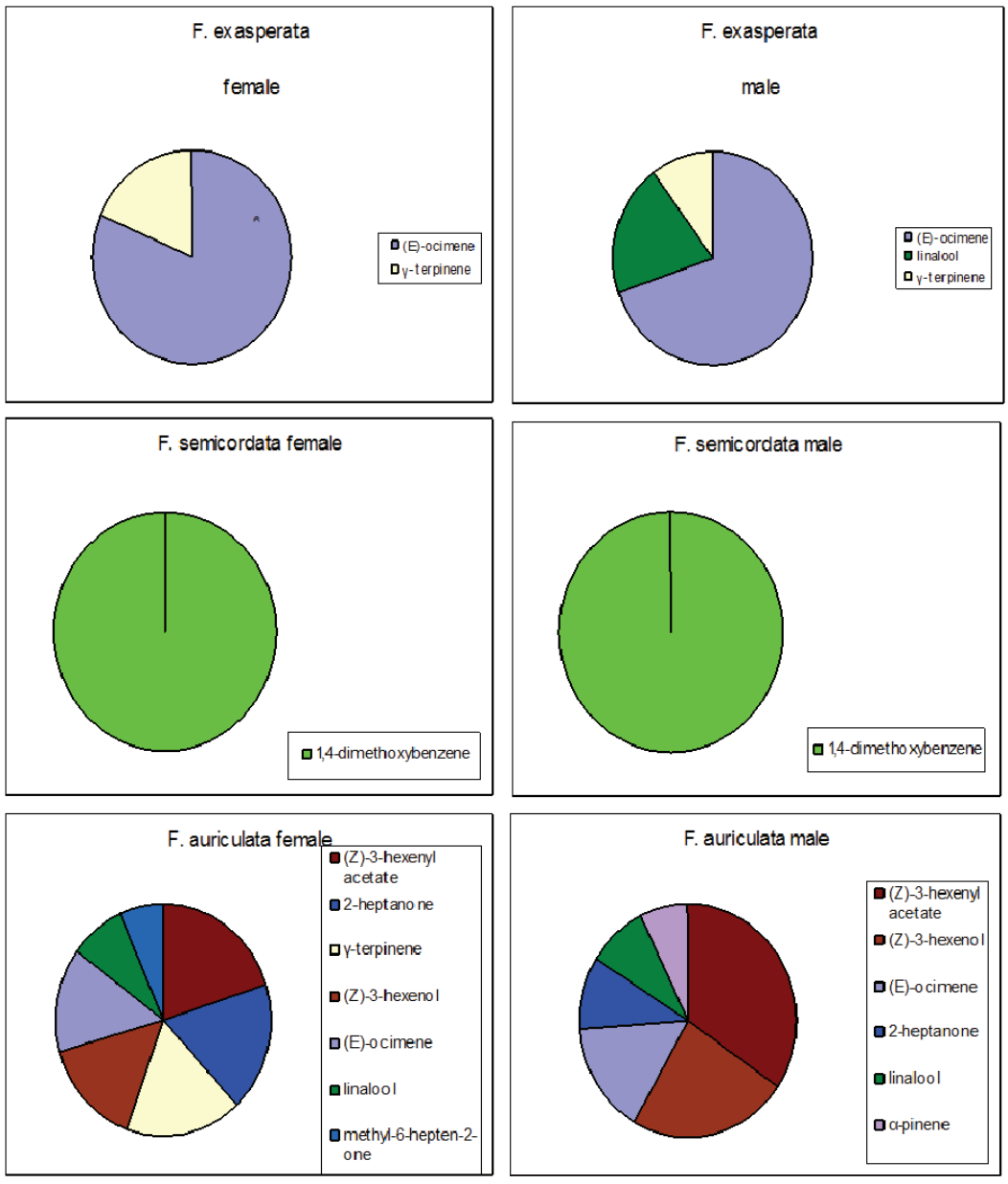
**□□□ure □□**Proportion of the main compounds (mean >5%) in the bouquet emitted at receptivity by both sexes of:

A) synchronous species (male and female figs flowering at the same time)

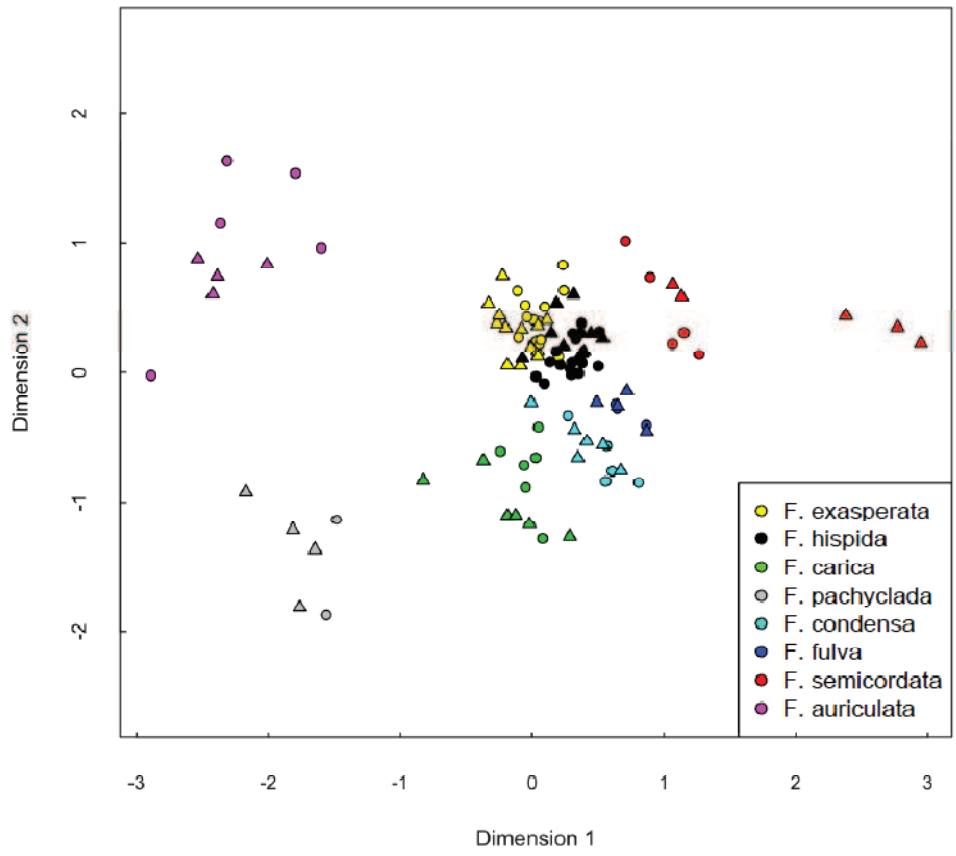
B) asynchronous species (male and female figs flowering separatly)



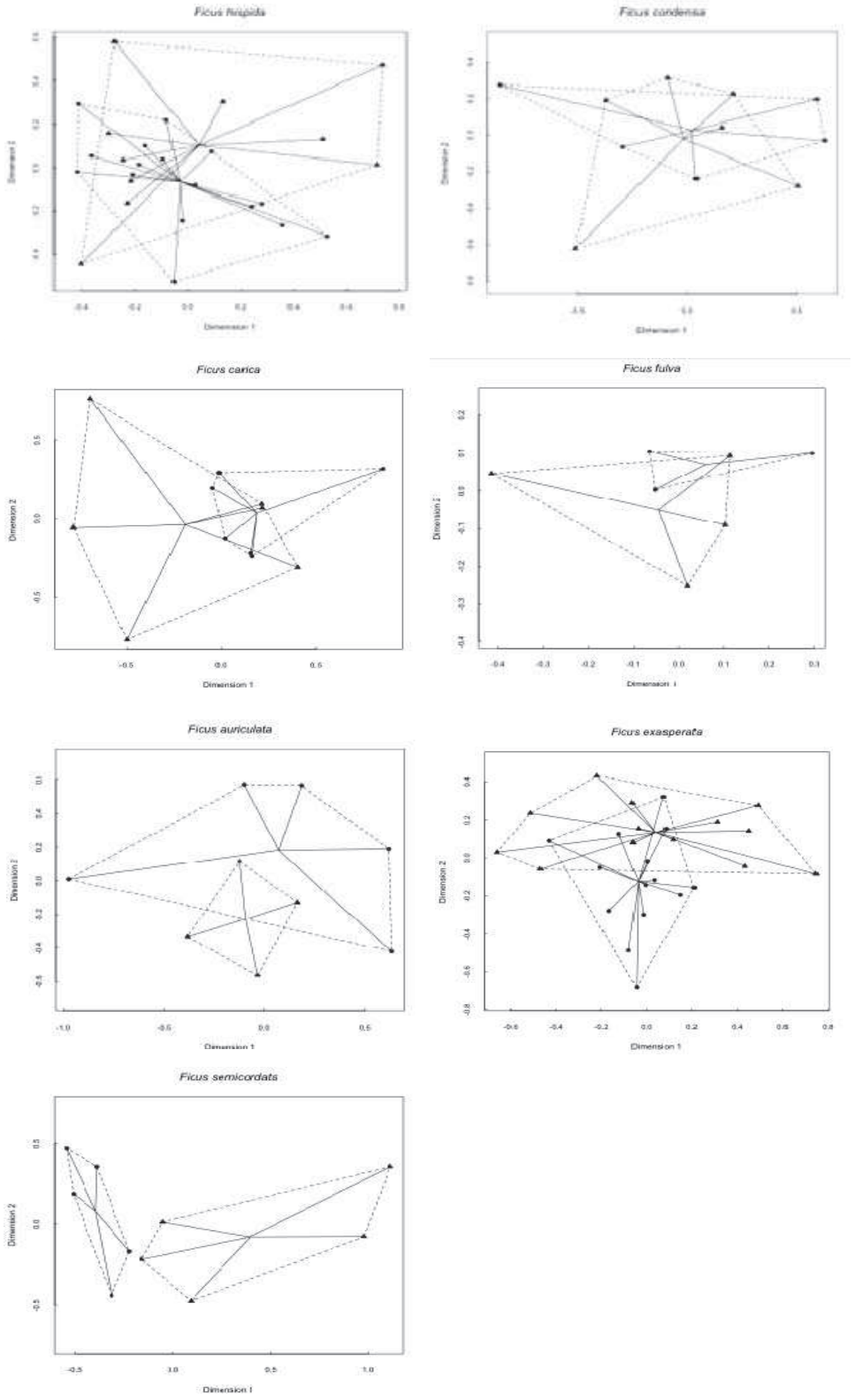
B)


F. exasperata
female
(E)-ocimene
γ-terpinene
F. exasperata
male
(E)-ocimene
linalool
γ-terpinene
F. semicordata female
1,4-dimethoxybenzene
F. semicordata male
1,4-dimethoxybenzene
F. auriculata female
(Z)-3-hexenyl acetate
2-heptanone
γ-terpinene
(Z)-3-hexenol
(E)-ocimene
linalool
methyl-6-hepten-2-one
F. auriculata male
(Z)-3-hexenyl acetate
(Z)-3-hexenol
(E)-ocimene
2-heptanone
linalool
α-pinene

**□□ure □□**Non-metric multidimensional scaling (NMDS) ordination of chemical composition of all studied species at receptive stage, based on Bray-Curtis distance, rotated by principal component analysis.



**□□ure □□**Non-metric multidimensional scaling (NMDS) ordination of chemical composition of both sexes of all studied species at receptive stage, based on Bray-Curtis distance, rotated by principal component analysis.



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# When males change their scents in presence of females: the case of the Mediterranean fig

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This manuscript is 'in prep' for a journal such as *Current biology* or *PLOS one*. The analyses are finished, and we want now to improve our writing to fit with the requirements of a general journal, such as the two sub-cited. For example, we planned to simplify the abstract to make it more easily understandable, and planned to submit this study soon.

## ABSTRACT

Intersexual mimicry is used by dioecious plant species to confuse and then force their pollinators to visit flowers of the non-rewarding sex. To fool their pollinated insects, plant can use different ways, and among them, the production of volatile compounds more or less identical between the two sexes. This mechanism of chemical mimicry has been approached theoretically, but received little attention in empirical studies. The Mediterranean dioecious fig species, *Ficus carica,* displays a unique flowering phenology due to its exclusively extra-tropical distribution, unique among the 800 worldwide fig species,. Fig production occurs firstly during spring only for male individuals, and secondly during summer with both sexes, which offers a very original opportunity to investigate scents emitted by male figs in presence or absence of female figs. Moreover, *F. carica* is engaged in specific and obligate pollination mutualisms with their pollinators, mostly permitted by specific attraction by a bouquet of volatile compounds emitted by figs. Since the three kinds of figs are attractive for pollinators, we investigated composition and quantities of emitted scents. Thanks to multivariate analysis, we showed that scents of two sexes of summer figs were not significantly different among them, but different to scents of spring male figs, and are also more variable to the latter. We also inaugurated electroantennography techniques with the tiny fig pollinators by identifying the compounds they detected. Previous differences between spring and summer scents are confirmed with electrophysiologically active compounds alone. Thus, we bring new insights in sexual chemical mimicry for dioecious species, in which flowers of a same male individual, to attract its unique pollinator, emits two different scents, according to the presence or absence of female blooming trees.

## An opportunity for testing sexual chemical mimicry

Mutualisms, which are cooperative interactions between species, are more and more studied by evolutionary biologists, who propose several hypotheses to explain their origin and maintenance [1]. Pollination mutualisms are ones of the most studied, notably between plants and insects [1]. A central component in ecology and evolution of these interactions concerns the mechanisms used by plants to attract pollinators, in which floral scents have been often shown as important mediators [2]. In the particular case of highly specific interactions, such as the nursery pollination mutualisms, where pollinators lay eggs within the inflorecence they pollinate, several studies showed that the chemical signals emitted by plants can be species- or developmental-stage specific and lead pollinators to the receptive flowers [reviewed by 3]. Even if recently intra- and inter- population differences of scents have been revealed, the scent emitted by one plant species to attract its pollinator remains globally constant [reviewed by 3], particularly in the fig/fig wasp interactions [4].

Most of the roughly 800 *Ficus* species are limited to tropical and subtropical areas, and are all engaged in specific and obligatory mutualism with their pollinators, which are attracted by a bouquet of VOCs emitted by the host plant, and then lay eggs within the ovules of receptive figs [5]. Half of them are (gyno)dioecious, where male figs classically produce pollen, and are as well the place where pollinators lay eggs. Female figs contain only female flowers, in which the pollinating wasps are not able to lay eggs (due to the length of the style). Consequently, female figs only produce seeds (and no pollinator), and so pollinators should avoid female figs where they die without laying eggs. There is thus a conflict of interests between the wasp and the female fig tree, which needs to be pollinated to produce seeds. Indeed, in dioecious *Ficus* spp., since figs of female trees offer no rewards (breeding sites) to pollinators, insects should thus be selected to avoid them. A selective pressure for the capacity to discriminate between male and female figs is therefore expected on the wasp's side,

although on the other side, male and female figs should be selected to emit the same signal to attract the pollinator, ensuring that the wasps enter male figs and disperse their pollen, but also enter female figs and pollinate their flowers. Such chemical mimicry between sexes has been firstly hypothesized by Grafen & Godfray [6], who explained that vicarious selection may be engaged in such case, to ensure enough resemblance between sexes, and thus constancy of gene flow. However, the climatic conditions under which the fig trees live can impact on the chemical mimicry [7]. Indeed, in some cases, related to environmental conditions, male and female figs do not flower synchronously, inducing a "no choice" case [7].

**A particular flowering phenology**

For tropical species, the very short lifespan of the wasps (generally a few hours) provide a constraint that leads to the particular phenology of the genus, an all-year-round flowering pattern. At all times of the year some trees bear figs at the pollinator-releasing stage and other trees bear receptive figs. However, one fig species exclusively occurs in temperate zones, the edible fig *F. carica*, naturally distributed in Europe around the Mediterranean basin. Even if its specific wasp (*Blastophaga psenes*) life span's also such short, the climatic conditions do not permit an all-year-round phenology. Thus, pollinator larvae overwinter in diapause in some male figs [8], and fig trees displayed a seasonal flowering phenology, with two distinct crops of figs: a first crop of male receptive figs (also called *profichis*) during spring, and a second crop during summer with male figs (also called *mammes*, in which the wasps overwinter) and female figs. Consequently, the female trees flowered only once a year and male trees twice a year.

This very original phenology of flowering constitutes a suited situation to investigate new insights on sexual chemical mimicry. Indeed, previous study on intersexual mimicry in

*Ficus* explained that chemical mimicry should be selected for species in which male and females flower synchronously, and absent for species where flowering is asynchronous [7; Hossaert-Mc Key et al, in prep]. Thus, in the case of *F. carica*, selection for a strict chemical mimicry between sexes should occur during summer, when pollinators may have to choose between male and female figs, which partially bloom synchronously and both require pollinator visit, as suggested by Patel et al. [7] and as tested and confirmed by the study performed by Grison-Pigé et al. [9], but should be released at spring. Consequently, at different period of the year, to attract the same unique and obligatory pollinator, male fig trees may emit different scent (according to the absence/presence of female flowering trees). However, to date, any case of such an intraspecific scent variation has never been reported. Moreover, we know that pollinators are attracted by the three types of figs produced by *F. carica* trees. Indeed, behavioral experiments showed that both female and summer male figs are attractive for the pollinator *B. psenes*, as well as spring figs [9, 10; C. Soler and M. Proffit, in prep].

In order to test if scents emitted by summer male figs are mimetic to the ones emitted by the female figs, whereas this chemical mimicry is released in the case of the scents emitted by spring male figs, we first investigated composition and emitted quantities of these bouquets of volatiles. We then verified our results by analyzing, for the first time in the fig/fig wasp interactions, the "electrophysiologically active bouquets", thanks to the compound identification by using electroantennography of the volatile compounds detected by pollinators.

**Difference in the three attractive emitted scents**

We explored how the scents emitted by the three kinds of figs may vary. Twenty-six volatile organic compounds (VOCs) were detected, and 23 identified (Table 1). Most of the VOCs are terpenes, common volatiles in the plant/insect chemical mediation [11]. We first performed a PCA on our global dataset about composition in VOCs (Fig. 1), which showed that, even if some overlap exists, scents of receptive spring male figs differed from both summer male and female fig receptive, as confirmed by a MANOVA performed on the coordinates of the first component of this PCA ($F_{(25;2)} = 13.572$; $p < 0.0001$). Indeed, LS means tests included in this MANOVA gave significant differences between scent emitted by spring male figs versus both those emitted by summer male and female figs (respectively, p= 0.0004 and p= 0.0005) while scents from summer male and female figs were not statistically different (p=0.9993). The volatile compounds which contributed the most to this first component are two terpenes (linalool and *β*-caryophyllene, for which contributions to the PCA are respectively 70% and 26%), which are as well the two main compounds of the bouquets (Table 1). This is congruent with previous studies on receptive figs of *F. carica* [9, 10], for which these compounds had high amounts in the receptive figs bouquets. Interestingly, these two compounds present higher variability of their relative proportion in the scents emitted by both summer figs, i.e. the ones in situation of selection for chemical mimicry, in comparison to spring male figs. As shown in the *Ophrys sphegodes* case [12], such variation may be highly selected to counter a possible pollinator learning ability to discriminate, and thus enhance stability of pollination by deceit.

Interestingly, a MANOVA performed on our global dataset do not give a global significant difference (Wilks' lambda: $F_{(50;2)} = 6.60$, p = 0.1403). Such result may be due to the similarity of scent emitted by female and summer male figs. Indeed, numerous compounds show different amounts between spring male figs versus both summer male and female figs (see

Table 1), and notably the two main ones of the bouquets, linalool and *β*-caryophyllene, although these differences don't seem important between female and summer male figs (see Table 1). Add to the previous ones, this result confirms our hypothesis of chemical mimicry between both types of summer figs.

Consequently, our study shows that, even to attract a same partner, on which is totally dependent for its reproduction, a same male fig tree can change its signal, in response to external biotic pressures, which corresponded here to the presence of receptive female figs. Such intraspecific variations of relative proportions of the same VOCs have never been documented hitherto for the interaction between fig and fig wasps. However, this innovative result needs to be confirmed by identification of active bouquets of VOCs.

**Identification of electrophysiologically active compounds**

To validate or invalidate this result, we determine compounds occurring in the bouquets of at least ¾ of the sampled individuals, because occurrence of each VOC would be highly selected if it plays a rule in the chemical mediation of such an obligate plant-insect interaction [3]. We selected by this criterion 6 different VOCs (linalool, linalool oxide, *β*-caryophyllene, germacrene D, *β*-bourbonene and *α*-copaene) to be used in electroantennography method. We determined that the four first VOCs are electrophysiologically active for *B. psenes* (6 individuals tested). Such identification of electrophysiologically active VOCs from an emitted bouquet of 26 VOCs by the specific pollinator of the Mediterranean fig constitutes a novel kind of result in the course of studies on chemical mediation occurring in fig/pollinator interaction [7]. Consider the signal detected by a fig pollinator opens innovative approach, which is needed to be applied in other interactions with other couples of mutualistic species to provide new insights in their chemical mediation [13].

Using the dataset composed by the relative proportions of the four physiological active compounds, a MANOVA indicated a significant global effect of the type of figs (Wilks' lambda: $F_{(23;3)} = 162$, $p < 0.0001$). Moreover, all these compounds present significantly different proportions between spring male versus both summer male and female bouquets, although summer male and female bouquets never present significantly different proportions, except for the germacrene D. For this VOC, relative amounts are significantly different between summer male versus both female and spring male, and not between female and spring male. Moreover, analysis on the total emitted quantities of VOCs showed no significant differences among the three types of figs ($X^2 = 2.5928$; $p = 0.2803$).

The main difference between the two types of bouquets (spring male VS summer male and female) is that the first crop is mainly composed by the monoterpene linalool, while *β*-caryophyllene is a sesquiterpene dominating the second crop. We propose here that this greater complexity of the bouquet results from selective pressure for inter-sex chemical mimicry. Indeed, since *β*-caryophyllene is to be oxydable more easily than linalool [14], its main importance in summer bouquet is pretty surprising. We consequently propose here that its higher presence in summer bouquet is not due to climatic conditions. Thus, the similarity between summer male and female bouquets cannot be explained by climatic conditions, but rather by intersexual chemical mimicry.

**Intra-individual variation in males scents**

To enhance the characterization of changes between scents emitted at receptivity by spring and summer male figs, we concentrated our analysis only on individual male trees (n = 5) where we were able to collect scent of both fig types during the same year. The MANOVA performed on the relative amounts of the active compounds of the bouquet showed that scents

are globally significantly different (Wilks' lambda: $F_{(6;3)} = 27.81$, p=0.0006) and different for each active compound, except for the linalool oxide (p=0.6754). This intra-sex variation is clearly visible on a PCA (Figure 2). We thus clearly showed here that each individual male tree emits a different scent at different period of the year.

**Confirmation of the hypothesis of sexual mimicry in deceptive dioecious species**

In this study, we demonstrated that even in an obligate pollination mutualism, the two mutualistic partners may communicate with different signals along the year in order to lead to the same result: their specific encounter. To our knowledge, such situation is unique in the world of plant-insect interactions. Documenting such difference in scent emitted by figs within same individuals improved our knowledge of the selective pressure exerted by the chemical mimicry. Indeed, pressure for chemical mimicry is the most common rule for dioecious *Ficus*, due to their life history, and notably their occurrence under tropical climate inducing synchronous flowering between male and female trees. In the uncommon case of the temperate species *F. carica*, this rule is respected during summer, when females and male figs bloom synchronously, but not when the male figs bloom alone in spring. Due to low temperatures during winter, pollinator should be protected by staying in diapause within the developing figs. However, wait for an entire year inside a fig is too long for the wasp [8], and render necessary a spring emergence, to ensure wasp population persistence. At that time, pressure for chemical mimicry is released since there are no female figs. Moreover, we demonstrated here that this process is associated with a change in relative proportion of the same VOCs emitted by summer male but also by a significant increase of the variability in the proportions of each active VOC, because of synchronicity with female figs. We thus confirmed the hypothesis of chemical mimicry by showing selection for similarity in signals allowing pollination by deceit [15].

## Conclusion

In this study, we highlighted the great role of pollinator as biotic agent acting as selection pressure on chemical mediation. We thus showed that a plant involved in an obligate mutualism can change its chemical mediation, even to attract the same partner. To our knowledge, this study is the first showing such a change in a highly strictly specific and obligatory mutualism, and confirm that the inclusion of floral chemistry should improve our understanding of plant–pollinator interactions in ecological and evolutionary time [16]. Since the primary impediment to more widespread investigation of floral volatiles is technological [16], we also innovate in studies on fig mutualism by identifying physiologically active VOCs for pollinators thanks to EAG techniques. Such studies with comparative analyses may lead new insights in other nursery pollination systems and other plant-insect interactions.

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## Experimental Procedures

### Studied species

*Ficus carica Linnaeus* (*Ficus, Ficus*) is (gyno)dioecious, like half of all roughly 800 *Ficus* species, pollinated exclusively by *Blastophaga psenes* Linnaeus (Agaonidae). 'Male' trees flower in end April – start of May ('spring male' figs) and July-August ('summer males' figs), but female trees only in June–July (see Fig. 1).

### Collection of Volatiles

We studied the volatile emissions at receptivity of 23 trees (10 females and 13 males) growing on the CNRS campus in Montpellier, France. We consequently extracted scents of figs of 10 female trees, 9 male trees in spring, and 8 male trees in summer. In order to sample summer males and spring males on the same trees, 5 males have been sampled twice (not every male trees sampled in spring produced enough summer male figs in summer for scent extraction). Fig floral stages were estimated by their physical characteristics, as open ostiole, softness and brightness.

For collection of odor samples, we used the Chromatoprobe head-space technique. Receptive figs were cut off from plants and were enclosed in a polyethylene terephtalate (Nalophan®) bag for 30 min. Then, the air was pulled out of the bag (flow rate: 200 mL min–1), over a filter (filled with 1.5mg of Carbotrap 20-40 and 1.5mg of Tenax 60-80), in which the compounds were trapped. Each collection lasted 5 min, as was done at any time between 11:00 and 15:00, when the emission of scent is the highest. One microlitre of a solution of internal standards (nonane and dodecane, 110ng of each) was added to each filter, before scent extraction, to allow quantification. The samples were then stored at -20°C until chemical analyses.

**Chemical Analyses**

The sampled were then analyzed by GC-MS, using a CP-3800 chromatograph (Varian) coupled with a Saturn 2000 spectrometer (Varian). The characteristics of the columns are the following ones: Varian, CP-Sil 8CB Low Bleed MS 30mx0.25mmI Dx0.25µm film Thickness. The injection is made with the injector 1079PTV (Programmed temperature Vaporizator) with Chromatoprobe equipment (Varian, Walnut Creek, USA). The injection is made at 40°C for 1 min, and then warm up for 3 min, until reaching 250°C, at the rate 200°C/min. The temperature program for the analysis was: 40°C for 3 min, ramped from 40°C to 100°C at 3.3°C$min^{-1}$ to 250°C, from 100°C to 140°C at 2.9°C$min^{-1}$, then 140°C to 180°C at 2.7 °C$min^{-1}$, and finally from 180°C to 250°C at 10°C$min^{-1}$. Temperature is still 250°C for the 8 following minutes.

Compound identification was based on matching of the mass spectra with NIST 98 MS and Adams 2007, and on confirmation by comparison of retention index (RI) with libraries and published data (Adams, 2007). Identification of some compounds was confirmed by comparison of both mass spectrum and RI with those of authentic standards. These analyses were performed at the "Plate-Forme d'Analyses Chimiques en Ecologie" (platform for chemical analyses in ecology) of "IFR 119 Montpellier Environnement Biodiversité", such as the electrophysiological ones.

**Data Analysis**

For the statistical analysis of the chemical blend, we ran Principal Component Analysis (PCA, covariance matrix, Stat Box v6.3) to compare patterns of scent composition of the three types of receptive figs. We then ran MANOVAs (SAS v9: MANOVA, proc glm, and LSMEANS with Tukey–Kramer multiple comparison tests) on the coordinates of the PCA, on relative

amounts of global VOCs dataset, and on relative amounts of electrophysiologically actives VOCs dataset.

**Electrophysiology**

To test the sensory capacities, for the first time, of these small (2mm long) fig wasps, we recorded antennal electrical response to compounds or solutions with a Syntech electroantennograph device (Hilversum, Netherlands). Each stimulus was being tested by depositing 1 µl of pure standard on a rectangle of filter paper (2 $cm^2$) placed in a glass Pasteur pipette. The stimulus was directed to the antenna by blowing an air pulse through the pipette into a tube with continuous air flow. Stimuli were released at least one-minute intervals to avoid receptor saturation. We recorded antennal responses of *B. psenes* (6 naïve individuals) to synthetic versions of (-)-trans-caryophyllene (purity: 99%), (±)-linalool (97%), linalool oxide (mixture of isomers, purity>97%), germacrene D (40%), (-)-*α*-copaene (95%) and *β*-bourbonene (the last COV was not tested because of its commercial unavailability). Each antenna was tested for all compounds in a random order. In each series of stimuli, before each compound injection, control stimuli were performed, consisting of an air flow through a glass Pasteur pipette containing a filter paper on which 1µl of solvent was firstly injected. The control stimulus triggered a small, but appreciable, EAG response and may be due to the presence of mechanoreceptors on the antennae. Antennal response to a stimulus was thus tested by comparing the amplitude of depolarization induced by the stimulus VOC with the corresponding control. A sign test (sign test, Stat Box v6.3) was then performed to check if the difference between both amplitudes was significant.

**Figure 1: PCA analysis on relative proportions of VOCs emitted by the three types of receptive figs of *F. carica* (component 1 explains 86% and component 2 explains 10% of variance)**

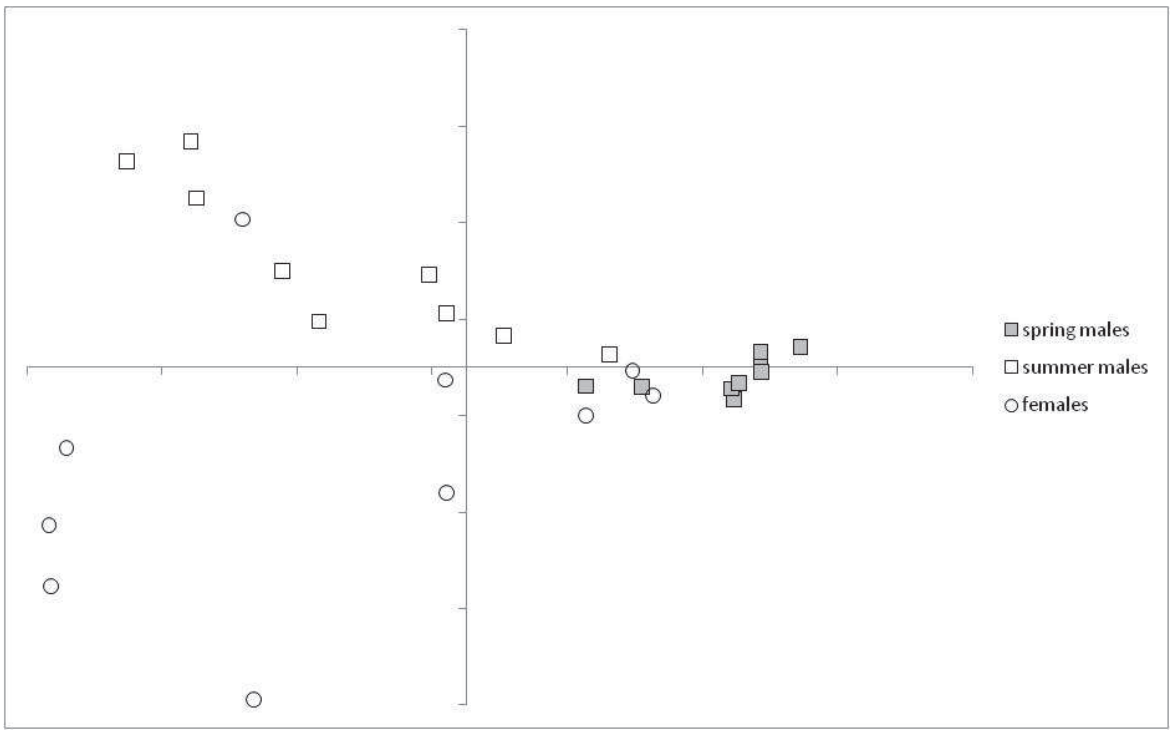

**Figure 1: PCA analysis on relative proportions of VOCs emitted by the two types of male receptive figs of *F. carica* (component 1 explains 89% and component 2 explains 9% of variance)**

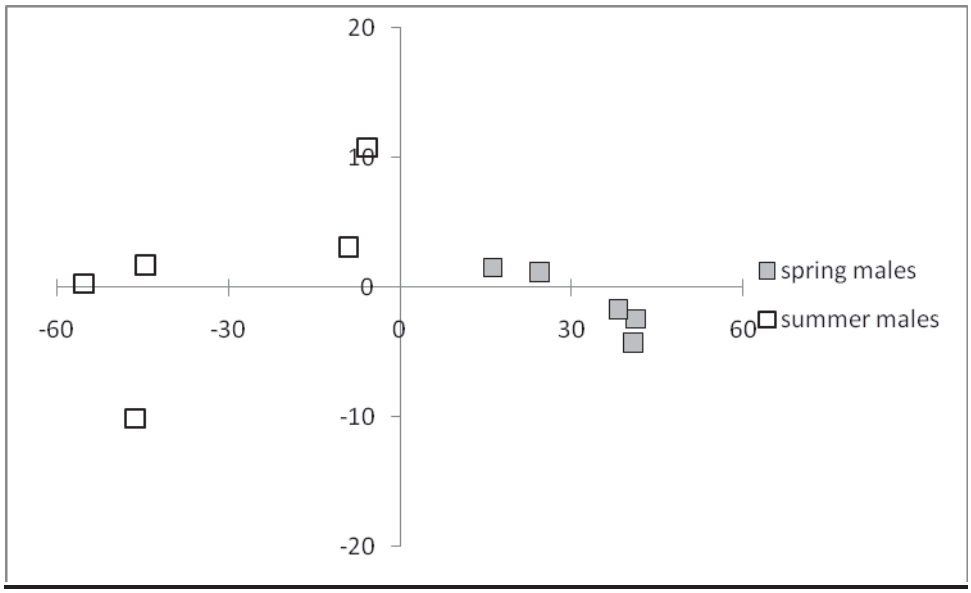

**Table 1: Relative proportions (means ± standard deviation) of VOCs in the three types of receptive figs of *F. carica*.**

| | profichi | | | mamme | | | female | | |
|---|---|---|---|---|---|---|---|---|---|
| **(Z)-3-hexenol** | 0.34 | ± | 0.53 | 0.00 | ± | 0.01 | 1.12 | ± | 1.50 |
| **(E)-2-hexenol** | 0.91 | ± | 2.33 | 0.00 | ± | 0.01 | 0.84 | ± | 1.52 |
| **alpha-pinene** | 0.29 | ± | 0.82 | 0.00 | ± | 0.01 | 2.22 | ± | 2.19 |
| **beta-pinene** | 0.19 | ± | 0.17 | 0.02 | ± | 0.07 | 2.61 | ± | 3.02 |
| **benzaldehyde** | 0.47 | ± | 1.00 | 0.16 | ± | 0.27 | 2.96 | ± | 4.94 |
| **(Z)-3-hexenyl acetate** | 0.72 | ± | 1.14 | 0.39 | ± | 0.77 | 0.08 | ± | 0.11 |
| **1,8 cineole** | 0.61 | ± | 0.99 | 0.12 | ± | 0.21 | 1.45 | ± | 2.42 |
| **acetophenone** | 0.41 | ± | 1.22 | 0.11 | ± | 0.15 | 0.25 | ± | 0.24 |
| **Z-ocimene** | 0.08 | ± | 0.12 | 0.35 | ± | 0.44 | 0.11 | ± | 0.20 |
| **E-ocimene** | 0.00 | ± | 0.00 | 0.58 | ± | 0.94 | 0.64 | ± | 0.86 |
| **linalool oxide1** | 2.37 | ± | 1.87 | 2.83 | ± | 1.65 | 3.70 | ± | 4.00 |
| **linalool oxyde 2** | 0.12 | ± | 0.20 | 0.05 | ± | 0.11 | 0.21 | ± | 0.25 |
| **linalool** | 86.10 | ± | 9.86 | 41.93 | ± | 18.45 | 35.92 | ± | 30.73 |
| **Peak 23.407** | 0.19 | ± | 0.45 | 0.52 | ± | 0.66 | 0.17 | ± | 0.22 |
| **methyl salicylate** | 2.15 | ± | 2.84 | 0.20 | ± | 0.29 | 0.11 | ± | 0.20 |
| **delta-elemene** | 0.14 | ± | 0.20 | 0.08 | ± | 0.13 | 0.10 | ± | 0.16 |
| **unknown a** | 0.19 | ± | 0.37 | 0.01 | ± | 0.02 | 0.11 | ± | 0.20 |
| **alpha-copaene** | 0.58 | ± | 1.21 | 0.89 | ± | 0.29 | 1.48 | ± | 1.89 |
| **beta-bourbonene** | 0.21 | ± | 0.58 | 1.69 | ± | 0.86 | 0.72 | ± | 0.68 |
| **beta-caryophyllene** | 1.99 | ± | 3.50 | 37.69 | ± | 17.31 | 24.57 | ± | 18.71 |
| **rotundene** | 0.58 | ± | 0.75 | 0.86 | ± | 1.03 | 1.11 | ± | 1.89 |
| **alpha-humulene** | 0.09 | ± | 0.09 | 0.21 | ± | 0.24 | 0.07 | ± | 0.15 |
| **unknown b** | 0.68 | ± | 1.17 | 2.29 | ± | 2.87 | 14.59 | ± | 14.52 |
| **germacreneD** | 0.47 | ± | 0.55 | 8.41 | ± | 4.89 | 4.13 | ± | 3.53 |
| **bicyclogermacrene** | 0.07 | ± | 0.20 | 0.51 | ± | 0.61 | 0.44 | ± | 0.48 |
| **delta-cadinene** | 0.04 | ± | 0.09 | 0.08 | ± | 0.13 | 0.28 | ± | 0.49 |

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# Signalling receptivity: Comparison of the emission of volatile compounds by figs of *Ficus hispida* before, during and after the phase of receptivity to pollinators

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**Abstract**
Figs and pollinating fig wasps are involved in highly specific mutualisms. Because associations between figs and their pollinating wasps are horizontally transmitted, partner encounter is a crucial step, and is mediated by the emission by receptive figs of the volatile compounds that are detected by the pollinator. However, pollinator attraction is probably not the only function of the volatile compounds produced by figs. Other likely functions include signalling to wasps that a fig has already been pollinated, and deterring or defending against visitors with negative effects on developing figs or the pollinators they contain. The functions of volatile compounds will also change over the course of fig development, and the composition of the odour bouquet is thus also likely to vary. However, this variation and its likely functional importance have rarely been studied. To address these questions, we investigated changes in the composition of the bouquet of volatile compounds, and in the rate of emission of odour components, before, during and after the phase of receptivity to pollinators in *Ficus hispida*, in Yunnan (China). This first study of the dynamics of variation in signal composition throughout the cycle of fig development, provides support for the hypothesis of chemical mimicry in dioecious figs.

**Keywords**: Fig-fig wasp mutualism, chemical mediation, volatile organic compounds, temporal variation

## 1. Introduction

In horizontally transmitted highly specialised mutualisms, partner encounter is a crucial phase in the functioning of the interaction. These include symbiotic protection mutualisms between myrmecophytic plants and their resident plant-ants (Heil and McKey, 2003) and obligate nursery pollination mutualisms. The best-studied examples are the interactions between *Yucca* plants and yucca moths (Dufaÿ and Anstett, 2003) and those between figs and their wasp pollinators (Cook and Rasplus, 2003). In contrast to interactions between plants and herbivores, where plants may escape by their lack of apparency (Feeny, 1976), plants have an interest in signalling their presence to mutualist associates. Signalling by the emission of chemicals, located via olfaction or by contact chemoreception, has been shown to be important in advertising host location and the presence of rewards for the partner in both ant-plant protection mutualisms (Brouat et al., 2000) and nursery pollination mutualisms (Dufaÿ et al., 2003). However, the plant's interest in advertising its location may be restricted to certain times. For example, in pollination mutualisms, attracting the pollinator is only necessary prior to pollination. Following pollination, it may be beneficial to reduce attractiveness of the flower to pollinators, or to other potential visitors, such as herbivores or parasites of developing pollinators or seed predators. Another way to prevent visits of non-mutualists is to emit repellent or camouflage compounds that deter these

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enemies (Schiestl and Ayasse, 2001; Kessler and Baldwin, 2006). However, information on post-pollination changes in the composition and total amounts of volatile compounds is available for only few insect-pollinated species (Arditti et al., 1973; Tollsten and Bergström, 1989; Tollsten, 1993; Schiestl and Ayasse, 2001; Theis and Raguso, 2005; Muhlemann et al., 2006). In these studies, a common trend seems to be a strong quantitative decline of the whole bouquet after pollination and a change in the ratios of compounds in flower scents.

In the interactions between figs and their pollinating wasps, chemical mediation of partner encounter is already known (van Noort et al., 1989; Hossaert-McKey et al., 1994; Gibernau et al., 1998; Song et al., 2001; Grison-Pigé et al., 2002). All of the ~750 *Ficus* species are associated with at least one specific species of pollinating agaonid wasp, which also strictly depends on its host *Ficus* for reproduction. When the fig is receptive, female pollinating wasps, attracted by specific volatile signals emitted by the host fig (Grison-Pigé et al., 2002), penetrate into the fig cavity through the ostiolar bracts (entrance of the fig). Once inside the fig, female wasps pollinate the female flowers and lay eggs in the ovaries of some of them. After several weeks, their offspring emerge in the fig cavity and the new generation of female wasps leaves the fig.

It has been shown that chemical signals emitted by receptive figs appear to enforce specificity of the interaction (Grison-Pigé et al., 2002). As in other specific pollination systems, such as *Silene latifolia* (Muhlemann et al., 2006) or *Ophrys sphegodes* (Schiestl and Ayasse, 2001), we should also expect variation in both the total quantity of odour components emitted and the relative concentrations of the different compounds, once the fig has been pollinated.

This variation is expected for several reasons. First, figs have long-lived inflorescences. The long lifespan of flowers has been interpreted as an adaptation to highly specialized pollination mechanisms (Schiestl and Ayasse, 2000) in which there is often huge temporal variation in pollinator availability (Khadari et al., 1995). The duration of receptivity of figs to pollinators in the absence of wasp visitation is longer then one week for several *Ficus* species (Khadari et al., 1995; Patel and McKey, 1998). After a sufficient number of foundress wasps enter the fig, the attractiveness of the figs to pollinators stops (Khadari et al., 1995; Patel and McKey, 1998). Post-pollination changes in fig traits detected by pollinators (e.g., scent) may signal the closure of the ostiole and the cessation of rewards for the pollinators. In contrast to figs, pollinating fig wasps often live only a few hours. We expect that figs attract pollinators only during the precise period when they can be entered and their ovules fertilized. Also, at that time in their development, figs should emit a very strong signal. Receptivity should thus be marked by sharp peaks in the production of volatile compounds. The production of volatile compounds by receptive figs has been shown to have low energetic cost (Grison-Pigé, 2001). Thus, in this system an induced change in quantities of volatile compounds produced and in their chemical composition, may be sufficient to advertise the end of the receptive period; complete cessation of the production of volatiles may not be necessary

In addition, many non-pollinating wasp species, also highly specific, exploit fig pollination mutualisms and also use chemical cues to locate their hosts (Proffit et al., 2007). These wasps, like pollinators, oviposit on figs at very precise stages of fig development (Kerdelhué and Rasplus, 1996; Peng et al., 2005). These non-pollinating wasps can discriminate among volatile chemicals emitted by figs at different developmental stages (Proffit et al., 2007). Qualitative composition of the bouquet may thus be expected to change over time, according to its function (e.g., attraction of pollinators, defence against herbivorous insects; attraction of seed dispersal vectors). However, few studies have investigated changes in chemical mediation before, during and after the phase of receptivity, or shown how these changes influence behaviour of insects associated with figs (Song et al., 2001; Proffit et al., 2007).

The objective of the present study was to characterize variation in quantity and composition (relative abundance, as well as qualitative differences) of volatile compounds emitted before, during and after receptivity (which corresponded here with pollination) in figs of *Ficus hispida*. We also wished to compare the bouquets of male and female figs in this dioecious fig species.

## 2. Materials and Methods

### *Study site and life history of the studied species*

The study was carried out at Xishuangbanna Tropical Botanical Garden (101°15'E, 21°55'N), Yunnan province, China, between November and December 2005. *Ficus hispida* (section *Sycocarpus*) is a functionally dioecious species. In dioecious fig species, female trees, whose figs contain only female flowers, produce seeds while functionally male trees produce pollen (male flowers) and pollinators (in ovaries of gall flowers) (Janzen, 1979; Kjellberg et al., 1987). The pollinator, *Ceratosolen solmsi marchali* Mayr (Chalcidoidea, Agaonidae), like other pollinating fig wasps, is attracted specifically to trees (both sexes) of its host species by volatile signals emitted by receptive figs (Chen and Song, unpublished data). In the functionally male figs, the pollinator lays eggs in some ovaries of the gall flowers and pollinates only these flowers, so that no seeds are produced. In female figs, pollinators are unable to deposit eggs, and ovules of pollinated flowers develop into seeds. Three species of non-pollinating fig wasps (NPFW) are also specifically

associated with *F. hispida* at Xishuangbanna (*Philotrypesis pilosa* Mayr, *Philotrypesis* sp., and *Apocrypta bakeri* Joseph, identified by J.-Y. Rasplus [pers. comm.]). These wasps oviposit from outside male figs into ovaries containing pollinator eggs (Abdurahiman, 1986; Peng et al., 2005; Proffit et al., submitted). Like the pollinators, females of NPFW are attracted to male trees of their host species by species-specific volatile signals emitted by receptive figs (Proffit et al., 2007; Proffit et al., submitted). After completing development, pollinators and NPFW mate inside the fig, then leave their natal fig in search of a receptive fig. Receptivity is thus a key period in fig development for the persistence of the fig-fig wasp mutualism. Mean duration of receptivity in *F. hispida* is the same in both sexes, 2.4±0.2 weeks, if figs stay unpollinated (Patel and McKey, 1998).

*Volatile collections*

Collecting sufficient amounts of volatile for analysis still can be a limiting factor in odour extraction, especially when individual figs are small and when figs in the appropriate stage are present in limited numbers on each plant. We conducted preliminary extractions to standardize our sampling protocol. In most of trees, for the different stages of development we studied, the number of figs per branch was too low to collect enough amount of volatile for each analysis. Thus because of the necessity to obtain concentrated extracts, odour collections were performed *ex situ*. In previously performed tests on several *Ficus* species, no significant differences were found in the blends of volatiles produced by figs *in situ* and *ex situ* (Grison-Pigé, 2001; M. Proffit, unpublished data). To allow a precise comparison of changes in the composition of odour bouquets over time, and to compare male and female figs in this respect, we thus combined samples for a given developmental phase from several individuals of each sex, taking into account the inter-individual variation between trees (Borges et al., submitted). For each developmental phase, we pooled samples from an equivalent number of figs originating from four individual trees of each sex. Figs in pre-receptive phase were placed in nylon bags to prevent oviposition by pollinators and parasites.

When figs reached receptivity (change in the physical aspect of the figs, opening of the ostiole, and fig wasps becoming attracted to the bagged figs), we experimentally introduced at least three pollinators into each fig tested, using the technique described by Hossaert-McKey and Bronstein (2000), and then marked the experimentally pollinated figs. Experimental introduction permits knowing the exact date figs were pollinated. In our study, receptivity was thus always followed, after a constant time interval, by pollination. For figs at receptivity, at pre-receptive phase, as well as all later developmental stages, we prepared one pooled sample of male figs and one of female figs for odour extraction for each developmental phase (Table 1). To remove environmental or technical effects, volatile chemicals of each sample were extracted using precisely the same method. For each development stage of the figs, female and male figs were extracted the same day.

Volatile compounds released by figs were collected by adsorption-desorption (dynamic headspace technique) (Turlings et al., 1991; Grison-Pigé et al., 2002; Dufaÿ et al., 2003). For odour collection, figs, between 14 and 60 figs (see Table 1), were enclosed in polyethylene terephtalate (Nalophan®) bags, which have been shown not to release volatiles. Airflow was maintained through the bags by two pumps. Entrance and exit flow rates (controlled by flowmeters) were 400 and 300 ml/min. The difference in flux ensured that the system was continuously purged, to compensate for the inevitable leaks, and that no contaminated outside air would enter the system. At the entrance of the bag, air was purified in a charcoal filter. At the exit, air flowed through a trap containing 30 mg of Alltech Super Q absorbent (ARS Inc., Gainesville, FL). Odour collection lasted 3 hours. Blanks were collected in parallel using an empty bag.

*Analysis of volatile compounds*

Trapped odour samples were stored at –18°C until analysis. Trapped volatiles were eluted with 150 µl of dichloromethane; two internal standards (nonane and dodecane, 200 ng $\mu l^{-1}$) were added to each sample for gas chromatography. Volatile compounds were analysed by injection in a CP-3800 (Varian Inc., Palo Alto, USA) gas

Table 1. Number of figs of *F. hispida* used for dynamic headspace extractions for male and female figs sampled at the seven different stages of fig development recognized (R-1: Pre-receptive stage, Receptivity and R+2, R+4, R+8, R+12 and R+16: two, four, eight, twelve and sixteen days, respectively, after pollination). For each developmental phase, we pooled samples from an equivalent number of figs originating from four individual trees of each sex.

| | R-1 | Receptivity | R+2 | R+4 | R+8 | R+12 | R+16 |
|---|---|---|---|---|---|---|---|
| Female | 50 | 24 | 31 | 28 | 35 | 32 | 14 |
| Male | 60 | 24 | 36 | 22 | 35 | 35 | 40 |

chromatograph with FID detector coupled with a Saturn 2000 mass spectrometer (Varian Inc., Palo Alto, USA). For both analyses a Varian column CP-SIL low blend MS (30 m, ID 0.25 mm, film thickness 0.25 µm, carrier with helium (at 1 ml/min) was used. The injector split vent was opened (1/4). Oven temperature was programmed at 50°C during 3 min; then increasing by 3°C $min^{-1}$ to 100°C, by 2.7°C $min^{-1}$ to 140°C, by 2.4°C $min^{-1}$ to 180°C and by 6°C $min^{-1}$ to 250°C. Component identification was based on computer matching of the mass spectra with NIST 98 MS library and on retention indices reported in the literature (Adams, 1995). By comparing spectra of each sample with the respective blank (empty bag, same day of collection), supposedly contaminant compounds were subtracted.

We estimated the total quantities of volatile compounds by using the internal standards (IS) method, by integrating the peak of every substance and dividing every peak area with the integrated IS peak area and multiplying by the amount of IS. We then compared the intensity of volatile emission for different stages of fig development. The relative proportions of the different compounds emitted were also determined for each sample. Principal component analysis (PCA, covariance matrix, StatBox) was used to compare patterns of scent composition among samples. We compared the relative amounts of all the compounds. Percentage similarity (Whittaker and Fairbanks, 1958) of odour composition for one stage among sexes was also estimated. The formula of the percentage of similarity (PS) for one stage of fig development is

$$PS = 1 - 0.5 \sum_{i=1}^{X} [p_{M,i} - p_{F,i}] = \sum \min (p_{M,i} - p_{F,i})$$

Where $p_{M,i}$ is the proportion of the compound $i$ for male (M), and there are $X$ compounds in the blend of male and female figs at this stage. $p_{F,i}$ is the same but for female (F).

## 3. Results

### *Temporal changes in total amount of volatile compounds emitted during fig development*

Temporal patterns of the emission of volatile compounds by figs of both sexes showed one major peak (at receptivity), then a decline in odour production which was sometimes followed by another peak of lower intensity (Fig. 1). Maximum volatile production was reached at full receptivity, when the ostiole opens and the bracts loosen to allow entrance of pollinating fig wasps. Once pollinators were experimentally introduced into a fig, the emission of volatile compounds by the fig decreased rapidly, reaching minimum values 12 to 16 days after pollination. This decrease in quantity of volatile production following pollination was all the more striking in that it occurred two days after pollination. Overall, there was no correlation between diameter of a fig and the quantity of odour components emitted ($r^2 = -0.14$, $P$=0.61). At receptivity, production of volatile compounds was two to four times higher than in other periods.

### *Changes in chemical composition of the bouquet of* F. hispida *over fig development*

The chemical composition of odour bouquets emitted by figs at the different stages is summarised for each sex in Table 2. The detected compounds are divided into three groups based on biosynthetic origin (Knudsen et al., 2006). Within each group compounds are listed in order of retention time in Table 2.

A total of 67 compounds were identified, with sesquiterpenes comprising the largest number of compounds. Depending on the stage of development, the odour blend of *F. hispida* figs comprised between 31 to 54 compounds, and 19 to 34 compounds with relative abundance greater than 1% (Table 2), these latter compounds accounting for 88 to 100% of the blend. Linalool, (E)-β-farnesene and indole were the most abundant compounds at receptivity (Table 2). For both male and female figs, the number of volatile compounds emitted increased around the period of receptivity, then declined rapidly after 2 days for female and 12 days for male. The maximal number of volatile compounds at receptivity was 53 in female figs, and 48 for male figs. The ratio between the number of volatile compounds present at receptivity and those present sixteen days after receptivity was similar in male and female figs (1.63 and 1.51, respectively). At the end of our collection period (16 days after pollination), the number of compounds was reduced to 33 in female figs and 32 in male figs.

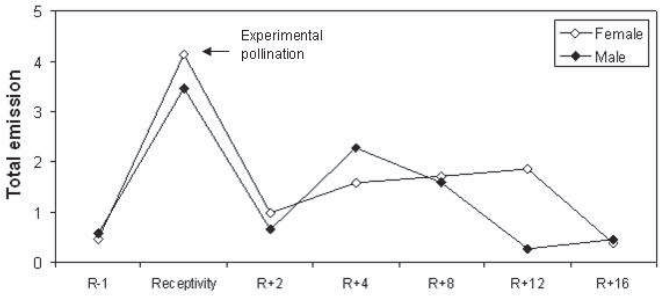
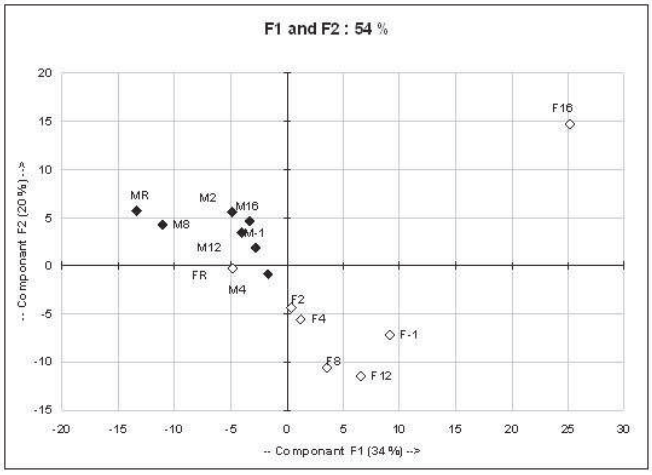



Figure 1. Temporal changes in the total amount of volatile compounds emitted by figs of *F. hispida* before, in ng/fig/hour, during and after pollination (R–1: Pre-receptive stage, Receptivity and R+2, R+4, R+8, R+12 and R+16: two, four, eight, twelve and sixteen days, respectively, after pollination).



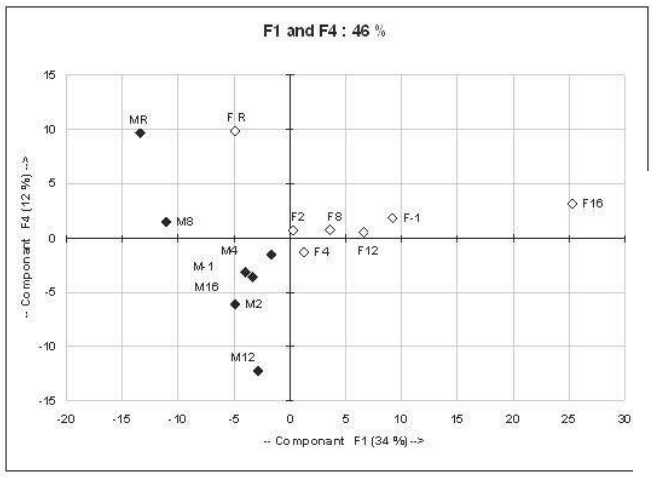




Figure 2. Plot of the principal component analysis (F1 X F2, and F1 x F4) based on the relative proportions of the different volatile compounds in blends of male (M) and female (F) figs of *F. hispida* at the seven different stages of fig development recognized (–1: pre-receptive stage, R: receptivity and 2, 4, 8, 12 and 16: two, four, eight, twelve and sixteen days, after pollination, respectively).

Components Analysis (Fig. 2) illustrates the variation in the chemical signature of the odour bouquet over the cycle of fig development for each sex. Although volatile composition varied substantially in figs of each sex over the course of development, principal components analysis showed that odours of male figs and those of female figs were quite well separated (Fig. 2). The first four principal components together explained 80% of the variance. The chemical signature of male figs and those of female figs remained quite distinct, except at receptive phase, when the signature of female figs fell within, or very close to, the space delimited by male figs. Only at receptivity and immediately afterwards were odours produced by the two sexes so similar. The odour of female figs at sixteen days after pollination was always in an extreme position within

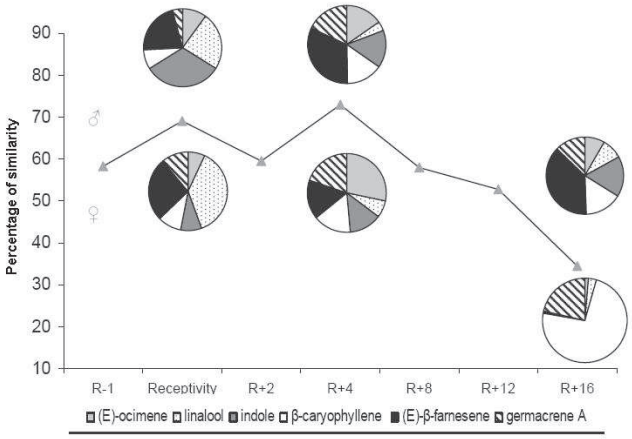



Figure 3. Temporal change in percentage of similarity between male and female figs of *F. hispida* over development. The pie charts present temporal change in the relative quantities of the major compounds (≥4.5% of the total blend) for male (top) and female (bottom) figs at three of the seven developmental stages (see Fig. 1 for legend).

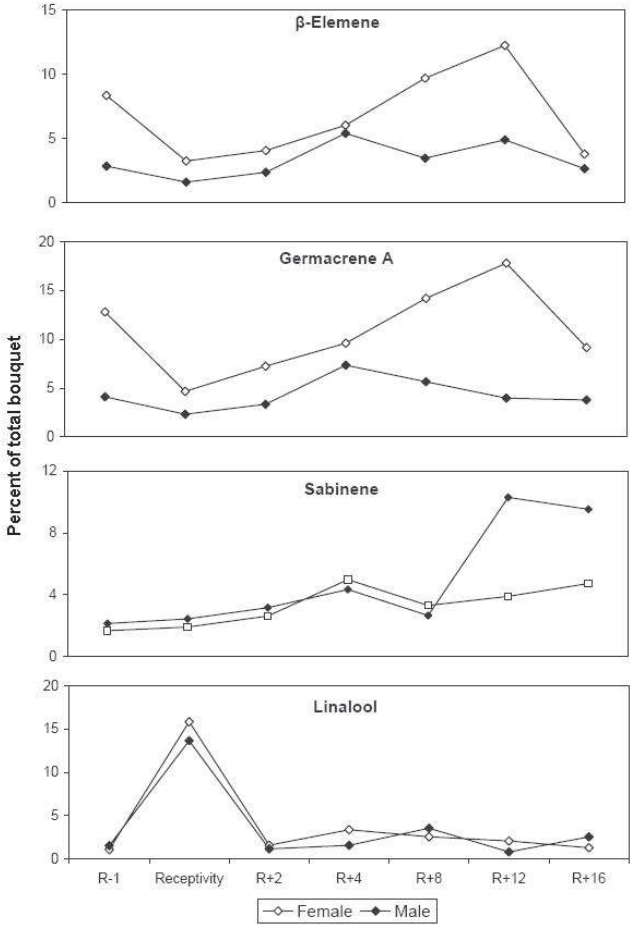



Figure 4. Temporal change in the relative proportions (% of total bouquet) of four major compounds, plotted separately for male and female figs of *F. hispida*.

Table 2. Relative amount of volatile compounds in male and female figs of *Ficus hispida*. The compounds detected in each species are divided into three groups based on general biosynthetic origin (Knudsen et al., 2006) and within all the groups in four classes. Within each class they are listed in order of their Kovat's retention index (RI).

| Compounds | | Female | | | | | | | Male | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IR | R-1 | Recep | R+2 | R+4 | R+8 | R+12 | R+16 | R-1 | Recep | R+2 | R+4 | R+8 | R+12 | R+16 |
| **Fatty acid derivatives** | | | | | | | | | | | | | | | |
| ethyl 2-methyl-butanoate | 827 | 0.53 | 1.28 | 1.66 | 0.23 | 0.51 | | | 0.42 | 1.76 | 3.28 | | | | 1.62 |
| (Z)-3-hexenol | 857 | 1.86 | 0.52 | 0.59 | | | | 0.90 | 1.24 | 0.60 | 1.57 | 0.49 | | | 3.24 |
| 2-heptanone | 892 | 0.30 | 0.47 | 0.94 | | | | | | 0.67 | 1.72 | | | | 2.53 |
| 2-hexyl acetate | 937 | 0.39 | 0.54 | 0.86 | | | | | | 0.69 | 1.82 | | | | |
| (Z)-3-hexenyl acetate | 1006 | 5.94 | 3.06 | 4.02 | 1.94 | 2.42 | 1.55 | | 3.53 | 3.22 | 7.92 | 4.50 | 1.28 | 0.90 | 4.22 |
| hexyl acetate | 1009 | 0.61 | 0.41 | 0.45 | | 0.65 | | | 0.39 | 0.60 | 1.38 | 0.68 | 0.34 | | 1.42 |
| (E)-2-hexenyl acetate | 1010 | 0.91 | | | | 0.42 | | | 0.39 | | 0.58 | 0.33 | 0.17 | | |
| butyl 2-methyl-butanoate | 1038 | | 0.08 | 0.29 | | | | | | 0.14 | 0.38 | | | | |
| decane | 1000 | | 1.09 | | | | | | | 1.07 | | | | | |
| undecane | 1100 | | | | | | | | | | 0.56 | | | 1.40 | |
| tetradecane | 1400 | 2.09 | 0.27 | 0.34 | 0.12 | | 0.32 | 5.43 | 1.13 | | 1.38 | 1.17 | 0.79 | 3.10 | |
| pentadecane | 1500 | 1.56 | 1.56 | 1.57 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| Total percent | | 14.19 | 7.72 | 9.15 | 2.29 | 4.00 | 1.87 | 6.33 | 7.10 | 8.75 | 20.59 | 7.17 | 2.58 | 5.40 | 13.03 |
| **Terpenes** | | | | | | | | | | | | | | | |
| Monoterpenes | | | | | | | | | | | | | | | |
| α-pinene | 933 | 0.63 | 1.56 | 0.68 | 0.99 | 0.73 | 1.02 | 1.36 | 0.77 | 1.33 | 1.30 | 1.45 | 0.70 | 2.39 | 3.21 |
| sabinene | 965 | 1.67 | 1.91 | 2.52 | 4.88 | 3.30 | 3.73 | 4.72 | 2.14 | 2.43 | 3.06 | 4.34 | 2.65 | 10.10 | 9.32 |
| β-pinene | 979 | 0.76 | 1.21 | 1.59 | 0.61 | 0.50 | 0.75 | 1.44 | 0.70 | 0.24 | 1.15 | 1.00 | 1.10 | 2.50 | 7.87 |
| myrcene | 990 | 0.50 | 0.09 | 0.16 | 0.49 | 0.23 | 0.39 | 0.69 | 1.36 | | 0.34 | 1.06 | 0.30 | 0.90 | 1.80 |
| p-cymene | 1023 | | 0.87 | | | | | 0.25 | | 0.26 | | | | | |
| limonene | 1029 | 1.49 | 2.90 | 1.35 | 0.57 | 0.50 | 0.39 | 9.37 | 1.15 | 1.23 | 3.33 | 1.70 | 0.39 | 2.24 | 3.10 |
| 1,8-cineole | 1034 | 1.64 | 2.46 | 1.96 | 4.73 | 3.49 | 4.83 | 0.46 | 1.86 | 2.89 | 2.86 | 4.00 | 4.58 | 8.80 | 2.06 |
| (Z)-β-ocimene | 1037 | 2.17 | | | 2.00 | 0.19 | 0.16 | 5.53 | 1.17 | | | 0.81 | | 0.40 | 2.96 |
| (E)-β-ocimene | 1047 | 3.59 | 2.90 | 2.73 | 13.62 | 7.09 | 5.10 | 0.57 | 4.92 | 5.51 | 3.25 | 6.22 | 5.90 | 15.08 | 2.43 |
| linalool-oxide | 1090 | 0.62 | 0.52 | 0.42 | 0.79 | 0.55 | 0.42 | 0.12 | 0.39 | 0.04 | | 0.55 | 0.45 | | |
| linalool | 1102 | 1.08 | 15.74 | 1.58 | 3.28 | 2.56 | 2.07 | 1.29 | 1.53 | 13.57 | 1.16 | 1.56 | 3.55 | 0.81 | 2.54 |
| 3,oxa-2,2,4-tri-methyl-4vinyl-cyclohexanone | 1108 | 1.28 | 0.28 | 0.88 | 0.96 | 0.68 | 1.24 | 1.74 | 0.68 | | | 0.59 | 0.39 | 0.33 | 1.70 |
| perillene | 1115 | 0.79 | 0.82 | 0.44 | 0.40 | 0.21 | 0.49 | | 1.55 | 1.11 | 1.03 | 0.50 | 0.43 | 0.09 | 1.69 |
| Total percent | | 16.22 | 31.26 | 14.31 | 33.32 | 20.03 | 20.59 | 27.54 | 18.22 | 28.61 | 17.48 | 23.78 | 20.44 | 43.64 | 38.68 |
| **Sesquiterpenes** | | | | | | | | | | | | | | | |
| unknown 1 | 1335 | 1.86 | 1.07 | 1.24 | 1.34 | 1.26 | 1.03 | 2.15 | 3.56 | 1.47 | 1.65 | 3.50 | 1.40 | 0.40 | 1.79 |
| clovene | 1351 | | | | | | | 0.35 | 0.58 | 0.44 | 0.34 | 0.62 | 0.28 | | |
| α-longipinene | 1360 | 1.08 | 0.68 | 0.70 | 0.82 | 0.75 | 0.55 | 1.67 | 1.78 | 0.87 | 1.33 | 1.83 | 0.75 | 1.61 | |
| unknown 2 | 1373 | | | 0.26 | 0.26 | 0.23 | 0.28 | | | 0.17 | | | | | |
| isoledene | 1376 | | 1.18 | 1.10 | 0.41 | 0.32 | 0.58 | | | 0.24 | 0.47 | 0.27 | 0.22 | | |
| α-copaene | 1379 | 0.95 | 0.45 | 0.12 | 0.47 | 0.47 | 0.79 | 1.70 | 2.32 | 0.82 | 1.46 | 0.80 | 0.83 | 0.93 | |
| daucene | 1381 | 5.06 | 2.26 | 2.71 | 1.84 | 2.28 | 1.13 | | | 0.79 | 1.13 | 1.16 | 0.89 | | 2.41 |
| bicycloopposit-4-ene | 1383 | 5.06 | 3.50 | 6.41 | 6.32 | 6.68 | 4.78 | 2.34 | 3.50 | 2.40 | 1.26 | 4.19 | 1.44 | 2.78 | |
| α-isocomene | 1392 | | 1.80 | 0.31 | 1.61 | 1.66 | 2.41 | 1.47 | 3.60 | 0.53 | 1.26 | 2.86 | 1.13 | 2.34 | |
| β-elemene | 1392 | 8.39 | 3.15 | 3.98 | 5.95 | 9.66 | 12.14 | 3.79 | 2.85 | 1.61 | 2.38 | 5.31 | 3.48 | 4.80 | 2.66 |
| β-longipinene | 1403 | | 0.52 | 0.49 | 0.50 | 0.42 | 0.35 | | | | | 1.10 | 0.79 | 0.31 | 1.42 |
| 7-epi-a-cedrene | 1403 | | 0.24 | 1.37 | 0.17 | | | | | | 0.16 | 0.38 | 1.57 | 0.48 | |
| unknown 4 | 1404 | | | 1.20 | | | | | 0.23 | | | | 0.76 | | |
| isocaryophyllene | 1409 | | 0.27 | | 0.17 | | | 0.33 | | 0.89 | 0.23 | 0.20 | 1.03 | | |
| cis-α-bergamotene | 1415 | | 0.95 | 2.32 | 0.24 | 0.20 | | | | 0.09 | | 0.16 | 1.29 | | |
| β-caryophyllene | 1423 | 9.64 | 3.91 | 5.94 | 7.45 | 4.90 | 6.56 | 30.91 | 7.92 | 4.18 | 5.08 | 6.00 | 3.86 | 4.76 | 4.40 |

Table 2. Continued.

| Compounds | | Female | | | | | | | Male | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IR | R-1 | Recep | R+2 | R+4 | R+8 | R+12 | R+16 | R-1 | Recep | R+2 | R+4 | R+8 | R+12 | R+16 |
| oppositadiene | 1423 | | | | | | | 0.58 | | | 0.38 | 0.71 | 0.51 | 0.89 | |
| unknow7 | 1430 | | | | | | | | 0.71 | | | | 0.80 | | |
| trans-α-berga-motene | 1435 | | 0.82 | 3.35 | 0.75 | 0.40 | 0.95 | 1.52 | 1.05 | | 0.30 | 1.15 | 1.48 | 0.44 | |
| aromadendrene | 1443 | | 0.30 | 0.91 | | | 0.49 | | 0.57 | | | 0.63 | 0.43 | | |
| (E)-β-farnesene | 1454 | 5.63 | 10.95 | 15.44 | 7.69 | 6.58 | 7.80 | 0.35 | 20.40 | 12.36 | 13.03 | 13.09 | 8.61 | 6.17 | 10.90 |
| α-humulene | 1458 | 1.27 | 0.43 | 0.16 | 0.88 | 0.51 | 1.35 | 3.03 | 1.22 | 1.36 | 1.05 | 1.41 | 0.70 | 2.67 | 1.62 |
| α-neoclovène | 1460 | 2.50 | 1.69 | 2.55 | 1.77 | 2.05 | 1.35 | 3.01 | 1.22 | 1.44 | 1.13 | 2.83 | 1.72 | 2.67 | |
| alloaromadendrene | 1464 | 1.98 | 0.57 | | | 5.33 | | | 2.24 | | 0.23 | 1.15 | 1.05 | | 2.02 |
| selina-7,11-diène | 1471 | 8.99 | 4.00 | 6.22 | 5.50 | 6.26 | 6.67 | | 0.70 | 0.80 | | | | | |
| amorpha-4,7-diene | 1476 | | 2.19 | 2.17 | 0.36 | 1.68 | 1.64 | | | 0.47 | 2.59 | 0.23 | | | 1.39 |
| γ-muurolene | 1477 | | | | | 0.45 | | 0.46 | | 1.14 | 0.86 | 0.48 | 0.84 | 0.71 | |
| α-curcumene | 1484 | 1.00 | 0.34 | | | 0.39 | | | 0.57 | | 0.35 | | | | |
| germacrene D | 1484 | 0.87 | | | | 0.34 | 0.58 | | 1.56 | | | | | | |
| β-selinene | 1492 | | 0.63 | | | | | 0.39 | 5.38 | 3.41 | 11.24 | | 8.48 | 8.38 | 3.17 |
| γ-amorphene | 1497 | | | 0.68 | | | | | 0.61 | 0.75 | 1.58 | 0.78 | | | |
| α-selinene | 1497 | | 0.54 | | 0.74 | 0.75 | 1.40 | 1.56 | 0.72 | 1.03 | 2.03 | | 2.06 | | |
| (E,E)-α-farnesene | 1506 | | | 0.42 | | | | | | | | | | | 2.91 |
| germacrene A | 1511 | 12.84 | 4.55 | 7.13 | 9.51 | 14.15 | 17.68 | 8.96 | 4.90 | 2.24 | 3.23 | 7.24 | 5.64 | 3.83 | 3.57 |
| δ-cadinene | 1521 | 1.07 | | | | 0.34 | 0.27 | | | | 0.62 | 0.59 | 0.59 | 0.64 | |
| β-sesquiphell-andrene | 1526 | | 2.59 | 4.56 | 1.74 | 1.40 | 1.89 | | | 0.99 | 2.12 | 1.70 | 2.20 | 0.22 | 2.10 |
| unknown 5 | 1529 | | 2.80 | 1.10 | | 0.33 | 0.29 | | 1.41 | 0.78 | 0.28 | 0.41 | 0.38 | | |
| α-alaskene | 1532 | | | 1.21 | | 0.34 | 0.21 | | | 0.57 | 0.23 | 0.31 | 0.21 | | |
| unknown 6 | 1613 | | 2.60 | | | | 0.06 | | | | | | | | 1.58 |
| Total percent | | 68.19 | 54.98 | 74.05 | 56.49 | 70.13 | 73.23 | 64.57 | 69.60 | 41.84 | 58.00 | 61.09 | 55.42 | 45.03 | 41.94 |
| **Shikimic acid derivatives** | | | | | | | | | | | | | | | |
| benzyl alcohol | 1030 | | 0.47 | | | | | | | 0.48 | | | | | |
| methyl benzoate | 1091 | | 0.38 | | | | | | | 0.50 | | | | | |
| indole | 1298 | 1.40 | 3.63 | 0.92 | 6.34 | 4.28 | 2.75 | | 3.52 | 18.26 | 2.37 | 6.40 | 20.00 | 4.37 | 4.79 |
| Total percent | | 1.40 | 4.48 | 0.92 | 6.34 | 4.28 | 2.75 | 0.00 | 3.52 | 19.24 | 2.37 | 6.40 | 20.00 | 4.37 | 4.79 |
| Total number of compounds | | 38 | 53 | 48 | 40 | 46 | 42 | 33 | 44 | 48 | 50 | 48 | 48 | 35 | 32 |

the space occupied by female figs. This pattern was not found in male figs, for which odour of male figs at sixteen days after receptivity remained within the space defined by the chemical bouquet of male figs at other stages of development.

*Similarity of the chemical signature among sexes*

Percentage similarity of volatile bouquets between male and female figs at the different stages showed a first peak (69.1%) at receptivity and a second, slightly higher peak (72.9%) four days after pollination (Fig. 3). However, different compounds were responsible for the similarity of the chemical signature at these two stages (Fig. 3), with germacrene A and indole replacing linalool after pollination. Percentage similarity declined rapidly after this post-pollination stage (4 days after) to reach the minimal value (34.4%) sixteen days after receptivity. Volatile bouquets were thus most divergent between male and female figs at the end of our sampling period 16 days after pollination.

*Temporal dynamics of emission of some major compounds*

Different odour components showed different patterns of variation over fig development. Fig. 4 presents some examples, drawn from among compounds that contributed strongly to the variation. Some compounds were abundant before receptivity, as for example β-elemene and germacrene A. In figs of both sexes, the receptive phase was marked by the emission of large quantities of linalool (Fig. 4), which was the predominant component of the bouquet at that phase. In both sexes, the stages soon after pollination are marked by an increase in concentrations of two compounds, β-elemene and germacrene A (Fig. 4), which continued to increase with fig development. While

also presenting a post-pollination increase in production, indole presented a different pattern, with male figs always producing higher concentrations of this compound than female figs, and with an apparent temporary decrease in odour production in the middle of the interfloral stage (Table 2). Finally, sixteen days after pollination male and female figs had very different chemical signatures, with female figs emitting higher concentrations of sabinene (Fig. 4).

## 4. Discussion

In this study, we have examined the dynamics of variation in the composition and emission rate of volatile compounds throughout fig development. As pointed out by Dufaÿ et al. (2003), the likely diversity of biological functions of odour components in nursery pollination mutualisms render inadequate a simple comparison of odours at two points in time, pollination and post-pollination. Other studies have produced evidence that pollinators are attracted only to receptive-phase figs (Chen and Song, unpublished data), and that post-pollination variations in odour emitted by figs are used by non-pollinating fig wasps to locate oviposition sites at a particular moment during the development of figs and pollinator larvae (Proffit et al., 2007). We show here quantitative and qualitative differences in composition of volatile compounds that can account for such stage-specific attraction. However, our results also show other changes in fig odours over time, suggesting additional potentially adaptive patterns of variation.

The composition of odour extracts of receptive figs of the species we studied, *F. hispida*, has already been reported by Song et al. (2001). Our results differ from those presented in that study, which used solvent extraction, a passive technique that recovers not only volatile compounds emitted by figs, but in addition chemicals present in the superficial cell layers of the plant. Our study, based on dynamic headspace extraction, reported only volatile compounds, which are potentially detectable by pollinators and other animals. Previous studies show that chemical compounds emitted by receptive figs of *F. hispida* are the same but varied greatly among plants in relative proportions, within *Ficus* populations (Borges et al., submitted; Proffit et al., submitted). In our study population, the odour bouquet is dominated by terpenes (13 monoterpenes and 39 sesquiterpenes, Table 2). Comparing the scent produced by receptive figs of *F. hispida* with those of other *Ficus* species (Grison-Pigé et al., 2001; 2002; Borges et al., submitted; Proffit et al., submitted), we found a similar blend. In each *Ficus* species, floral scents have fewer than 50 compounds belonging to four different chemical classes: monoterpenes, sesquiterpenes, fatty acid derivatives and shikimic acid derivatives. One to three main compounds always represent 20% to 92% of the total blend (Grison-Pigé et al., 2001; 2002). These main compounds are widespread in floral scents of plants of different families (Knudsen et al., 2006), but they generally differ among fig species (Grison-Pigé et al., 2001, 2002). By controlling the synthesis and emission of volatile compounds to produce a strong and specific signal during receptivity, figs can advertise their availability to pollinators. The post-pollination decline in odour production we found is consistent with results of other studies on *Ficus* (Grison-Pigé et al., 2002), on other nursery-pollination mutualisms (Muhlemann et al., 2006) and other pollination mutualisms (Arditti et al., 1973; Tollsten and Bergström, 1989; Tollsten, 1993; Schiestl and Ayasse, 2001; Theis and Raguso, 2005).

Our results also reveal three other striking phenomena. First, although female and male figs tend to have quite distinctive odours throughout much of their development (Fig. 2), at receptivity their odour bouquets seem similar (Borges et al., submitted). This pattern is consistent with the hypothesis that in dioecious figs, selection results in reciprocal chemical mimicry between male and female figs (Grafen and Godfray, 1991; Patel et al., 1995; Anstett et al., 1997). In our study, odours produced by figs of the two sexes were most similar precisely at the point in the cycle when it is important for the functioning of the system that pollinators be unable to distinguish between figs of the two sexes (Patel et al., 1995; Grison et al., 2001). In both female and male receptive figs, the odour bouquet is dominated by the production of large amounts of linalool. Linalool is a frequent component of many floral odours (Knudsen et al., 2006). Gibernau (1997) showed that a mixture of linalool with two monoterpene compounds (linalyl oxides and benzyl alcohol) in certain proportions was necessary and sufficient to attract *Blastophaga psenes*, the specific pollinator of *F. carica*. Alone, linalool is thus unlikely to ensure specific attraction of the pollinator of *F. hispida*. However, in receptive figs of this species, linalool is associated with lower concentrations of other compounds, such as indole and ocimene, and the composition of this bouquet could ensure the specificity of attraction (Proffit et al., submitted). Not only the qualitative composition, but also the quantitative strength of the message, showed no apparent difference between male and female figs.

The second striking phenomenon found in our study was the shift in composition of the odour bouquet following pollination. Male and female figs underwent similar changes, so that although they continued to resemble each other, different compounds were responsible for this resemblance. In figs of both sexes, there was an increase in the concentration and relative proportions in the bouquet of germacrene A and indole. Together with changes in the physical structure of the figs likely to be perceptible by pollinators (e.g., closing of the ostiole), the shift in composition of the odour bouquet could constitute a signal

that repels pollinating fig wasps. Compounds that are emitted at a higher flux rate following pollination are likely candidates as repellent compounds, that function either to prevent additional pollinator visits or to deter enemies such as seed predators (Raguso, 2003). Immediately after pollination, as at receptivity, the interests of male and female figs, and the function of the odours they emit, are similar. While they are likely candidates for such roles, the deterrent function of germacrene A and indole remains to be demonstrated (Chen and Song, unpublished data).

The third striking result of our study was the increasing divergence between the odour bouquets of male and female figs as they developed. This divergence can be seen from day 8 after pollination onwards, and the separation in chemical signature between the two sexes was maximal 16 days after pollination (Fig. 2). Percentage similarity between odour bouquets of male and female figs also reached its minimum observed value at this stage of development, which corresponded to the time in the cycle when the odour bouquets of female figs included the smallest number of compounds. In *F. hispida*, figs at this stage are nearing the end of the interfloral phase and will soon reach maturity, when they must attract frugivorous vertebrates that disperse their seeds (mainly bats in the case of *F. hispida*). Divergence in the odour bouquets of female figs at this stage might be linked to changes associated with this specific biological function (or in the related function of avoiding seed predators), which is not performed by male figs (Lambert, 1992; Patel and McKey, 1998; Dumont et al., 2004).

This first study of the dynamics of variation in signal composition throughout the cycle of fig development has provided support for the hypothesis of chemical mimicry in dioecious figs, and has demonstrated the change, in both male and female figs, in the composition of the odour bouquet following pollination. Our study also suggests many interesting perspectives that should be pursued in more detailed studies. Male figs must protect the developing pollinators. What defensive roles, if any, are played by odour compounds? Do developing figs produce compounds that deter parasites of pollinators? Alternatively, is there selection against production of distinctive compounds that might attract these enemies? In this study, sampling of female figs ceased before their maturity and so further studies should sample mature female figs, to test the prediction that they produce distinctive odours specifically attractive to frugivorous vertebrates (R. Borges, pers. comm.).

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# CHAPITRE 2 :
# Travaux Expérimentaux

## PARTIE C :

## *PARFUM DE FIGUES :*

## MÉDIATION CHIMIQUE & DISPERSION DES GRAINES

**Articles associés :**

Manuscrit 9 : **Soler C.**, Bessière J.-M., Schatz B. and Hossaert-McKey M. Scent as a component of dispersal syndromes: a comparative analysis in the genus *Ficus*. *In prep.*

Manuscrit 10 : **Soler C.**, Chen J., Bessière J.-M., Schatz B. and Hossaert-McKey M. Mutualistic partners require convergence and then divergence of fig scents. *In prep.*

Dans sa revue sur la biologie des figuiers, Janzen (1979) pose la question suivante : « Qui mange des figues ? ». Sa réponse est simple : « Tout le monde ! ». Comme explicité dans l'introduction de ce manuscrit, de par leur fructification continue sur l'année, les figuiers tropicaux constituent une ressource essentielle pour les frugivores en saison sèche. En étudiant les données de frugivorie disponibles sur 260 espèces de *Ficus* (≈30% des espèces décrites), Shanahan et al., (2001) ont mis en évidence que plus de 1200 espèces d'oiseaux et mammifères étaient « figovores », en plus de quelques espèces de poissons et de reptiles. Au total, ces espèces se répartissent en plus de 90 familles et de 520 genres différents. Une telle diversité d'espèces animales se nourrissant de figues s'explique notamment par la diversité du genre *Ficus,* tant en termes de morphologie, de distribution, ou encore de valeurs nutritives des fruits de ces espèces. Il est aussi intéressant de noter l'existence de forts chevauchements au sein des guildes de frugivores associées aux *Ficus* ; mais il est possible d'y distinguer des spécialistes, certains animaux de cette guilde n'étant qu'occasionnels (Shanahan *et al.*, 2001). De plus, une seconde phase de dispersion peut parfois s'ajouter. C'est le cas chez *Ficus microcarpa* dont les graines ingérées et disséminées par des oiseaux seront ensuite transportées vers d'autres sites par des fourmis attirées par l'élaïosome[9] présent sur les graines de ce figuier (Kaufmann *et al.*, 1991). Chez le figuier méditerranéen et quelques autres figuiers tropicaux, les figues tombées au sol par barochorie peuvent aussi faire l'objet d'une dispersion secondaire par des fourmis granivores, et ce même en l'absence d'élaïosome sur les graines (B. Schatz, comm. pers.). Parmi les oiseaux, les perroquets et les pigeons semblent être les plus gros consommateurs de figues d'espèces tropicales (mais aussi d'importants prédateurs de graines) ; chez les mammifères il s'agit surtout des primates, des écureuils et des chauves-souris frugivores.

[9] Appendice charnu attaché aux graines de certaines espèces de plantes, riche en lipides et en protéines, il est responsable de l'attraction de fourmis, et représente ainsi une adaptation typique à la myrmécochorie.

Au cours de cette thèse, nous nous sommes intéressés à l'attraction olfactive de la guilde des frugivores par les figuiers, et plus précisément au lien entre identité du frugivore principal, stade phénologique, et parfums des figues. L'association entre les différentes espèces de figuiers et leurs disperseurs montre que la chiropterochorie (dispersion des graines par les chauves-souris) est un processus rencontré chez une majorité des espèces tropicales de figuiers (Hodgkison *et al.*, 2003), suggérant l'existence d'un flux de gènes sur de longues distances pour ces plantes (Alvarez *et al.*, 2010). Une espèce telle que *Ficus hispida* est connue pour une dispersion des graines principalement assurée par des chiroptères, et pour l'émission, au stade de dispersion, d'un bouquet caractérisé par de grandes quantités de dérivés d'acide gras, des COVs associés à la fermentation des fruits (Hodgkison *et al.*, 2007; Borges *et al.*, 2008). Ces deux traits écologiques pourraient être associés en supposant que ces COVs soient responsables de l'attraction des chauves-souris frugivores, et soient des indices phénologiques du degré de maturité des fruits, similairement à ce que nous avons démontré précédemment lors de la pollinisation. Ainsi, une trop forte concentration de ces COVs serait le signe d'une trop grande maturité du fruit, ce qui a un effet répulsif sur les chiroptères (Sanchez *et al.*, 2004). De surcroît, Hodgkison et al. (2003) ont démontré que, contrairement à d'autres espèces animales, le passage dans le tractus digestif de chiroptères ne réduisait en rien la capacité germinatrice des graines. Cette dernière est même augmentée dans certains cas (Heer *et al.*, 2010; Albrecht, comm pers). L'interaction entre figuiers et chiroptères est ainsi particulièrement intéressante à étudier, sachant, en outre, qu'il est pertinent de considérer ces deux groupes d'espèces comme des espèces clefs de voûte dans le fonctionnement des écosystèmes. En effet, si ce rôle est déjà connu pour les figuiers, ce ne sont pas moins de 15% des espèces végétales d'une forêt malaise qui dépendent de chiroptères pour leur dispersion ou leur pollinisation (Hodgkison *et al.*, 2003).

L'ensemble des espèces frugivores constitue ainsi un groupe fonctionnel important dans la dynamique forestière tropicale, en permettant la dissémination des graines, et en assurant ainsi la régénération des arbres et des lianes. Il a effectivement été démontré que, dans les fragments forestiers de taille réduite des monts Usambara, en Tanzanie, la diminution de l'abondance des espèces frugivores, et la disparition de bon nombre d'entre elles, entrainaient une diminution de la régénération des arbres par déficit de dispersion (Cordeiro and Howe, 2001). Ce groupe fonctionnel primordial dépend à son tour de l'existence de ressources suffisantes en fruits tout au long de l'année pour assurer sa survie. En raison de leur rôle clef de voûte sous les tropiques, il a même été proposé que la très forte diversité des espèces frugivores observée dans le sud-est asiatique serait liée à l'abondance locale des *Ficus* (tant au niveau spécifique que numérique), alors qu'inversement, le faible nombre d'espèces frugivores présentes à Madagascar s'expliquerait par la plus faible abondance de *Ficus* (Goodman & Ganzhorn, 1997).

Dans ce contexte, l'île de Madagascar est un cas très intéressant pour l'étude des relations plantes/frugivores, car 96% des espèces de plantes y sont endémiques (Schatz, 2001), et que la guilde associée de frugivores est très peu diversifiée (Goodman & Benstead, 2003). En fait, au delà même de la faible densité de frugivores disperseurs de graines, c'est la structure de cette guilde qui est originale. En effet, sur l'ensemble des oiseaux malgaches, seuls 8% sont frugivores, ce qui représente un pourcentage exceptionnellement faible (Fleming *et al.*, 1987). De même, les chauves-souris frugivores ne sont représentées à Madagascar que par trois espèces (appartenant à trois genres différents, Goodman *et al.*, 1997). En fait, le groupe principal d'animaux frugivores présents à Madagascar est constitué par les lémuriens. Dans ce contexte malgache particulier vis-à-vis de la dispersion, les *Ficus* pourraient constituer la principale ressource expliquant la forte densité en lémuriens dans la région de Ranomafana-Andringitra

(Johnson, 2004). Cependant, les lémuriens forestiers se déplaçant exclusivement au sein du couvert forestier, leur intervention sera quasi inexistante dans les processus de nucléation par formation de nouveaux bosquets forestiers autour d'arbres attirant des frugivores. Même s'ils sont représentés par un faible nombre d'espèces, les chauves-souris et les quelques oiseaux frugivores ont donc un rôle important à jouer dans la régénération de la forêt, en dispersant les graines en milieu forestier et en apportant une partie de ces graines, de la forêt vers des zones plus ouvertes, où ces figuiers peuvent aussi se développer, comme en témoigne la présence actuelle d'arbres isolés (Carrière *et al.*, 2002). Par conséquent, le chevauchement existant entre différentes espèces et groupes d'espèces de frugivores peut être particulièrement élevé dans le cas malgache. En outre, la situation malgache permet également d'analyser les conséquences en cascade de la présence prépondérante d'un groupe de disperseurs de graines sur les caractéristiques de plantes donnant des fruits charnus, et sur l'abondance et le comportement des autres espèces consommant ces fruits. Par ailleurs, il est intéressant de constater que quelques espèces de *Ficus* sont à la fois présentes sur le continent africain et sur Madagascar. Cette particularité dans la répartition géographique de ces espèces rend possible, et particulièrement pertinente, l'étude des convergences évolutives concernant les syndromes de dispersion, et notamment l'émission de COVs attractifs en réponse à la pression de sélection due à la dispersion par les lémuriens.

Dans le premier article de cette section (article n°9), nous nous sommes intéressés aux odeurs émises par les figues fruits, comme caractère à prendre en compte dans l'étude des syndromes de dispersion. Notre objectif a été de tester si, à différentes espèces zoochores de figuiers, était associée l'émission d'odeurs différentes, au stade de dispersion. Cependant, en accord avec la littérature, notre attendu n'était pas aussi strict que pour l'interaction figuier/pollinisateur, où la spécificité est extrême, mais il correspondait plutôt à

la mise en évidence des différences dans les odeurs émises selon des groupes d'espèces, comme les oiseaux et les mammifères. Nous avons mis en évidence l'existence de différences significatives dans les odeurs émises entre les figuiers dispersés majoritairement par les oiseaux, et ceux dispersés majoritairement par les mammifères. Plus étonnamment, une différence existe également entre les bouquets volatils d'espèces dispersées majoritairement par les lémuriens, et ceux des espèces dispersées par les chauves-souris. Cette différence se perçoit même à un niveau intraspécifique, dans le cas de *F.* lutea, qui émet un bouquet différent en Afrique du Sud (où ses graines sont majoritairement dispersées par des chauves-souris) et à Madagascar (dispersion par des lémuriens). De plus, nous avons aussi démontré que les COVs impliqués pour ces deux derniers groupes de disperseurs sont majoritairement des dérivés d'acides gras, alors que les espèces de figuiers ornithochores émettent principalement des terpènes. Enfin, nous avons également déterminé que les figuiers ornithochores émettent des quantités totales plus faibles d'odeurs que les autres. Nous avons donc montré qu'il existait des différences à plusieurs niveaux dans l'odeur émise, et donc l'appartenance de l'odeur dans le syndrome de dispersion des figuiers. Nous démontrons ainsi l'existence d'une pression de sélection pour une certaine spécificité du message au stade dispersion, comme au stade réceptivité, malgré un degré de spécificité moindre.

L'article n°10 reconsidère les implications de la dioécie, mais dans le contexte de la dispersion. Pour la phase de pollinisation, nous avons montré précédemment l'existence d'un mimétisme chimique intersexuel des bouquets olfactifs à réceptivité, permettant la pollinisation par tromperie. *A contrario*, pour la dispersion, il serait logique que la sélection naturelle favorise une divergence entre la composition chimique des bouquets des deux sexes. En effet, il serait pertinent que les frugivores soient attirés par les figues femelles uniquement, c'est-à-dire les figues fruits qui contiennent les graines. En outre, la consommation des figues mâles serait même, d'une part

coûteuse en temps pour les disperseurs, et d'autre part coûteuse pour le figuier, puisqu'il y aurait alors consommation du pollen et des pollinisateurs. En testant cette hypothèse de divergence intersexuelle des bouquets au stade dispersion, nous avons pu mettre en évidence des différences significatives entre sexes, dans le bouquet olfactif émis chez quelques espèces de figuiers dioïques, et donc l'existence d'une inversion des pressions de sélection sur le bouquet olfactif entre les stades de réceptivité et de dispersion.

# Scent as a Component of Dispersal Syndromes: a Comparative Analysis in the Genus *Ficus.*

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Before publication, we still have to perform several other analyses. We first want to complete our dataset, notably by integrating *Ficus lutea* results in the global dataset. Moreover, we want to analyze in more details the chemistry (work on family of compounds) and diversify our statistical approach. If our results are confirmed by these further steps, we planned to submit to journals such as *Animal Behaviour* or *Biotropica*.

## ABSTRACT

Many studies of plant/frugivore interactions have focused on identifying traits adapting plants to different seed-dispersing animals, but almost no work has examined the role of fruit scents in differential attraction. We used the well-known example of Ficus species to focus on this neglected element of dispersal syndromes. *Ficus* trees are displaying an unique phenology of a year-round fruiting and they have been considered as keystone resources for frugivores. Figs of some species are eaten primarily by birds, others by bats and others mammals, notably lemurs in Madagascar. Little is known about the specific attraction of these frugivores by fig species when they are mature. Recent works have shown that mutualisms between figs and their specialized pollinating wasps are chemically mediated. The emission of specific volatile signals by figs and their perception by the pollinator ensure encounter of the mutualists. To explore whether fig/frugivore interactions are also chemically mediated, we used adsorption/desorption methods to study scents emitted by mature fruits of several fig species from different tropical regions. We examined whether the identity of the main seed disperser (bats/birds/lemurs) was associated with differences in the quality and quantity of the scents emitted by figs at the seed-dispersal stage. We found consistent differences between scents emitted by mammal- and bird-dispersed fig species, and interestingly even between scents emitted by bat- and lemur-dispersed fig species. Our results suggest that differences among taxa of seed dispersers have influenced the evolution of the dispersal syndromes of fruits in which chemical mediation plays an important rule.



## KEY-WORDS

Scent, dispersal, frugivores, *Ficus*, syndromes, plant-animal interactions

## INTRODUCTION

Seed dispersal is a critical phase in the life cycle of most terrestrial plants (Howe and Smallwood 1982; Nathan and Muller-Landau 2000). Indeed, plants that fail to disperse are unlikely to reproduce (Harms et al. 2000; Howe and Miriti 2000). Thus, seed dispersal is geographically widespread and occurs in all plant taxa (Herrera and Pellmyr 2002). For example, up to 90% of woody plants in tropical forests disperse via the consumption of fruits and subsequent defecation of their seeds by birds and mammals; these plant species produce a variety of fruits that attract a wide variety of seed-dispersing frugivores (Howe and Smallwood 1982). Given large differences in how frugivorous taxa perceive fruits and disperse seeds, and similarly large differences in fruit traits among plant taxa, many studies have hypothesized the existence of “dispersal syndromes” to describe the co-occurring sets of fruit traits matched to the behavior, physiology, and morphology of different types of frugivores (Jordano 1995; McKey 1975; Van der Pijl 1982). In fact, these syndromes are often called upon to demonstrate how frugivores have shaped the evolution of fruit traits (Kalko et al. 1996; Lomascolo et al. 2010).

The unique phenology of figs (*Ficus* spp.), with year-round fruiting providing resources during the lean season to a huge variety of frugivorous species, explains that figs are often described as keystone species in tropical ecosystems (Shanahan et al. 2001). Observations in tropical rain forests have revealed a broad diet overlap between birds and mainly diurnal mammals when feeding on figs (Shanahan et al. 2001; Breitwisch 1983). This has led to the widespread belief that interactions between figs and their vertebrate seed dispersers are relatively generalized, as characterized by large crops of low-quality fruits consumed and dispersed by diverse

and taxonomically disparate groups of frugivores. However, two different types of figs are generally described in the literature (Kalko et al. 1996). Diurnally dispersed figs are often characterized by visually conspicuous fruit displays, particularly bright colors that stand out against surrounding vegetation (e.g., orange and red) while nocturnally dispersed species are often characterized by distinctive odors rather than colors (Kalko et al. 1996; Lomascolo et al. 2010; Shanahan et al. 2001; Borges et al. 2008; Hodgkison et al. 2007; Lambert and Marshall 1991). These adaptations in fruit display and chemistry correspond closely with the sensory capabilities and foraging behavior of both groups of disperser—as birds and diurnal mammals have well-developed color vision, and locate fruits primarily by sight (Dominy et al. 2001; Schaefer et al. 2004). In contrast, fruit bats and other nocturnal mammals have an acute sense of smell, and locate fruits primarily by olfaction (Kalko et al. 1996; Hodgkison et al. 2007; Korine and Kalko 2005; Sanchez et al. 2006). Thus, many fig species are likely to be more specialized in their interactions with species groups of frugivores than previously thought (Lomascolo et al. 2010; Borges et al. 2008). These observations have conducted to consider two main types of dispersal syndromes: the bird-dispersed species syndrome (fruits are conspicuous, little, positioned on branches and with few scent) and the bat-dispersed species syndrome (fruits are dull, big, caulichorous and with a strong smell) (Kalko et al. 1996). But interestingly, scent is often a neglected element of the description of these dispersal syndromes, and fruits are only describe as having a strong versus no or few smell (Borges et al. 2008). In fact, only two studies have really examined the scent emitted by mature figs, i.e. identify the VOCs emitted and their relative amounts (Borges et al. 2008; Hodgkison et al. 2007), and another one just quantify the emitted amount without

identification (Lomascolo et al. 2010). Moreover, these studies were performed in limited geographic areas and so the associated frugivores guild should be restrained.

In this study, we examined the scent emitted by figs at dispersal stages from different world areas, where the associated guilds of frugivores are different. We notably investigate this question in Madagascar, offering the advantage to have *Ficus* species dispersed by prosimians, and then testing the hypothesis of the existence of particularities of primate-dispersed fruits. Thus, we performed a multispecies analysis to test the hypothesis of distinct dispersal syndromes, focusing on a precise ecological trait, the emitted scent. By trying to characterize common ecological traits in relationship with the identity of the main disperser, our results allowed us to check the generalization of this hypothesis in the figs/dispersers interaction.

## MATERIAL & METHODS

### Material

We studied the volatile emissions of 55 trees, from 11 different species: *F. tiliifolia, F. auriculata, F. montana, F. sur, F. trichopoda, F. microcarpa, F. aurea, F. macrophylla, F. hispida, F. exasperata* and *F. lutea*. *Ficus montana* trees were growing in greenhouses on the Leeds University campus in England, and all others *Ficus* trees have been sampled in their natural habitat, in different tropical areas in America, Africa and Asia. All the trees of a same species have been sampled in a same population, except for *F. lutea* which have been sampled in South Africa and in Madagascar. Some important details on the studied species, as the identity of the

main disperser, are summarized in Table1. For the dioecious species, only female figs have been used. Stage of dispersal for figs was estimated by physical characteristics of figs, as softness and “easy-to-pick-up”.

**Collection of Volatiles**

For collection of odor samples, we used the Chromatoprobe head-space method. Mature figs were cut off from plants and were enclosed in a polyethylene terephtalate (Nalophan®) bag for 30 min. Then, the air was pulled out of the bag (flow rate: 200 mL min–1), over a filter (filled with 1.5mg of Carbotrap 20-40 and 1.5mg of Tenax 60-80), in which the compounds were trapped. Each collection lasted 5 min, and was done at any time between 11:00 and 15:00. One microlitre of a solution of internal standards (nonane and dodecane) was added to each filter, before scent extraction, to allow quantification. The samples were then stored at -20°C until chemical analyses.

**Chemical Analyses**

The sampled were then analyzed by GC-MS, using a CP-3800 chromatograph (Varian) coupled with a Saturn 2000 spectrometer (Varian). The characteristics of the columns are the following ones: Varian, CP-Sil 8CB Low Bleed MS 30mx0.25mmIDx0.25µm film Thickness. The injection is made with the injector 1079PTV (Programmed temperature Vaporizator) with Chromatoprobe equipment (Varian, Walnut Creek, USA). The injection is made at 40°C for 1 min, and then warm up for 3 min, until reaching 250°C, at the rate 200°C/min. The temperature program for the analysis was: 40°C for 3 min, ramped from 40°C to 100°C at 3.3°Cmin–1 to 250°C, from 100°C to 140°C at 2.9°Cmin–1, then 140°C to 180°C at 2.7 °Cmin–1,

and finally from 180°C to 2500°C at 10°Cmin–1. Temperature is still 250°C for the 8 following minutes.

Compound identification was based on matching of the mass spectra with NIST 98 MS and Adams 2007, and on confirmation by comparison of RI with libraries and published data (Adams 2007). Identification of some compounds was confirmed by comparison of both mass spectrum and GC retention indices with those of standards. These analyses were performed at the “Plate-Forme d’Analyses Chimiques en Ecologie” (platform for chemical analyses in ecology) of “IFR 119 Montpellier Environnement Biodiversité”.

**Data Analysis**

For the statistical analysis of the chemical blend, we performed Principal Component Analysis (PCA, covariance matrix, Stat Box v6.3) to compare patterns of scent composition of the different fig species. We then performed a MANOVA (SAS v9: Manova, proc glm, and LSMEANS with Tukey–Kramer multiple comparison tests) on the relative amounts of the main compounds of the bouquets to test for “disperser-identity” effect.

## RESULTS

More than 200 different VOCs were found in the bouquets emitted by mature figs of the eleven studied species. These VOCs belong to four different families of compounds: monoterpenes, sesquiterpenes, benzenoids and fatty acid derivatives. Fatty acid derivates proportions were high in bouquet of mammal-dispersed species (up to 80%; see Table 2), indicating high quantity of fermentable soluble

carbohydrates in fruits. (Borges et al. 2008; Hodgkison et al. 2007; Dudareva et al. 2006; Sanchez et al. 2004).

To test if the emitted scents were different for mammal-dispersed species and bird-dispersed species at the dispersal stage, we first performed a Principal Component Analysis (Fig. 1) on the relative amounts of VOCs in the bouquet of 10 fig species (all the studied species, except *F. lutea*, see after). This PCA shows a clear spatial separation of the scents emitted by mature figs of the *F. tiliifolia*, the lemur-dispersed species from all the other studied species. Moreover, all the bird-dispersed species scents are grouped together. All the out-of-ellipses points are the scents of individuals of bat-dispersed species (Fig. 1). According to this PCA, especially according to the second component, we thus have 3 classes of scents, each corresponding to species dispersed by a different frugivore (bat, bird or lemur). We confirmed this result by a MANOVA performed on the relative amounts of VOCs dataset. Indeed, the "disperser identity" effect was significant ($F_{32;13}$= 9.37; *p<0.0001)*. Moreover, bird-dispersed species emit lower quantity of VOCs than mammal-dispersed species (Kruskall-Wallis test: *p*= 0.03), but there were no significant difference of the total amount of VOCs emitted by bat- and lemur-dispersed species (Kruskall-Waliis test: *p*= 0.10) (see Table 2).

To test if the disperser identity has an effect on the composition of the scents emitted by the mature fruits of a same *Ficus* species, we then performed similar analyses on VOCs emitted by figs of *F. lutea*. Indeed, this species is mainly dispersed by bats in South Africa, although it's dispersed by lemurs in Madagascar. The figure 2 corresponds to the PCA performed on the relative amounts of VOCs emitted by mature figs of *F. lutea*. There is a clear separation of the scents emitted by South African trees and Malagasy trees. The result of this PCAs was confirmed by a

MANOVA performed on the relative amounts of VOCs dataset, where the “disperser identity” effect was significant ($F_{3;5}$= 14.69; *p= 0.0065)*.

## DISCUSSION

In this study, we confirmed previous results on scents and dispersal syndromes, by finding significantly different scents among bird- and bat-dispersed species (Lomascolo et al. 2010; Borges et al. 2008). Moreover, we have been able to highlight for the first time that scents emitted by bat-dispersed figs are different of those of lemur-dispersed figs, the latter being thus identified as a distinct dispersal syndrome.

The bird-dispersed species scents are mainly constituted by terpenes, although the major VOCs of mammal-dispersed species are fatty acid derivatives. The high amounts of this fatty acid derivatives in the bouquets of mammal-dispersed species indicate high amounts of sugar in the fruit composition (Borges et al. 2008). Consequently, the presence of these fatty acid derivatives, as well as their amounts, could be used by lemurs as indicators of the degree of ripeness of the fruits, as it has been shown for bats (Hodgkison et al. 2007; Sanchez et al. 2006). The presence of low volatility compounds (such as sesquiterpenes) in the bouquets of mammal-dispersed figs may be used to help in the precise location of the fruits, as suggested by Hodgkison et al. (2007). The terpenes are the main volatiles of the bouquets of the bird-dispersed species. Since they are common floral volatiles (Dudareva and Pichersky 2006; Knudsen et al. 2006), it seems difficult they can be used alone by birds for location of ripe figs. Above all, the global quantities of scents emitted by the bird-dispersed species are quite low, which make it even more difficult to be used as

a signal. Although some birds use olfaction (for example in foraging or selfmedication; Bonadonna et al. 2006; Petit et al. 2002), the role of dispersal-phase VOCs in attracting or repelling birds is completely unexplored (Borges et al. 2008). Our results require to be confirmed by behavioral experiments with birds; however, the similarity in the scent composition (see Fig. 1) for the bird-dispersed species may thus confirm that scent is not a cue for birds. Indeed, if the selective pressures on scents are released, since it is not used as a signal, the emitted scent may be due to metabolic by-products, which may be similar for all the studied species. On the contrary, mammals represent an important selective pressure on the scent emitted. Indeed, our unique lemur-dispersed species emits scents clearly different from those of the studied bat-dispersed species, even if the class of the major VOCs of the bouquets is the same (fatty acid derivatives). This is especially clear in the case of *F. lutea,* which differs in the scent composition depending of the frugivores. Thus, our results indicate that the isolation of Madagascar from the African continent with a new guild of frugivores affected also the composition of mature figs volatile compounds, even at an intraspecific level.

In summary, our results indicate that to attract different frugivores, *Ficus* emit different scents. The dispersal-syndrome hypothesis is thus supported by our results, which demonstrate that frugivores identity has an important effect on the evolution of fruit traits in the genus *Ficus,* and suggests that they may, at least partially, be responsible for the immense diversity of fleshy fruits produced by figs and other animal-dispersed worldwide plants.

**Table 1:** Main ecological traits of the sampled *Ficus* species.

| Species | Reproduction type | Originate from | Sampled in | Dispersed mainly by |
|---|---|---|---|---|
| *F. aurea* | monoecious | America | America | Bird |
| *F. auriculata* | dioecious | Asia | Asia | Bat |
| *F. exasperata* | dioecious | Asia | Asia | Bird |
| *F. hispida* | dioecious | Asia | Asia | Bat |
| *F. lutea* | monoecious | Africa | Africa (2 sites) | Bat or lemur |
| *F. macrophylla* | monoecious | Oceany | America (Hawaii) | Bird |
| *F. microcarpa* | monoecious | Asia | Asia | Bird |
| *F. montana* | dioecious | Asia | Europe | Bird |
| *F. sur* | monoecious | Africa | Africa | Bat |
| *F. tiliifolia* | monoecious | Africa | Africa | Lemur |
| *F. trichopoda* | monoecious | Africa | Africa | Bat |

**Table 2.** Means of global quantities and of relative amounts of the main VOCs emitted by mature figs of the studied species.

| | *F. auriculata* | *F. hispida* | *F. sur* | *F. trichopoda* | *F. aurea* | *F. exasperata* | *F. macrophylla* | *F. microcarpa* | *F. montana* | *F. tiliifolia* |
|---|---|---|---|---|---|---|---|---|---|---|
| | bat-dispersed species | | | | bird-dispersed species | | | | | lemur-dispersed species |
| Global amounts (ng/fig/h) | 6.23 | | | | 0.53 | | | | | 8.62 |
| (E)-ocimène | 0.00 | 0.05 | 11.96 | 2.05 | 0.00 | 32.32 | 0.00 | 0.00 | 0.00 | 0.19 |
| (Z)-3-hexenyl acetate | 1.38 | 0.05 | 0.29 | 0.75 | 2.15 | 3.46 | 6.13 | 0.00 | 0.12 | 0.07 |
| 2-heptyl acetate | 0.00 | 22.42 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 5.64 |
| alpha copaène | 0.00 | 0.12 | 0.10 | 0.45 | 0.45 | 0.28 | 0.00 | 12.89 | 0.08 | 0.18 |
| alpha-pinene | 0.00 | 0.09 | 0.04 | 0.33 | 0.04 | 3.46 | 0.39 | 0.00 | 7.68 | 0.17 |
| beta-caryophyllene | 0.09 | 3.22 | 16.66 | 3.06 | 0.14 | 0.24 | 0.17 | 20.43 | 0.26 | 0.20 |
| ethyl caproate | 9.30 | 0.00 | 0.00 | 0.00 | 0.63 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Ethyl hexanoate | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.05 | 0.00 | 0.00 | 1.37 | 8.17 |
| ethyl-2-methyl butanoate | 4.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 5.10 | 0.00 |
| EthylButyrate | 1.25 | 0.00 | 0.00 | 0.00 | 0.63 | 0.00 | 0.00 | 0.00 | 0.00 | 14.66 |
| gamma-terpinène | 1.88 | 0.00 | 0.00 | 0.00 | 0.00 | 21.70 | 0.00 | 0.00 | 0.00 | 0.00 |
| heptan-2-ol | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 15.12 |
| isobutyl acétate | 16.10 | 63.37 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| limonene | 0.00 | 0.00 | 0.48 | 2.12 | 0.08 | 5.60 | 2.55 | 2.62 | 0.50 | 0.57 |
| LIPID NI a | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 31.27 | 0.00 |
| methyl butanoate | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 9.72 | 0.00 |
| Methyl butyrate | 0.76 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 19.60 |
| methyl hexanoate | 6.04 | 0.00 | 10.07 | 3.36 | 0.00 | 0.00 | 11.77 | 0.00 | 0.56 | 17.29 |
| methyl octanoate | 0.73 | 0.00 | 5.67 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.85 | 2.61 |
| methyl-caproate | 29.98 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.56 | 0.00 |
| NI 1 | 0.00 | 0.00 | 0.43 | 0.26 | 1.07 | 0.00 | 1.59 | 13.18 | 0.00 | 0.00 |
| NI 2 | 0.00 | 0.00 | 0.00 | 0.38 | 2.37 | 0.00 | 0.01 | 10.14 | 0.00 | 0.00 |
| NI 3 | 0.00 | 0.00 | 9.34 | 9.62 | 13.70 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| NI 4 | 0.00 | 0.00 | 0.02 | 0.30 | 5.68 | 0.00 | 0.49 | 0.00 | 0.00 | 0.00 |
| NI 5 | 0.00 | 0.00 | 1.56 | 0.19 | 0.00 | 0.00 | 6.56 | 0.00 | 0.00 | 0.00 |
| NI 6 | 0.00 | 0.00 | 0.00 | 0.00 | 13.21 | 0.00 | 8.88 | 0.00 | 0.00 | 0.00 |
| NI 7 | 0.00 | 0.00 | 10.43 | 4.35 | 0.72 | 0.00 | 24.53 | 0.00 | 0.00 | 0.00 |
| NI8 | 0.00 | 0.00 | 0.00 | 9.82 | 1.22 | 0.00 | 1.51 | 0.00 | 0.00 | 0.00 |
| NI9 | 0.00 | 0.00 | 8.12 | 9.85 | 0.74 | 0.00 | 4.80 | 2.70 | 0.00 | 0.00 |
| NI10 | 0.81 | 0.14 | 0.00 | 2.15 | 0.00 | 0.00 | 0.00 | 11.33 | 0.00 | 0.00 |
| p-cymène | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 5.40 | 0.00 | 0.00 | 0.00 | 0.00 |
| pentyl acetate | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 10.46 | 0.00 |
| pentyl furane | 0.00 | 0.00 | 0.00 | 7.42 | 0.00 | 0.00 | 0.14 | 0.00 | 0.00 | 0.00 |

**Figure 1:** PCA analysis on relative abundance of VOCs produced by mature figs of 10 different species: 5 are bird-dispersed (yellow and red points) and 5 mammal-dispersed (blue and green squares). The green ellipse plots together the scents of the lemur-disperser species, and the red ellipse those of the bird-dispersed species.

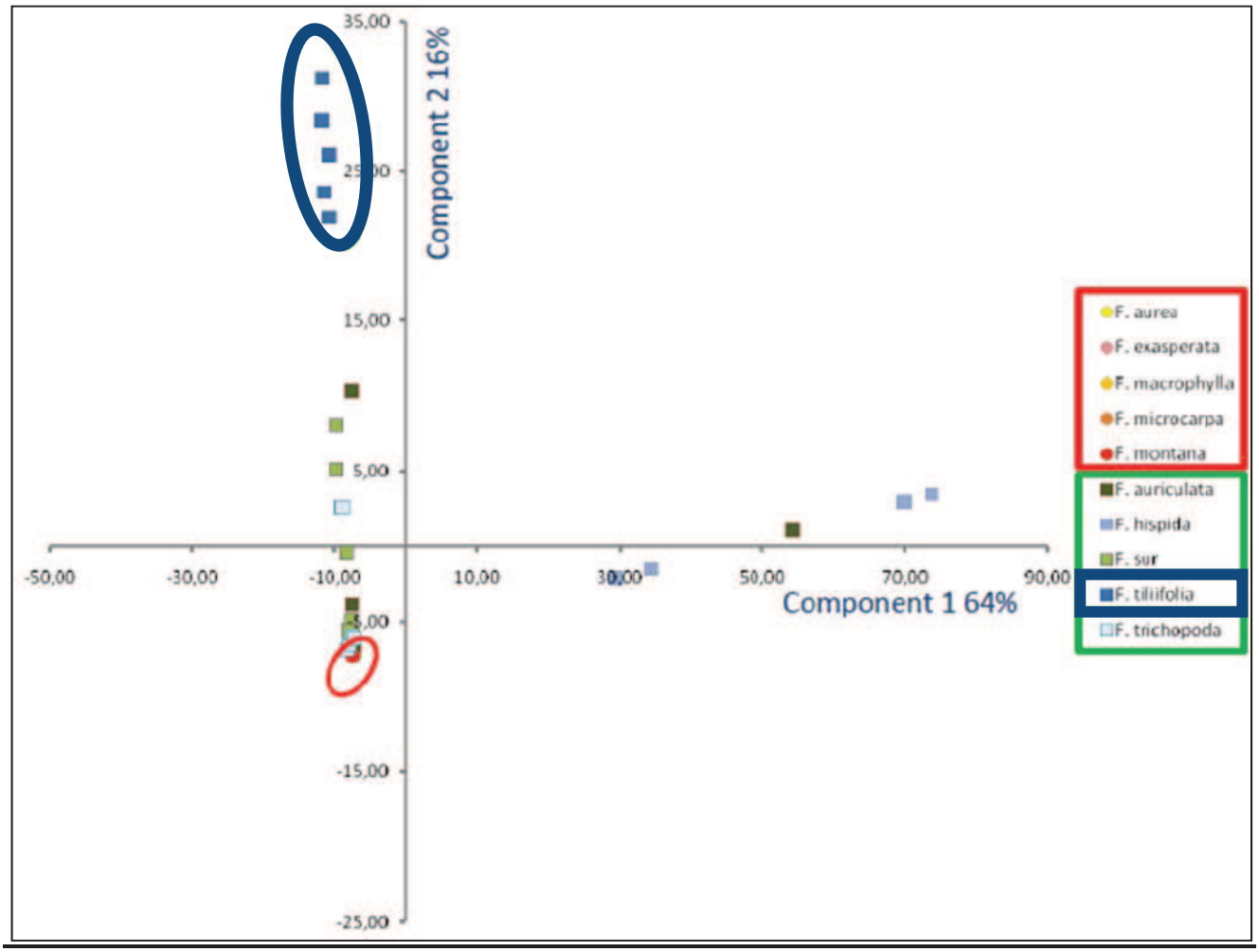
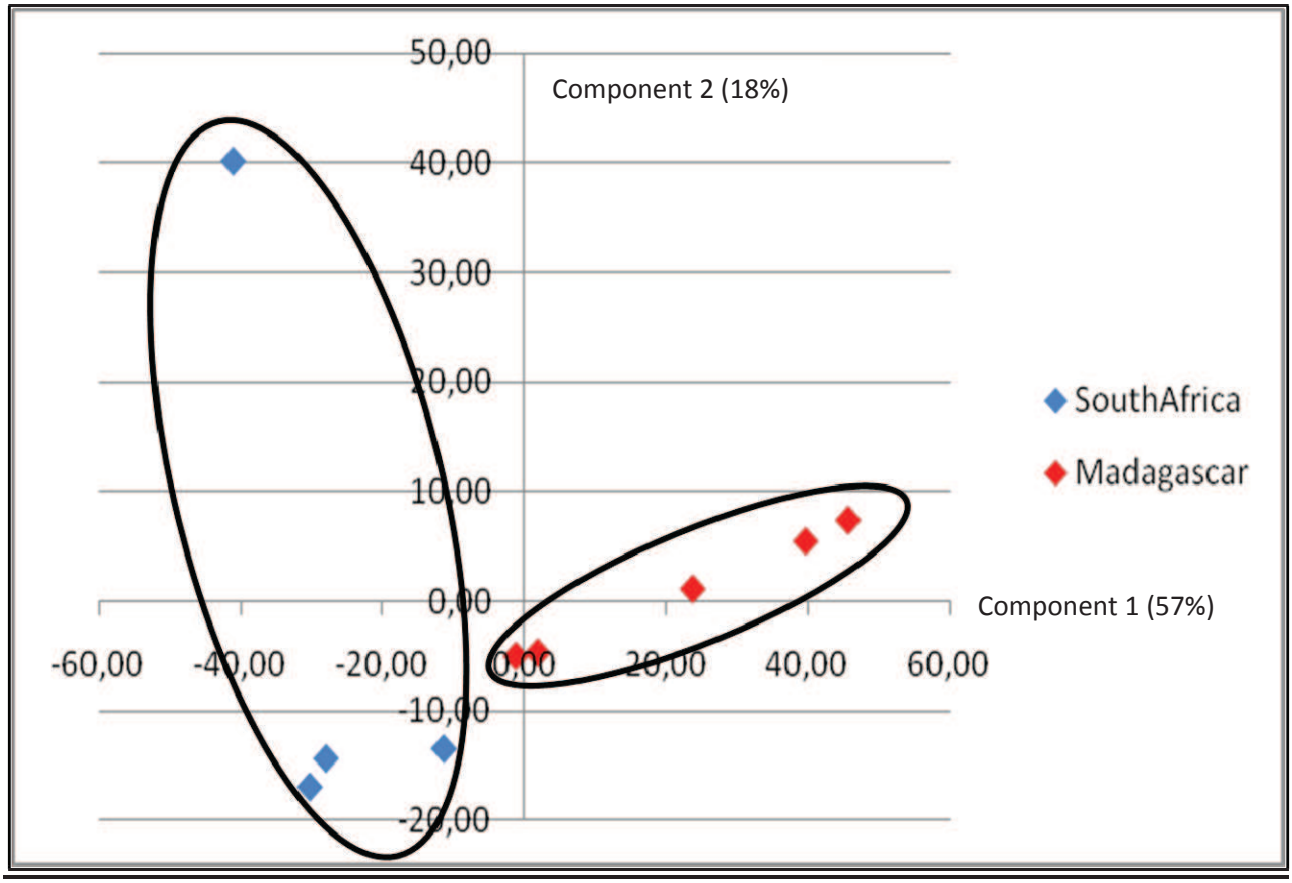


**Figure 2:** PCA analysis on relative abundance of VOCs produced by mature figs of *F. lutea* from Madagascar and from South Africa.



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# Mutualistic partners require convergence and then divergence of fig scents

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In this work still in preparation, we analyzed the scent of two dioecious *Ficus* species at receptivity and dispersal developmental phases. We are completing our dataset in order to integrate supplementary species before publication (at least 2, maybe 3 or 4), which will be a good point for our paper compared to previous studies. Our aim is notably a more detailed investigation on the chemistry of emitted scents in order to highlight common patterns in the different studied species. We were thinking of submitting this work for publication to *Ecology Letters* or *Journal of Evolutionary Biology*.

## ABSTRACT

Webs of interdependencies created by mutualisms play key roles in the maintenance of biodiversity and the functioning of ecosystems. As in many other interspecies interactions, most mutualisms are mediated by chemical signals. All *Ficus* species are well known to be engaged in mutualisms with both pollinators and seed-dispersing animals. Indeed, fig / fig wasp mutualisms are one of the best studied cases of “nursery pollination mutualism”, in which the plant benefits from pollination and offers breeding sites to the pollinating insects. The encounter between figs and fig wasps is possible thanks to a specific chemical message emitted by receptive figs. About the half of the roughly 800 *Ficus* species are dioecious, i.e., trees are functionally male or female. Seeds production requires that pollinating wasps enter and pollinate female figs, in which they die without laying eggs. Mechanisms for the evolutionary stability of dioecious fig/pollinator mutualisms are still unclear. One very appealing hypothesis holds that in dioecious figs, individuals of each sex are selected to produce odours that are very difficult for wasps to distinguish from those produced by the other sex. Moreover, due to their year-round fruiting (related to the needs for year-round flowering to maintain pollinator populations), fig trees are often the only available resource for frugivorous animals, especially during the lean season, making them keystone species in tropical ecosystems. Some pioneer studies started to demonstrate the important role of chemical mediation for the attraction of several and diverse species of fig seed dispersers. Consequently, at the dispersal stage, we should expect divergence in scents emitted by figs of both sexes, with only female figs attracting their mutualist seed dispersers. Indeed, the male figs, in which wasp larvae have been developing, should not be attractive to frugivores which might not consume the newly emerging pollinators, which would have a negative effect on the tree’s fitness. Using scent samples obtained by headspace adsorption/desorption method, we demonstrate here for two dioecious figs species that evolutionary forces led to convergence in scent at the pollination stage, and to divergence scents at the dispersal stage.



## KEY-WORDS

*Ficus*, scent, pollination, seed dispersal, mutualism, convergence, divergence, evolutionary forces

## Introduction

In plant-animal interactions, most of the studies performed on the role of Volatile Organic Compounds (VOCs) are on floral scents, and showed that these bouquets are implicated in pollinator attraction in several group of species (Grison-Pigé et al. 2002a; Ayasse 2006; Knudsen et al. 2006; Raguso 2008). In contrast, similar research on volatile compounds responsible for the attraction of seed dispersal frugivorous to mature fruits are still scarce (Dudareva et al. 2006). However, most of these studies showed that scent could be responsible of frugivores attraction without identifying the compounds responsible for this attraction (Kalko *et al.* 1996; Laska & Seibt 2002; Sanchez *et al.* 2006). To our knowledge, only two recent papers properly both analyzed the scent released by fruits and its implication in frugivores attraction (Hodgkison *et al.* 2007; Borges *et al.* 2008). Furthermore, the differences in VOCs production between receptive flowers and mature fruits for the same species in relation to the attraction of pollinators or dispersers has been investigated only once, largely due to the research priorities of investigators who are either pollination or seed dispersal specialists (Borges et al. 2008).

In the *Ficus* (Moraceae) system, flowers are borne inside the syconium, which will ripen to form a fig fruit. This system is unique because the syconium is the same physical unit of attraction for both pollinators and seed dispersers. The highly species-specific pollinating fig wasps use VOCs produced by figs to find pollen-receptive figs (Ware *et al.* 1993; Hossaert-McKey *et al.* 1994; Gibernau *et al.* 1998; Grison-Pigé *et al.* 2002a; Chen & Song 2008). Because the fig system is a nursery pollination system (Hossaert-McKey et al. 2010) and VOC signatures vary during the fig developmental cycle (Proffit et al. 2008), pollinators and seed dispersers might be attracted to the fig only during appropriate time windows thanks to distinct volatile

bouquets. In dioecious fig species, in which pollinators can only breed within gall figs produced by male trees, and seeds are only produced within seed figs on female trees (Verkerke 1989), there should be selection on gall and seed figs to be indistinguishable from each other at the pollination stage (Grafen & Godfray 1991; Patel *et al.* 1995; Hossaert-McKey et al, in prep; Ashman 2009), and for dispersal agents to be attracted to seed figs rather than gall figs in the dispersal stage (Patel & McKey 1998; Dumont *et al.* 2004; Borges *et al.* 2008). Although the pollinators of fig species are closely related to each other, fig seed dispersers are phyletically diverse, including mainly a huge range of birds, as well as mammals including bats, rodents and primates (reviewed by Shanahan et al. 2001).

These features of the fig pollination and seed dispersal system present unique constraints on the chemical ecology of these mutualisms. In this paper, we present data comparing differences between the VOC signatures of the receptive (pre-pollination) and dispersal phase of two dioecious fig species. Based on the ecology of figs and their mutualistic partners, we hypothesized in two dioecious fig species *Ficus montana* and *Ficus auriculata* that volatile signatures emitted by figs should be convergent at the receptivity stage, and then divergent between sexes at the seed dispersal stage, as well as distinct from each other between these two developmental phases.

## □ATER□A□□□□ET□ODS

### S□□□□□□□□□□□□□□

*Ficus montana* trees were growing in greenhouses on the Leeds University campus in England, and *Ficus auriculata* trees have been sampled in their natural habitat, in

XTBG, Yunnan, China. *Ficus montana* is pollinated by *Lipporhopalum tentacularis* (Grandi) and is a bird-dispersed species, whereas *Ficus auriculata* is pollinated by *Ceratosolen emarginatus* (Mayr) and bat-dispersed. Fig stages were estimated by physical characteristics of figs, as for example open ostiole, softness and brightness for receptivity, softness and “easy-to-pick-up” at dispersal.

**C**

We studied the volatile emissions of 42 trees (6 females and 6 males, for each developmental stage, for *Ficus Montana* [so 24 different trees have been sampled], 9 females and 9 males for *Ficus auriculata*, with 4 or 5 individuals by stages). The collections of odor samples have been done by Chromatoprobe head-space method. First, figs were cut off from plants and enclosed in a polyethylene terephtalate (Nalophan®) bag, for 30 min. Then, the air was pulled out of the bag (flow rate: 200 mL min–1), over a filter (filled with 1.5mg of Carbotrap 20-40 and 1.5mg of Tenax 60-80), in which the compounds were trapped. Each collection lasted 5 min, as was done at any time between 11:00 and 15:00, when the emission of scent is the highest. One microlitre of a solution of internal standards (nonane and dodecane) was added to each filter, before scent extraction, to allow quantification. The samples were then stored at -20°C until chemical analyses.

**CA**

The sampled were then analyzed by GC-MS, using a CP-3800 chromatograph (Varian) coupled with a Saturn 2000 spectrometer (Varian). The characteristics of the columns are the following ones: Varian, CP-Sil 8CB Low Bleed MS 30mx0.25mmI

Dx0.25µm film Thickness. The injection is made with the injector 1079PTV (Programmed temperature Vaporizator) with Chromatoprobe equipment (Varian, Walnut Creek, USA). The injection is made at 40°C for 1 min, and then warm up for 3 min, until reaching 250°C, at the rate 200°C/min. The temperature program for the analysis was: 40°C for 3 min, ramped from 40°C to 100°C at 3.3°Cmin–1 to 250°C, from 100°C to 140°C at 2.9°Cmin–1, then 140°C to 180°C at 2.7 °Cmin–1, and finally from 180°C to 2500°C at 10°Cmin–1. Temperature was still 250°C for the 8 following minutes.

Compound identification was based on matching of the mass spectra with NIST 98 MS and Adams 2007, and on confirmation by comparison of RI with libraries and published data (Adams 2007). Identification of some compounds was confirmed by comparison of both mass spectrum and GC retention indices with those of standards.

**D□□□A□□□□□□**

For the statistical analysis of the chemical blend, we performed Principal Component Analysis (PCA, covariance matrix, Stat Box v6·3) to compare patterns of scent composition of the two developmental stages of both fig species. We then performed a MANOVA (SAS v9: Manova, proc glm, and LSMEANS with Tukey–Kramer multiple comparison tests) on the relative amounts of the main compounds of the bouquets to test for sex effect or stage effect.

## RES□□TS

To compare if volatile signatures of receptive and dispersal phases were different, we first performed PCAs on the relative amounts of the VOCs emitted by

each species (Fig. 1). According to these PCAs, both species seem to emit different scent at receptivity and dispersal stage. A MANOVA performed on the relative amounts dataset (VOCs>3%) show that the scent emitted is stage-specific for both studied species (*F. montana*: $F_{(9;14)}$= 3.30; $p$ = 0.02; *F. asperifolia*: $F_{(9;8)}$= 1.05 ; $p$ =0.04).

To test if evolutionary forces led to convergence in scent at the pollination stage and to divergent scents at the dispersal stage between sexes, we first performed PCAs on the relative amounts of VOCs of the emitted bouquets by species and by stages (Fig. 2). For both species, male and females scents are graphically distinct at dispersal stage, whereas the PCAs do not show a separation between the scents emitted by male and female figs at receptivity stage. To confirm these results, we then performed MANOVA on the relative amounts dataset. For the four cases (2 developmental stages for 2 species), we used the main compounds of the bouquet (mean>3%). For both species at receptivity, scent emitted by males was not significantly different of those emitted by females (*F. montana*: $F_{(5;6)}$= 1.21; $p$ = 0.41; *F. asperifolia*: $F_{(6;2)}$=2.27 ; $p$ = 0.33). On the contrary, for both species at dispersal stage, scents emitted male and female figs are significantly different (*F. montana*: $F_{(6;5)}$= 8.55; $p$ = 0.02; *F. asperifolia*: $F_{(2;5)}$=5.46 ; $p$ = 0.05). The dispersal scents were mainly composed terpenes (male figs) and by fatty acid derivatives (females figs), thought at receptivity the emitted bouquets are dominated by terpenes for both sexes.

## **D□SC□SS□O□**

Our results showed the expected differences between the VOCs of receptive and dispersal stages of figs. Indeed, Proffit et al (2008) demonstrated that figs do

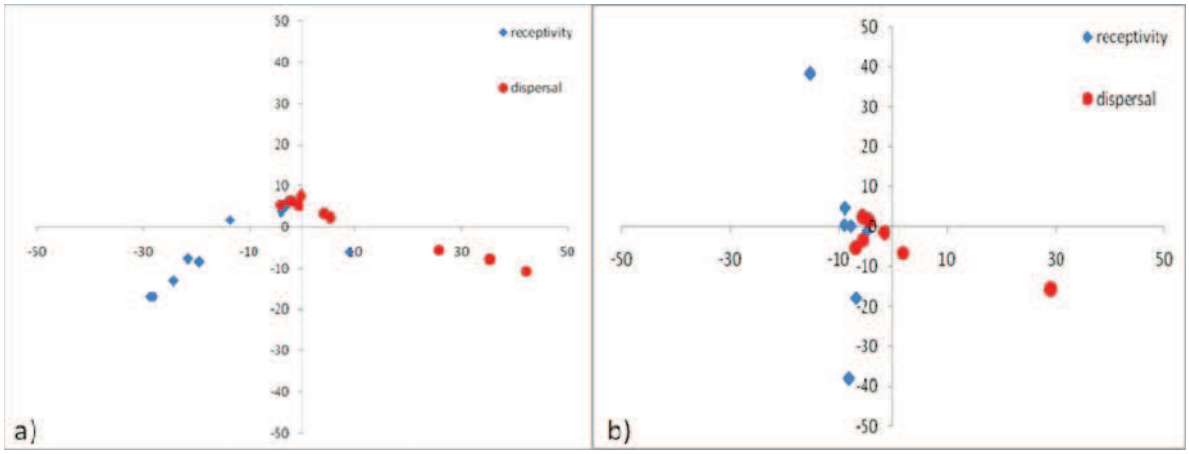
differ in VOC profile between pre-pollination and post-pollination stages, since a clear message is selected at receptivity to guide the pollinator. This message is mainly composed by terpenes, as it has been shown in previous studies (Gibernau *et al.* 1998; Grison-Pigé *et al.* 2002b; Chen & Song 2008). A distinct message is emitted at the dispersal stage, which may be used by certain frugivores to localize the fruits (female figs) and may use the presence of fatty acid derivatives as a cue of the degree of ripeness of the fruits (Kalko *et al.* 1996; Sanchez *et al.* 2004; Korine & Kalko 2005; Dudareva *et al.* 2006).

Our results demonstrate at one and the same time that scents emitted by the two fig sexes are convergent at the pollination stage, but divergent at the dispersal stage. The hypothesis of 'chemical mimicry' between sexes at receptivity (Grafen & Godfray 1991; Patel *et al.* 1995; Hossaert-McKey *et al.* 2010) is thus confirmed, as already shown in some other studies (Borges et al. 2008; Chen et al. 2009). Concerning the volatile compounds emitted by figs at dispersal stage, our results differ from a previous study on figs. Indeed, Borges et al (2008) found the same divergence of scents between male and female figs at maturity when they are dispersed by bats, but not when they are bird-dispersed species. In our study, scents are divergent among sexes at dispersal stage, whatever the identity of the seed disperser, so even in the case of the bird-dispersed species, *F. montana*. This question then needs to be studied in more important number of bird-dispersed species, and to be correlated case by case with the olfactive abilities of bird species involved fig seed dispersion (Leroy 1987). However, Borges et al. (2008) hypothesized that the synchrony of the crops may play an important rule to explain these differences: for their bat-dispersed species (*F. condensa*), dispersal stage figs of both sexes are available simultaneously, contrary to their bird-dispersed species

(*F. exasperata*). Indeed, selection for difference is expected to be greater in dioecious species that produce seed and gall figs simultaneously in the same season (Lambert 1992), since synchrony induces the possibility of choice by animals. Such explanation does not fit with our case, in which the bat-dispersed species (*F. auriculata*) have male and female figs that do not always bloom synchronously. In fact, the peak periods of bearing fruits showed once on the male trees and twice on the female trees, which peak periods do not overlap (Y-Q. Peng, unpublished data). In addition, such difference for scents emitted by bird-dispersed species could also be explained in one hand by the method for collection of VOCs used by Borges et al. (2008), which is less sensitive than our (R. Borges and M. Hossaert-McKey, pers. comm.), and in other hand because of the very low quantity of scent emitted by the bird-dispersed species studied by Borges et al. (2008), likely to explain that they potentially missed some information. Otherwise, distinct emitted scent at dispersal stage between male and female figs were not surprising when we examined the difference between these two ecological types of figs. Indeed, female figs are fruits, while male figs are not fleshy and made of galls and dead insects. They were thus expected to emit different scents, which may be reinforced by selective pressures. Indeed, the consumption of male figs by frugivores may have a bad impact on fig trees fitness, and should be avoided.

In this study, using scent samples for two dioecious fig species, we show that evolutionary forces led to stage-specific scents, as well as to convergence in scent at the pollination stage and to divergent scents at the dispersal stage. We need now to confirm these results working on the signal, i.e. the portion of the message perceived by the mutualistic partner.

□□□□□□□□PCA analysis on relative abundance of VOCs produced by figs of *Ficus montana* (a) and *Ficus auriculata* (b). Receptivity scents are represented by blue diamond, and dispersal scents by red circles.



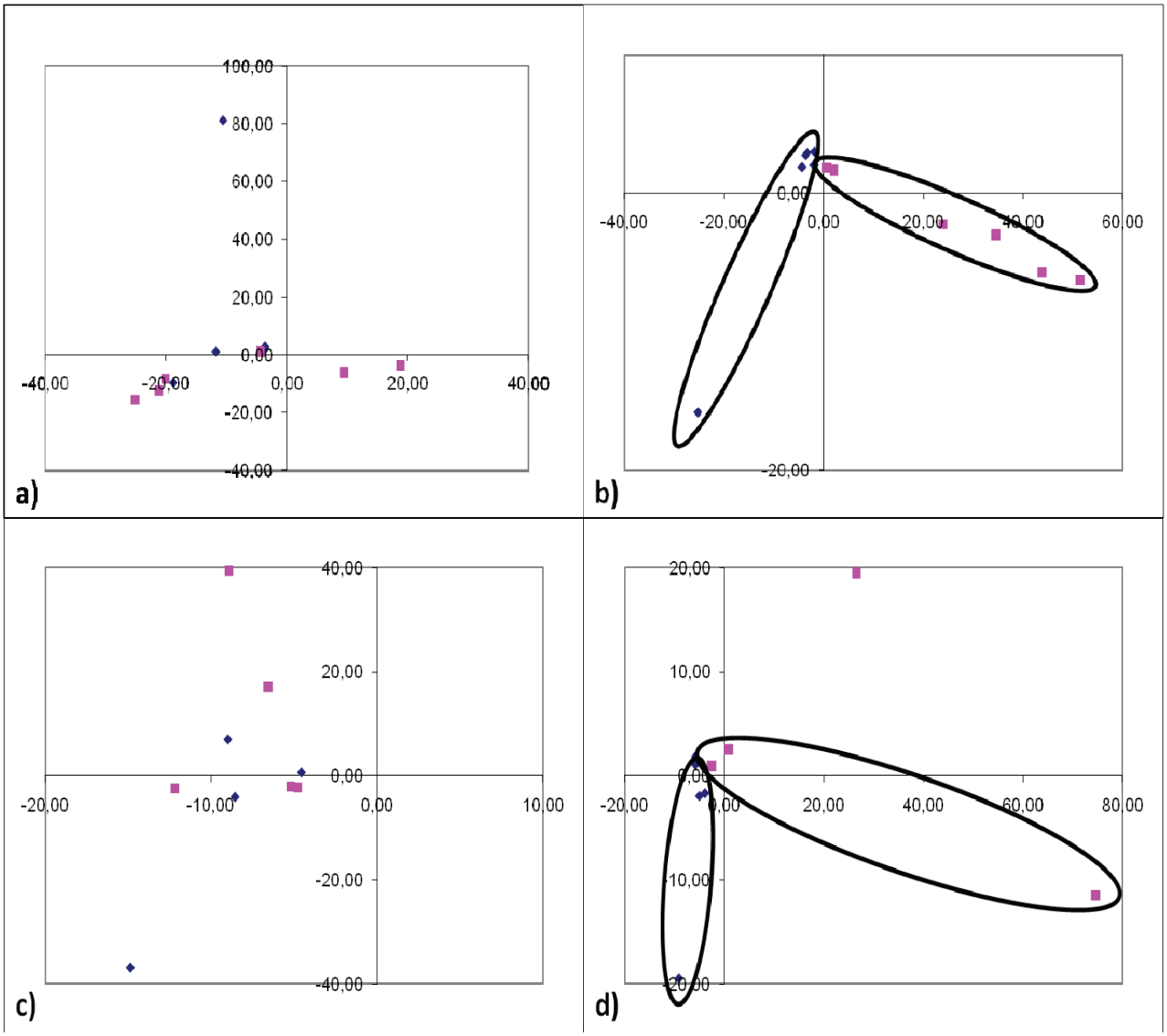
PCA analysis on relative abundance of VOCs produced by figs of (female scents are represented by pink squares, male scents by blue diamond):

a) *Ficus montana* at receptivity stage (first component explains 38% and second component 19% of the total variation observed).

b) *Ficus montana* at dispersal stage (first component explains 34%, and second component 19% of the total variation observed).

c) *Ficus auriculata* at receptivity stage (first component explains 38% and second component 18% of the total variation observed).

d) *Ficus auriculata* at dispersal stage (first component explains 34% and second component 19% of the total variation observed).



## RE□ERE□CES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# DISCUSSION

Au cours de cette thèse, nous avons étudié le rôle des contraintes et des pressions biotiques dans la médiation chimique des interactions entre le figuier et ses partenaires mutualistes. Nous allons maintenant les discuter afin d'en extrapoler les implications valables pour la communication interspécifique et son évolution en général.

## A) *Parfums de figues* : contraintes liées à la médiation chimique

Dans le cadre de la relation figuier/pollinisateur, nos travaux montrent que les contraintes phylogénétiques sont peu ou pas importantes dans l'émission du message. De plus, le message chimique semble peu influencé par des contraintes environnementales, même s'il reste marqué par des événements biogéographiques forts tels que l'apparition d'une barrière montagneuse induisant l'isolement de certaines populations, comme dans le cas des Ghâts. Les caractéristiques des plantes (morphologie, couleur, etc.) peuvent présenter une grande variabilité (Endress, 1994), notamment celles impliquées dans l'attraction de partenaires mutualistes, comme les bouquets volatils émis par les fleurs réceptives. Cependant, peu de travaux abordent le rôle des contraintes environnementales sur ces variations. En effet, la majorité des études sur ces bouquets a été réalisée selon une approche d'écologie de la pollinisation, privilégiant leur rôle majeur dans l'attraction du pollinisateur (Dobson, 1994; Dudareva & Pichersky, 2006; Knudsen *et al.*, 2006). En conséquence, ces études accordent bien souvent une prépondérance aux effets de la sélection naturelle (dans l'adaptation à un pollinisateur) pour expliquer la variance phénotypique observée, négligeant généralement la plasticité phénotypique (Majetic *et al.*, 2009). De nombreux travaux ont montré des cas d'évolutions convergentes des bouquets olfactifs pour attirer un groupe spécifique d'espèces de pollinisateurs, (comme par exemple les syndromes de sphingophylie; Schlumpberger & Raguso, 2008), ou des cas d'isolement reproducteur par attraction de pollinisateurs différents (Waelti *et al.*, 2008). Même si ces études mettent ainsi en évidence le rôle prépondérant

du pollinisateur comme pression de sélection, elles n'excluent en rien le fait que la plasticité phénotypique puisse expliquer, au moins partiellement, la variance phénotypique observée ! Et que cette dernière puisse aussi s'exprimer en réponse à des variations dans l'environnement. Dans notre étude, l'expression de cette plasticité n'est pas à exclure. Cependant, si elle existe, elle est toujours masquée par la variation interindividuelle des bouquets émis, si bien qu'aucune variation interpopulationnelle n'est visible. En fait, pour exclure l'existence d'une telle variation, il faudrait réaliser une expérience de « *common garden* »[10] qui, pour les espèces du genre *Ficus*, requiert au minimum une quinzaine d'années de mise en place, difficilement réalisable… surtout au cours d'une thèse !

Au delà de cette plasticité phénotypique répondant à des variations environnementales, la variation des bouquets olfactifs peut aussi trouver des explications dans la variation génétique des individus et des populations. Cette dernière peut non seulement être due à la sélection, mais aussi être liée à l'histoire de vie des espèces, et refléter leur histoire phylogénétique. En effet, la mise en place et l'activation enzymatique des voies de biosynthèse sont codées génétiquement. La chimie des bouquets volatils a été utilisée dans quelques cas pour clarifier des situations où la taxonomie était mal établie - notamment dans des genres où les espèces étaient pollinisées par le même groupe d'insectes (Azuma *et al.*, 1997; Dobson *et al.*, 1997). L'article n°3 nous a permis de montrer qu'il n'existait pas d'inertie phylogénétique sur les bouquets émis à réceptivité par les *Ficus*, contrairement à des résultats préliminaires sur ce système, mais qui concernaient un plus faible nombre d'espèces, et où moins de sous-genres étaient représentés (Grison-Pigé, 2001; Soler, 2007). Il apparait donc que la variation interspécifique des bouquets volatils émis à réceptivité par les figuiers, toujours détectable malgré une forte variation interindividuelle, n'est pas à mettre en relation avec la phylogénie

[10] Cette expérience consisterait à cultiver différentes espèces, sous les mêmes conditions contrôlées, afin de s'affranchir de l'effet des variabilités environnementales, tout en préservant la variabilité génétique.

des espèces. Le plus étonnant ici est que le codage des bouquets de COVs pour des objectifs phylogénétiques permet d'obtenir de meilleurs résultats en considérant la présence/absence, plutôt que la quantité relative des COVs. En effet, la spécificité des bouquets est généralement due à des proportions relatives de COVs communs, mais rarement à la présence d'un composé dominant unique (Gibernau *et al.*, 1998; Grison-Pigé *et al.*, 2002; Chen *et al.*, 2009; Proffit & Johnson, 2009; Hossaert-McKey *et al.*, 2010). Une première hypothèse évoquée pour l'absence d'inertie phylogénétique dans les odeurs florales est l'impact des changements de pollinisateurs au cours du temps: passer d'un pollinisateur utilisant l'odeur pour détecter la plante, à une autre espèce, utilisant d'autres types de signaux, implique une perte de traçabilité phylogénétique (Levin *et al.*, 2003). Ceci ne peut cependant pas s'appliquer aux figuiers, de par l'utilisation du bouquet par les Agaonidés et la coévolution *Ficus* / Agaonidés au cours d'une histoire commune très ancienne (de 60 à 100 millions d'années selon Cook & Rasplus, 2003). Une seconde hypothèse évoquée dans la littérature est l'existence d'un phénomène s'apparentant à des 'harmoniques olfactives'; en d'autres termes, les contraintes phylogénétiques seraient visibles sur des groupes de COVs. Il serait intéressant de regarder si une telle inertie existe, en regroupant les COVs en fonction de leur appartenance à des voies de biosynthèses, ou par les enzymes de synthèses correspondantes. En effet, sachant qu'une enzyme ne donne pas forcément qu'un seul produit et / ou n'utilise pas forcément qu'un seul substrat, il serait possible d'obtenir des résultats différents, en supposant néanmoins que l'identification des enzymes impliquées soit possible et suffisamment précise. Un dernier mode de regroupement très pertinent nécessiterait d'importants travaux en électro-antennographie, suivis de tests comportementaux avec les composés détectés par les insectes. En effet, il est fortement possible que les COVs non utilisés par l'insecte dans la détection de sa plante hôte soient davantage soumis à une certaine inertie phylogénétique, puisque moins soumis à sélection. Si de tels travaux mettaient en évidence que des COVs du

bouquet se dégagent de la contrainte phylogénétique lorsque ce sont des éléments du signal utilisé par l'insecte, cela montrerait alors l'importance centrale du pollinisateur comme pression de sélection. Sachant en outre que les coûts métaboliques associés à la production des COVs ne semblent pas non plus importants dans l'évolution du bouquet odorant (Euler & Baldwin, 1996; Grison-Pigé *et al.*, 2001b), ces éléments convergent vers l'idée que la principale force dans cette évolution du message émis par les *Ficus* corresponde à celle exercée par leurs partenaires mutualistes.

## B) ***Parfums de figues* : médiation chimique & pollinisation**

En étudiant la médiation chimique de l'interaction *Ficus* / pollinisateurs, nous avons pu mettre en évidence que le pollinisateur représente une pression de sélection importante sur le bouquet, tant au niveau intraspécifique qu'interspécifique.

Au delà de leur fonction d'attraction du pollinisateur, les bouquets de COVs émis par les figues à réceptivité ont aussi un rôle dans le maintien de la spécificité pré-zygotique de l'interaction *Ficus* / pollinisateurs, comme suggéré pour la première fois par Ware et Compton (1994), et vérifié depuis par plusieurs auteurs (Grison *et al.*, 1999; Proffit & Johnson, 2009). Le bouquet volatil joue donc un rôle de barrière au changement de partenaire - d'hôte pour l'insecte - et donc à l'hybridation. Les cas d'hybridation sont très rares chez les *Ficus* (Parrish *et al.*, 2003), et le rôle de l'odeur dans ce cas n'a jamais été étudié. Dans le cas particulier d'un croisement interspécifique en conditions contrôlées, nous avons montré que les bouquets émis à réceptivité par les *Ficus* ne semblaient pas suffisants, par eux-mêmes, pour être de véritables barrières à l'hybridation. Les bouquets volatils sont des éléments de filtres floraux, qui doivent être combinés à d'autres, comme la morphologie ou la phénologie, pour jouer un rôle de barrière pré-zygotique. Dans notre cas, bien qu'ils aient été forcés, les filtres floraux ne sont pas des barrières

totales, puisqu'il y a des hybrides fertiles, ce qui laisse supposer d'ailleurs que, dans notre modèle, les barrières post-zygotiques sont faibles. Le cas étudié montre que l'hybridation n'a été possible que dans un sens (paternité de *F. montana* uniquement possible) puisque les pollinisateurs ne peuvent pondre ni dans les figues de *F. asperifolia*, ni dans celles des hybrides. Cependant, notre étude représente un cas de « forçage écologique », puisque nous réunissions, dans un même espace clos (serres de culture), deux espèces normalement allopatriques. Il n'existe donc pas de pression naturelle sur les bouquets leur permettant d'être aisément discriminables par le pollinisateur, de même que pour les autres éléments des filtres floraux du syndrome de pollinisation. Dans cette étude, on peut s'étonner qu'il y ait pollinisation spontanée de *F. asperifolia*, alors que nos analyses montrent que les odeurs émises sont différentes de celles de *F. montana*. Il est possible que le pollinisateur spécifique de *F. montana* - *Liporrhopalum tentacularis* – utilise, au moins partiellement, des COVs communs aux deux espèces pour localiser sa ressource. Nous ne pouvons donc pas conclure actuellement avant d'avoir réalisé, d'une part, des électro-antennogrammes permettant d'établir quels COVs sont perçus par l'insecte dans le message émis par le figuier et, d'autre part, des tests comportementaux avec des mélanges synthétiques des COVs précédemment identifiés, de façon à établir quels COVs sont attractifs pour le pollinisateur. Si le signal détecté par l'insecte est distinct pour les deux espèces, il est possible qu'il y ait un effet « confinement » : beaucoup d'arbres des deux espèces se retrouvent dans l'espace confiné des serres de culture, où leur densité est bien plus élevée qu'en conditions naturelles, entrainant des hybridations inhabituelles, comme décrit dans des zoos pour certains animaux comme les tigrons et ligrons (Lovely, 2009). Les antennes de l'insecte pourraient en particulier être saturées, rendant ainsi possible la confusion entre les deux espèces de *Ficus* par le pollinisateur. Par ailleurs, la confusion dans la médiation chimique peut aussi résulter de composés de surface de la figue, qui rendrait possible la visite des figues réceptives de *F. asperifolia* par *L. tentacularis*. Là encore, nos espèces étant naturellement allopatriques, il n'y

a pas de pression particulière pour que ces composés de surface soient différents entre ces deux espèces. Cette hypothèse nous semble plus probable, et, pour la tester, il faudrait, après s'être assurés de la non-attractivité à distance de *F. asperifolia*, analyser les composés de surface des deux espèces. Plus généralement, une meilleure compréhension du rôle de la médiation chimique dans les processus d'hybridation chez les *Ficus*, implique de réaliser d'autres études similaires, avec notamment deux espèces sympatriques. Cependant, maintenir des populations de *Ficus* en serres, ainsi que celles de leur pollinisateur, n'est pas chose aisée….

Ces travaux sur le rôle de la médiation chimique dans l'interaction figuier/pollinisateur, nous ont également permis de mettre en évidence des pressions de sélection au niveau intraspécifique, dans le cas des espèces dioïques. Ainsi, une sélection vicariante agit sur les bouquets émis à réceptivité par les figuiers dioïques aboutissant à un mimétisme chimique entre les deux sexes. Cette pression pour le mimétisme peut cependant être relâchée dans des cas d'asynchronie forte de la phénologie des deux sexes, puisque, pour le pollinisateur, le choix vers l'un ou l'autre n'est alors plus possible. Cette particularité apporte un nouvel éclairage sur la question de l'honnêteté du signal dans les mutualismes. Du fait de leur coût élevé, certains signaux ne peuvent pas provenir d'individus tricheurs, et constituent donc un signal honnête de la qualité de l'émetteur. Les messages chimiques émis par les *Ficus* ont effectivement un coût : si la production des COVs n'est pas coûteuse en terme de carbone (Grison-Pigé *et al.*, 2001b), d'autres coûts indirects y sont associés, comme celui dû à la large communauté de prédateurs opportunistes et de parasites spécifiques qui usurpent ce message olfactif (Schatz *et al.*, 2003; Proffit *et al.*, 2007; Schatz *et al.*, 2008; Proffit *et al.*, 2009; Schatz & Hossaert-McKey, 2010). De surcroît, la relation même avec le pollinisateur représente un coût, puisque ce dernier utilise les tissus végétaux pour le développement de ses larves (Galil & Eisikowitch, 1971). En outre, en

étant vecteur de pollen, il peut également transmettre des maladies et des pathogènes (Grimont *et al.*, 1979; Herre, 1990). Ce dernier aspect peut d'ailleurs être une raison du changement du bouquet pré / post réceptivité mis en évidence dans l'article n°8. Le message émis peut donc plutôt être considéré comme honnête, ce qui ne semble *a priori* pas étonnant dans le cadre d'un mutualisme spécifique et obligatoire. Cependant, les figuiers femelles des espèces dioïques trichent (Dufaÿ & Anstett, 2003) avec leurs pollinisateurs, puisque ceux-ci ne peuvent pas pondre dans les figues femelles alors qu'il les pollinisent. Cette tricherie permet néanmoins la production de graines, et donc le maintien du mutualisme. Dans le cas particulier de *F. semicordata*, les coûts associés à l'émission du message sont moindres, puisqu'il s'agit d'un cas de canal privé, donc d'un cas où le message n'est pas usurpé (article n°2). Dans cet exemple, le mimétisme intersexuel du message n'est pas parfait (Chen *et al.*, 2009), mais, comme un seul COV – le 4-methylanisole - est suffisant par lui–même pour attirer le pollinisateur, la pression pour le mimétisme sur les autres COVs est relâchée. *A contrario*, dans le cas des espèces monoïques, qui représentent l'état ancestral chez les figuiers, les mêmes coûts associés sont présents, mais pas la tricherie. Les coûts semblent même plus élevés, puisque d'une part, les fleurs qu'utilise le pollinisateur pour pondre sont fonctionnelles, et pourraient donc donner des graines, et que d'autre part, la communauté d'hyménoptères associée est encore plus large (en moyenne, 12 espèces de parasites associées à une espèce de figuier monoïque, contre 3 chez une espèce dioïque; Kerdelhué & Rasplus, 1996). L'apparition de la dioécie aurait donc permis l'apparition de la tricherie dans l'interaction figuier/pollinisateur. Plusieurs hypothèses ont été proposées pour expliquer l'apparition de la dioécie (reviewées et complétées par Harrison & Yamamura, 2003), et la possibilité de tricher pourrait en être une nouvelle.

L'interaction, hautement spécifique et obligatoire, dans laquelle les figuiers sont engagés représente donc une pression de sélection importante

sur la médiation chimique. Nous allons maintenant discuter de la pression de sélection que peut présenter une interaction mutualiste plus lâche, celle avec les frugivores disperseurs de graines.

## C) *Parfums de figues* : médiation chimique & dispersion des graines

Comme explicité dans les deux articles correspondants de la thèse (articles n°9 et 10), nous avons pu mettre en évidence deux pressions majeures sur la composition du bouquet émis par les figues au stade de dispersion : l'identité du disperseur impliqué, ainsi que la nécessité pour les figues femelles de se distinguer des mâles dans le cas des espèces dioïques. Tout comme pour les bouquets émis à réceptivité, les pressions biotiques semblent donc être des éléments clefs dans l'évolution de la médiation chimique.

Le premier de ces deux articles met en évidence un résultat original : la distinction entre odeurs émises pour attirer des primates et celles pour attirer des chiroptères, ce qui confirme de plus leurs différences avec les espèces ornithochores. Chez les primates, il a été montré que l'olfaction pouvait être un sens important, quoique ces études aient surtout porté sur son rôle dans le choix d'un partenaire sexuel ou dans le comportement social (Dominy *et al.*, 2001; Charpentier *et al.*, 2010). Peu d'études existent sur son rôle dans la recherche alimentaire, et elles se concentrent surtout sur le comportement, et non sur la nature des odeurs impliquées (Dominy *et al.*, 2001; mais pour un exemple, voir Hiramatsu *et al.*, 2009). Il semble que les primates utilisent l'olfaction en complément d'autres sens, notamment lorsqu'ils sont déjà à proximité de la ressource (Laska *et al.*, 2007). Ces données bibliographiques les distinguent donc des chiroptères, connus pour utiliser l'odeur de fruits pour les localiser à distance (Thies *et al.*, 1998; Bianconi *et al.*, 2007). Chez les primates, l'orientation, lors de la recherche alimentaire, semble principalement basée sur l'utilisation de la mémoire spatiale (Janson, 2007;

Erhart & Overdorff, 2008). Cette dernière a également été démontrée chez les chiroptères (Winter & Stich, 2005), suggérant qu'elle peut se combiner à la mémoire olfactive, chez le genre *Carollia* notamment (Thies *et al.*, 1998). Chez les lémuriens, l'utilisation de la mémoire spatiale a également été clairement mise en évidence (Erhart & Overdorff, 2008), notamment chez les microcèbes (Luhrs *et al.*, 2009).

Au cours de cette thèse, nous avons réalisé quelques expériences de comportement sur *Microcebus murinus*, dans les locaux du MNHN à Paris, en collaboration avec Pierre-Yves Henry. Nous avons notamment testé leur réponse comportementale à des odeurs synthétiques de fruits (synthèse de l'odeur de *F. tillifolia*, prélevée lors d'une mission précédente à Madagascar). Les résultats obtenus n'étaient que préliminaires et représentaient uniquement une phase de mise au point pour faciliter les expériences *in natura* à Madagascar. Ces dernières n'ont malheureusement pas pu être réalisées (Cf. avant-propos). De plus, au cours de ces expériences, nous n'avons pas mis en évidence une attraction des microcèbes par l'odeur de *F. tiliifolia,* même s'il était évident qu'ils détectaient l'odeur (cette absence d'attraction s'explique sans doute par leur naïveté, ces animaux étant nés en captivité, et n'ayant donc jamais été confrontés aux odeurs des *Ficus* malgaches). En ce qui concerne les Eulemurs, nos observations de terrain ont mis en évidence que l'odorat était utilisé localement, c'est-à-dire sur l'arbre portant des fruits mûrs, pour discriminer, parmi les fruits, ceux qui étaient suffisamment mûrs, sans l'être trop. Il est possible que, comme cela a été montré chez les chiroptères (Korine & Kalko, 2005), les lémuriens puissent également éviter des fruits infestés par des parasites (des nématodes principalement) grâce à l'odeur. Il n'existe donc pas à l'heure actuelle de preuve d'un comportement de repérage à distance des fruits par olfaction chez les lémuriens. D'autres expériences restent nécessaires pour clarifier ce point, car les lémuriens semblent bien utiliser leur rhinarium pour déterminer la direction de la source odorante (R. Corlett, comm.pers.).

Cependant, l'analyse des bouquets olfactifs des espèces de figuiers dispersées par les lémuriens montrait des valeurs élevées en dérivés lipidiques, qui sont des COVs très légers, et donc détectables à longue distance. L'analyse d'odeurs d'autres fruits malgaches (hors du genre *Ficus*), dont les lémuriens se nourrissent également, montrait par contre des taux élevés en terpènes (C. Lemarchand & C. Soler, résultats non publiés), correspondant plutôt à des COVs floraux (Knudsen et al., 2006). Cependant, de récentes études (R. Hodgkison, comm. pers.) ont mis en évidence l'attraction de certaines espèces de Phyllostomidées (espèces américaines frugivores de chiroptères) par des bouquets volatils de figues fruits composés principalement de terpènes. Dans notre cas, les espèces chiroptérochores émettaient principalement des dérivés lipidiques, mais notre étude concernait des chiroptères frugivores de l'ancien monde, à savoir des Ptéropodidées. Ces questions, sur l'identité des composés impliqués et leur rôle attractif, restent donc ici en suspens, mais devraient être complétées grâce à d'autres missions de terrain à Madagascar.

Le second article (N°10) sur les odeurs au stade de dispersion montre quant à lui qu'un changement des pressions de sélection (convergence puis divergence des bouquets, respectivement à réceptivité et au stade dispersion), s'opère sur les bouquets volatils émis chez les figuiers dioïques. Si de tels résultats avaient déjà été mis en évidence par Borges et al. (2008), nos conclusions diffèrent cependant en un point. Le point commun entre ces deux études est la mise en évidence de pressions de sélection favorisant l'émission d'un message différent par les infrutescenses mâles et femelles, lorsque le disperseur qui se déplace pour aller se nourrir sur les figues femelles utilise le bouquet volatil émis pour localiser sa ressource. Alors que Borges et al. (2008) ont mis en évidence que la pression pour la divergence des bouquets pouvait être relâchée dans le cas d'une espèce ornithochore, *a contrario*, dans notre cas, la pression pour divergence est maintenue, quelle

que soit l'identité du disperseur. Ces deux études étant les seules, à notre connaissance, concernant les bouquets volatils émis par les « fruits » mâles et femelles d'espèces dioïques, nous ne pouvons pas établir de comparaisons avec des espèces n'appartenant pas au genre *Ficus*. En fait, il peut même paraitre surprenant que cette odeur puisse être similaire chez les deux sexes. En fin de la phase de maturation, les figues mâles contiennent des restes du pollen et des galles d'où les insectes ont émergé, alors que les figues femelles sont des fruits charnus. Sachant que les fruits produisent généralement une odeur assez caractéristique, principalement composée de dérivés lipidiques issus de la fermentation des sucres qu'ils contiennent (Dudareva *et al.*, 2006), il semble étonnant que des figues mâles, sans tissus charnus, puissent produire une odeur similaire à celles des femelles. En fait, l'espèce ornithochore analysée par Borges et al. (2008), *Ficus exasperata*, émet des quantités extrêmement faibles de COVs. De plus, la technique utilisée par ces auteurs est peu sensible, et laisse supposer l'existence d'un biais technique, particulièrement pour la capture des COVs très légers (R. Borges, Comm. Pers.). Cependant, sur le terrain, nous avons parfois rencontré des espèces où, pour un odorat humain, les figues mâles avaient un peu de tissu charnu d'où émanait une odeur fruitée. Chez les figuiers monoïques, les fruits sont charnus et contiennent les insectes. La production de pulpe dégageant l'odeur de fruits des figues mâles pourrait alors être un caractère dérivé de l'état ancestral qu'est la monoécie, tout comme la présence occasionnelle de certaines fleurs femelles fonctionnelles (c'est-à-dire pouvant donner des graines), parfois observée chez des figues mâles.

Plus généralement, il est évident que seule l'analyse comparative avec d'autres espèces de figuiers permettra d'aboutir à des conclusions plus robustes sur ce thème, finalement assez négligé, de l'odeur des figues mûres, et de l'évolution de la médiation chimique lors de la phase de dispersion des graines chez les figuiers monoïques et dioïques.

## D) *Parfums de figues* : évolution de la médiation chimique d'interactions mutualistes

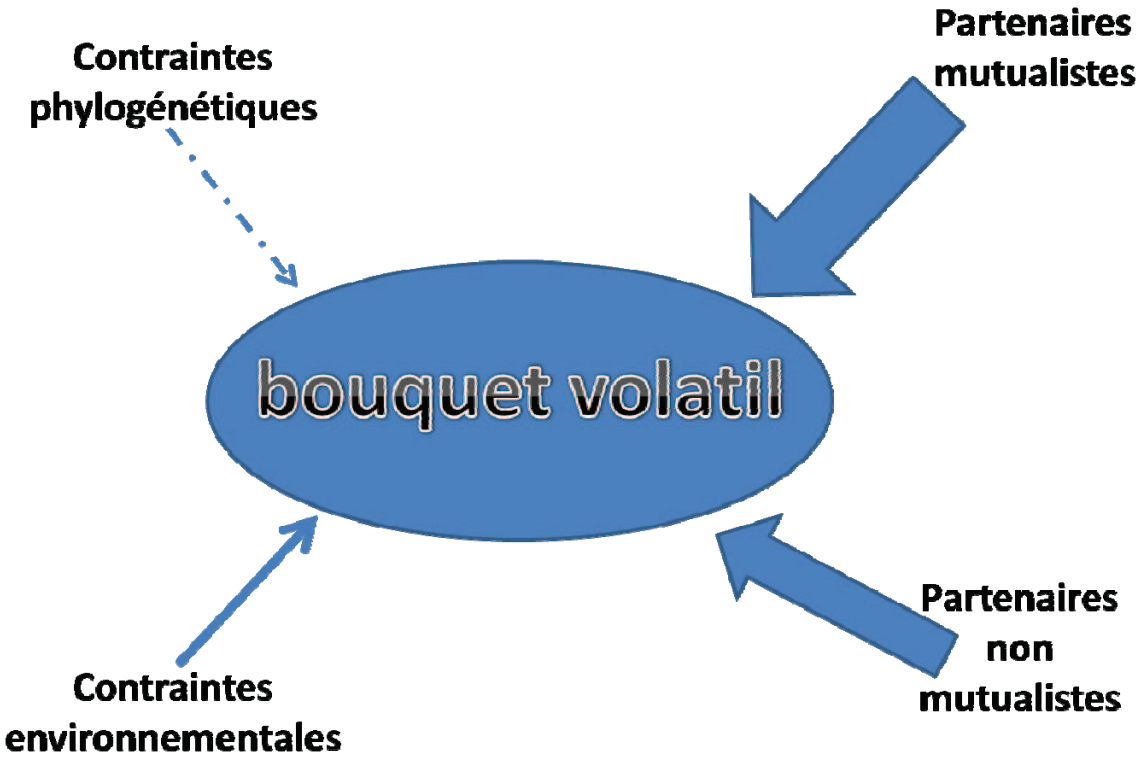
Après l'étude des messages émis par les figues à différents stades phénologiques, et la mise en évidence du rôle des partenaires mutualistes dans l'évolution de ces messages, il est important de se placer du côté du partenaire mutualiste, afin de comprendre si le signal fait lui aussi l'objet d'une évolution quant à sa perception et son utilisation. Cette analyse est indispensable pour confirmer le rôle du partenaire mutualiste en tant que pression de sélection, et justifie les tests comportementaux réalisés au cours de cette thèse (protocoles en annexe 3). Il faut tout d'abord noter que le signal sera intégré différemment chez les insectes et les vertébrés, du fait de la différence fondamentale de leurs organes olfactifs.

Les insectes ont des antennes couvertes de sensilles, renfermant de la lymphe qui est mise au contact de l'air ambiant par des pores dans la cuticule. Les COVs s'y infiltrent et sont captés par des protéines, qui assurent leur transport jusqu'aux récepteurs membranaires des dendrites nerveux déclenchant un stimulus nerveux. C'est la présence de ces protéines de transport et de ces récepteurs qui détermine qu'un individu détecte ou non chaque COV (Caissard, 2007) Les expériences d'électro-antennographie ont pour objectif de déterminer la liste des COVs ainsi détectés par les espèces d'insectes testés ; elles devront être complétées par des tests comportementaux pour établir l'interprétation réalisée par les insectes généralement répartie en trois catégories : attraction, répulsion ou indifférence (voir nos articles 5 & 7).

Chez les vertébrés, l'air inspiré passe sur la muqueuse nasale, et donc sur l'épithélium olfactif, dont les neurones olfactifs se terminent par des cils sensoriels porteurs de protéines chimio-réceptrices transmembranaires (Eisenberg & Kleiman, 1972). Ces protéines ne sont pas sensibles à un COV

particulier, mais plutôt à une combinaison d'un groupement chimique particulier et d'une conformation en trois dimensions du COV (Müller-Schwarze, 2006). Chaque neurone olfactif n'exprime qu'une seule protéine chimioréceptrice ; tous les neurones olfactifs exprimant la même protéine chimioréceptrice se rejoignent dans un même glomérule qui envoie donc au cerveau l'information d'un seul type de récepteur (Lledo *et al.*, 2005). Le cerveau associe alors une odeur à chaque combinaison d'informations, avec des seuils de détection différents (Müller-Schwarze, 2006). Les tests expérimentaux consistent à présenter des odeurs, naturelles et / ou mélanges synthétiques, et à établir la réponse comportementale des individus et des espèces testées. Les tests comportementaux réalisés au cours de cette thèse, notamment ceux sur les lémuriens et les chauves-souris, sont encore à poursuivre. Cependant, ces tests préliminaires de comportement tendent à confirmer les résultats obtenus par l'analyse des messages. Au cours de cette thèse, nous avons montré que les messages émis par les figues, et sur lesquels se fondent leurs partenaires mutualistes pour les repérer, étaient principalement façonnés par des pressions de sélection d'origine biotique (Cf. Fig. 10). Même si nous nous sommes concentrés sur la médiation chimique des interactions avec les partenaires mutualistes, il est important d'interpréter ces données en considérant également l'impact des non-mutualistes. En effet, l'interaction entre la figue et son pollinisateur étant basée sur un échange de ressources, des structures comme les galles et les larves du pollinisateur constituent des ressources exploitables pour les parasites, qui seront alors de véritables parasites du mutualisme, c'est-à-dire des deux partenaires à la fois (Proffit, 2007). Ces parasites peuvent ainsi usurper le message émis par une figue, comme montré dans notre article n°11 (Annexe 1). Il en est de même lors de l'interaction figuier/frugivore : des prédateurs de graines peuvent se nourrir des fruits sans assurer la dispersion des graines, comme dans le cas des animaux granivores (Shanahan *et al.*, 2001). Encore non étudiée à ce jour, l'usurpation du signal, au stade dispersion, est fort probable, mais reste à démontrer.

**Figure 10 :** Schéma récapitulatif des pressions existantes sur les bouquets volatils émis par les figuiers et responsables de l'attraction des mutualistes pollinisateurs et disperseurs de graines (la taille des flèches correspond à l'importance de la pression correspondante).



La médiation chimique pour attirer pollinisateur et disperseur de graines permet aux figuiers d'assurer leur reproduction. Le cas de la pollinisation par tromperie chez les figuiers dioïques illustre cependant l'existence d'une co-évolution antagoniste entre figuiers et pollinisateurs. De plus, nous avons montré que le figuier tendait à diminuer les coûts liés à la détection par les partenaires non mutualistes, par exemple en modifiant le message émis avant et après les stades de réceptivité et de dispersion. Cependant, des travaux récents ont montré que les parasites spécifiques (Proffit, 2007) et les fourmis prédatrices (Schatz & Hossaert-McKey, 2010) sont capables non seulement de détecter cette variation de la médiation chimique, mais aussi de la contourner en exploitant les phases où les ressources qu'ils recherchent sont les plus abondantes. Dans leur revue sur les conflits dans les mutualismes *nursery pollination*, Dufaÿ et Anstett (2003) ont montré qu'une fonction écologique donnée amenait aux mêmes conflits évolutifs, même pour des interactions ayant évolué indépendamment. La résolution de ces conflits engendre des mécanismes proximaux différents, malgré la similarité des pressions de sélection engagées.

L'étude de la médiation chimique entre figuiers et mutualistes fait apparaitre, ici, une force déterminante dans l'évolution de ces interactions, que résume la théorie de la « Reine Rouge », une métaphore issue d'un épisode du livre de Lewis Carroll (1871), au cours duquel Alice et la Reine Rouge se lancent dans une course effrénée sans que le paysage alentour ne change. La Reine Rouge explique qu'il faut courir pour rester à la même place, ce qui symbolise à présent la nécessité d'une évolution permanente des espèces pour maintenir leur valeur reproductive, même lorsque le milieu abiotique ne change pas (Van Valen, 1973). Les résultats obtenus ici sur l'évolution de la médiation chimique, dans le contexte de l'interaction mutualiste entre figuiers dioïques et pollinisateurs, illustrent bien cette idée d'un environnement biotique qui se modifie en permanence. En effet, si le pollinisateur détecte une différence entre les figues mâles et femelles, le

figuier devra la masquer pour maintenir la pollinisation des femelles. Similairement, la faune endémique malgache représente un nouvel environnement biotique pour les figuiers auquel ils doivent s'adapter. Ainsi, la communication entre deux individus reflète les forces sélectives façonnant l'interaction elle-même.

# CONCLUSION & PERSPECTIVES

***PARFUM DE FIGUES :***

**UN EXEMPLE DE**

**L'ÉVOLUTION DE LA COMMUNICATION**

L'étude de la médiation chimique des interactions figuiers/mutualistes nous a permis de mettre en évidence la pression de sélection que représente un partenaire sur le message assurant leur rencontre régulière. De surcroît, nous avons montré que les pressions façonnant ce message façonnent l'interaction elle-même. Etudier le mode de communication entre deux individus met donc en évidence les forces évolutives qui régissent leur interaction. L'analyse complète d'une communication requiert de comprendre la façon dont les systèmes communicants interagissent (études des processus neurobiologiques, physiologiques et cognitifs), la nature de leurs différences et de leurs similitudes, et l'impact de cette communication sur la valeur reproductive des individus et des espèces impliquées. A l'heure actuelle, le point le plus souvent négligé, dans l'étude d'exemples de communication, est la manière dont le signal est perçu et intégré par le receveur, un point qui reste également important de compléter dans notre étude. En effet, terminer les expériences de comportement nous apparait comme la perspective majeure à ce travail.

Dans le cadre de nos travaux sur la pollinisation, il est primordial d'apporter des informations quant aux COVs détectés (tests électro-physiologiques) et réellement attractifs (tests comportementaux) pour les pollinisateurs (ainsi que pour les non-pollinisateurs). De plus, il serait intéressant de comprendre le rôle des composés de surface des figues afin de savoir s'ils complètent le rôle des bouquets volatils. La notion de coûts physiologiques associés à la production des COVs est également à poursuivre. Dans le cadre de nos travaux sur la dispersion, il est primordial de distinguer le signal du message, et de comprendre quelles parties du message utilisent les différents frugivores. Il serait également pertinent de poursuivre les travaux afin de vérifier l'absence d'inertie phylogénétique sur les messages émis à dispersion, comme nous l'avons montré au stade de réceptivité. Enfin, il serait intéressant de comparer nos résultats à d'autres interactions, mutualistes ou non, utilisant un mode de communication différent, en particulier pour vérifier que les

pressions s'exerçant sur un message non chimique sont similaires. En effet, quel que soit le type de signal employé (et la façon dont il est perçu), il semble que l'émetteur aura toujours un compromis à réaliser entre la perception par des récepteurs améliorant sa valeur sélective, et la perception par des parasites ou tricheurs non désirés… Par exemple, à notre connaissance, aucune étude n'existe sur l'usurpation des signaux par sonar : Est-ce qu'un groupe de dauphins peut se faire repérer par une orque prédatrice lors d'échanges de communications sociales ?

La communication, quel que soit son mode, peut être vue comme un ciment assemblant entre eux différents éléments d'un réseau, au sein d'un réseau plus large qu'est l'écosystème. Dans la nature, de nombreuses formes de communication différentes coexistent, et de nombreux facteurs peuvent expliquer une telle diversité. Nous proposons par ce travail que la pression sélective exercée par les interactions entre l'ensemble des organismes vivants de l'écosystème soit une cause majeure de cette diversité, et un des moteurs évolutifs de la communication au cours du temps.

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# ANNEXES

# ANNEXE 1:

**Manuscrit 11**

Proffit M., Chen C., **Soler C.**, Bessiere J.-M., Schatz B. and Hossaert-McKey M. 2009.
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# Can chemical signals, responsible for mutualistic partner encounter, promote the specific exploitation of nursery pollination mutualisms? – The case of figs and fig wasps

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**Abstract**

In nursery pollination mutualisms, where pollinators reproduce within the inflorescence they pollinate, floral scents often play a major role in advertizing host location and rewards for the pollinator. However, chemical messages emitted by the plant that are responsible for the encounter of mutualist partners can also be used by parasites of these mutualisms to locate their host. Each species of *Ficus* (Moraceae) is involved in an obligatory nursery pollination mutualism with usually one pollinating fig wasp (Hymenoptera: Chalcidoidea: Agaonidae). In this interaction, volatile compounds emitted by receptive figs are responsible for the attraction of their specific pollinator. However, a large and diverse community of non-pollinating chalcidoid wasps can also parasitize this mutualism. We investigated whether the chemical message emitted by figs to attract their pollinator can promote the host specificity of non-pollinating fig wasps. We analysed the volatile compounds emitted by receptive figs of three sympatric *Ficus* species, namely, *Ficus hispida* L., *Ficus racemosa* L., and *Ficus tinctoria* G. Forster, and tested the attraction of the pollinator of *F. hispida* (*Ceratosolen solmsi marchali* Mayr), and of one species of non-pollinating fig wasp [*Philotrypesis pilosa* Mayr (Hymenoptera: Chalcidoidea: Pteromalidae)] to scents emitted by receptive figs of these three *Ficus* species. Analysis of the volatile compounds emitted by receptive figs revealed that the three *Ficus* species could be clearly distinguished by their chemical composition. Behavioural bioassays performed in a Y-tube olfactometer showed that both pollinator and parasite were attracted only by the specific odour of *F. hispida*. These results suggest that the use by non-pollinating fig wasps of a specific chemical message produced by figs could limit host shifts by non-pollinating fig wasps.

## Introduction

As in many other pollination systems, plants involved in nursery pollination mutualisms, where the reward for pollinators is breeding sites within the flowers or inflorescences (reviewed by Dufaÿ & Anstett, 2003), emit volatile compounds to attract their mutualistic partner (Ware et al., 1993; Hossaert-McKey et al., 1994; Dufaÿ et al., 2003; Dötterl et al., 2006; Okamoto et al., 2007). These chemical messages emitted by the plant are important signals in communication between flowering plants and pollinators (for review see Raguso, 2008) and may contribute to reproductive isolation between sympatric, closely related species (Levin et al., 2001). However, like other mutualistic interactions between plants and insects,

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nursery pollination mutualisms are often exploited by parasites of the mutualism (Kerdelhué & Rasplus, 1996; Pellmyr et al., 1996; Gaume & McKey, 1999; Bronstein, 2001; Després & Cherif, 2004). These parasitic species exploit the resources or services involved in the mutualism but do not provide any benefit (Bronstein, 2001). In many cases, for these parasites as well as for the mutualistic partners, interaction with their host is species-specific and obligatory; locating their host is therefore crucial. In nursery pollination mutualisms, the chemical messages emitted to attract pollinators (Grison-Pigé et al., 2002b; Dötterl et al., 2006; Proffit et al., 2008) could thus also be used as species-specific signals by parasites of the interaction.

The interactions between figs and fig wasps provide an excellent model for the study of host location by parasites of nursery pollination mutualisms. Figs are urn-shaped inflorescences, closed by imbricate bracts that form the ostiole. The female flowers of all *Ficus* species (more than 750 species worldwide) are pollinated by mutualistic fig wasps that feed exclusively on the developing ovule tissues of their host (Galil & Eisikowitch, 1968; Janzen, 1979). *Ficus* species are found mainly in tropical habitats, and many species often co-occur in one area. As the density of individual species is often quite low (Anstett et al., 1997) and flowering is usually asynchronous among trees, the message emitted by each fig species must be efficient over considerable distances and must attract the associated pollinator species. Female pollinating wasps transport fig pollen by carrying it from their natal fig to a receptive one, usually on a different tree (Kjellberg et al., 2001), using volatile compounds emitted by the receptive fig to locate it (Ware et al., 1993; Hossaert-McKey et al., 1994; Grison-Pigé et al., 2002b). They lay eggs in the ovaries of a fraction of the flowers they pollinate, thereby initiating the formation of galls, each corresponding to the development site of one pollinator larva (Galil & Eisikowitch, 1968). A large and diverse community of non-pollinating chalcidoid wasps that do not carry pollen also mature within the ovaries of fig inflorescences (Compton & van Noort, 1992; Cook & Rasplus, 2003). Unlike pollinators, most non-pollinating fig wasps (NPFWs) oviposit into the ovaries of female flowers by inserting their ovipositor through the fig wall, and hence without entering the figs. While many NPFWs are gall-makers or parasitoids, some of them are cleptoparasites of the pollinator (or of other gall-makers). Cleptoparasite larvae feed from the gall tissues (which pollinators have initiated) and develop at the expense of the pollinator larvae. Consequently, these parasites will reap the benefits of the mutualism while delivering no benefits in return. Most of these NPFWs are assumed to be specifically associated with a single *Ficus* species. However, studies of NPFWs are hindered by poorly developed taxonomy, and their biodiversity and ecology are consequently poorly known (Cook & Rasplus, 2003). A previous study showed that NPFWs use volatile compounds emitted by figs to locate their host and that the signal they used is specific to the fig developmental stage to which they are restricted (Proffit et al., 2007).

In this study, we focussed on *Ceratosolen solmsi marchali* Mayr (Hymenoptera: Chalcidoidea: Agaonidae), the species-specific pollinator of the gynodioecious fig *Ficus hispida* L. (Moraceae), and on a cleptoparasite of the *F. hispida*/*C. s. marchali* mutualism, *Philotrypesis pilosa* Mayr (Hymenoptera: Chalcidoidea: Pteromalidae) (Abdurahiman, 1986). In this system, pollinator and parasite oviposit at the same stage of fig development (Peng et al., 2005; Proffit, 2007) and, as for all NPFWs, their emergence from the natal fig is synchronous with pollinator emergence. Both wasps are attracted by volatile chemicals emitted by the host fig at receptivity (Proffit et al., 2007; Chen & Song, 2008). Nothing is known, however, about the specificity of chemical mediation of the interactions between the fig, its pollinating wasp, and the parasite of the mutualism. To examine this question, we performed chemical analyses, using volatile compounds emitted by receptive figs of *F. hispida* and of two sympatric non-host *Ficus* species, as well as bioassays on *C. s. marchali* and on *P. pilosa*. The aim of this study was to investigate if chemical mediation reinforces the specificity and evolutionary stability of fig/fig wasp interactions.

## Materials and methods

### Study site and species used

The study was carried out at the Xishuangbanna Tropical Botanical Garden (21°55′N, 101°15′E), Yunnan Province, China, in December 2005 and, only for *Ficus racemosa*, in March 2006. We collected and analysed odours released by receptive figs of *F. hispida* (subgenus *Sycomorus*, section *Sycocarpus*; gynodioecious) and two other sympatric species, *Ficus tinctoria* (subgenus *Ficus*, section *Sycidium*; gynodioecious) and *F. racemosa* (subgenus *Sycomorus*, section *Sycomorus*; monoecious). For the two gynodioecious species, only male figs were used. We also investigated the attraction of *C. s. marchali* and *P. pilosa* to odours of receptive figs. The pollinator *C. s. marchali* lives for a much shorter period than *P. pilosa* (a few hours vs. more than 1 week) and usually oviposits only in one fig, whereas NPFWs oviposit in several figs over the course of their life.

Like all gynodioecious *Ficus* species, *F. hispida* is functionally dioecious, with some trees bearing only female figs and producing only seeds while other trees bear

hermaphrodite inflorescences that produce only wasps (pollinators and NPFWs) and pollen, and thus are functionally male (Kjellberg et al., 1987). In China, *F. hispida* is pollinated by *C. s. marchali*. Three species of NPFWs [identified by J.-Y. Rasplus as *P. pilosa*, *Philotrypesis* sp., and *Apocrypta bakeri* Joseph (all Pteromalidae)] oviposit from outside the fig (on male trees only) (Peng et al., 2005; Proffit, 2007). At any time of year, some *F. hispida* trees are producing figs and both wasp reproduction and seed production thus occur year-round (Patel & Hossaert-McKey, 2000). In monoecious fig species, all figs contain functionally male and female flowers and produce both wasps and seeds [for a detailed account of the fig-fig wasp cycle, see Galil & Eisikowitch (1968) and Kjellberg et al. (2005)].

#### Collection and tentative identification of volatile compounds

Volatile compounds released by figs were collected by adsorption-desorption (dynamic headspace) technique (Grison-Pigé et al., 2001; Tholl et al., 2006). Because of the necessity to obtain concentrated extracts (the number of figs per branch was very low) and to obtain extracts from conditions comparable to those in the insect behavioural tests (see below), odour collections were performed ex situ. In previously performed tests on several *Ficus* species, no significant differences were found in the blends of volatiles emitted by figs in situ and ex situ (Grison-Pigé, 2001, M Proffit, unpubl.). Figs in pre-receptive phase were placed in nylon bags to prevent oviposition by pollinators and NPFWs. For odour collection, receptive figs were taken from the tree and enclosed in bags made of polyethylene terephtalate (Nalophan®; Kalle Nalo, Wiesbaden, Germany). The developmental stage of the figs could be precisely determined from their external characteristics. Indeed, at the same time as female flowers reached sexual maturity and became receptive, the cavity inside the fig grew and the ostiole opened to allow pollinator visitation. Figs then became softer compared to the stages of development before and after receptivity and pollinators were attracted to the bagged figs. At the entrance of the Nalophan bag, air was conducted through a Teflon tube and purified using two glass tubes filled with activated charcoal (Supelco, Bellefonte, PA, USA). On the other side of the bag, at the exit, a trap containing 30 mg of Alltech Super Q adsorbent (ARS, Gainesville, FL, USA) was placed inside the bag and connected to a plastic tube. Airflow was maintained through the bags by two pumps. Entrance and exit flow rates (controlled by flowmeters) were 400 and 300 ml/min, respectively. Odour collection was always performed under natural light and ambient temperature ($20.4 \pm 1.7$ °C) between 12:00 and 15:00 hours, corresponding to the insects' period of maximum activity during our field season. During this period, depending on the number of trees in receptive stage, we collected one sample from each of six individuals of *F. hispida*, three individuals of *F. tinctoria*, and five individuals of *F. racemosa* (two in December 2005 and three in March 2006). During the collections, in order to collect an amount of scent sufficient for analysis, numbers (mean ± SD) of figs in the sample were adjusted according to fig diameter: for *F. hispida*, $19 \pm 1$, for *F. racemosa* $45 \pm 19$, and for *F. tinctoria* $262 \pm 49$.

Trapped odour samples were stored at –18 °C until analysis. Trapped volatiles were eluted with 150 µl of dichloromethane; two internal standards (nonane and dodecane, 200 ng/µl) were added to each sample for gas chromatography (GC). Volatile compounds were analysed by injection in a CP-3800 (Varian, Palo Alto, CA, USA) GC with a flame ionization detector (GC-FID) and in a GC coupled with a mass spectrometer (GC-MS) Saturn 2000 (Varian). For both analyses, a Varian® CP-SIL low bleed MS column [length 30 m, inside diameter 0.25 mm, film thickness 0.25 µm, with helium as carrier gas (1 ml/min)] was used. The injector split vent was opened (1/4). Oven temperature was held at 50 °C during 3 min; then increased by 3 °C/min to 100 °C, by 2.7 °C/min to 140 °C, by 2.4 °C/min to 180 °C, and by 6 °C/min to 250 °C. The MS interface was 270 °C and the ion trap worked at 170 °C. Mass spectra were taken at 70 eV (in EI mode).

The GC-FID and GC-MS data were processed using the Saturn Software package 5.52 (Varian). Retention index (RI) of each compound was calculated according to Van den Dool & Kratz (1963). Tentative identification of compounds was based on matching of the mass spectra with NIST 98 MS and MassFinder 3 libraries, and on confirmation by comparison of RI with MassFinder 3 libraries and published data (Adams, 2001). Identification of some components was confirmed by comparison of both mass spectrum and GC retention data with those of standards.

In addition, for each sample, we estimated the total quantities of volatile compounds by using as a scale the peak areas of both internal standards (IS) in GC-FID chromatograms. Peak areas of all the compounds were determined, divided by IS peak area, and multiplied by amount of IS. The relative proportions of the compounds emitted by figs were determined for each sample using GC-FID chromatograms.

#### Statistical analysis of the volatile blends

We first compared the intensity of volatile emission among the three species. For the *Ficus* species studied here, seasonal variation may affect the quantity of volatile compounds emitted by figs, but not the relative proportions of the different compounds in the blend (Grison-Pigé et al.,

2002a, M Proffit, unpubl.). For this reason, the extractions performed on *F. racemosa* in March 2006 were not used for the quantitative comparison of total emission. Differences among the three species in the number and the total amount of compounds emitted were tested using general linear models [Proc GLM, type 3 SS, combined with pairwise Tukey tests (SAS, 2003, Release 9.01; SAS Institute, Cary, NC, USA)]. The residuals of the variables were normally distributed (for all, Shapiro test, $P>0.5$).

To compare scent composition among samples, statistical analyses were performed using PC-ORD (McCune & Mefford, 1999). First, a data matrix of pairwise comparisons among samples was composed. The data matrix consisted of comparisons of scent composition (using the relative proportions of all compounds) among samples based on the Bray-Curtis distance index (Bray & Curtis, 1957), ranging between 0 and 1. This index was used here because, as for faunal and floral communities, in inter-specific comparison of floral scents more than half of the entries in the matrix are zero (Bray & Curtis, 1957). Variation in patterns of scent composition among species was tested for significance with a multi-response permutation procedure (MRPP) on the data matrix. MRPP tests a priori defined groups against random groups in ordinate space. A non-metric multidimensional scaling was used to find the best two-dimensional representation of the distance matrix. To evaluate how well the particular configuration produces the observed distance matrix the stress value is given. The smaller the stress value, the better is the fit of the reproduced distance matrix to the observed. In order to determine if some compounds were significantly different among the volatile profiles of the three species studied, we used Dufrêne & Legendre's (1997) method of indicator species to calculate 'indicator compounds'. This method is used in analysis of species composition of communities. Here, 'indicator compounds' are defined as the most characteristic compounds in the scent of each species, found mostly in a single species and present in the majority of the individuals of that species. For each compound, the analysis following the method of Dufrêne & Legendre (1997) was used to calculate an observed indicator value (IV) for each species, combining information on the presence and abundance of the compound within the species. In addition, we also tested, for each compound in each species, whether the observed IV differed from a random IV using a Monte Carlo test with 1000 randomizations.

#### Insect behavioural tests

We tested whether individuals of *C. s. marchali* and *P. pilosa* were attracted to volatile compounds emitted by receptive figs from the three sympatric *Ficus* species, using a glass Y-tube olfactometer, in which only chemical cues were available to guide the wasps (Schatz et al., 2003; Tooker et al., 2005). Previous studies, using four-arm olfactometers, six-arm olfactometers, and wind tunnels, have shown that wasps behave more naturally in Y-tube olfactometers than in the other kinds of apparatus tested, in which wasps often remained immobile on the glass walls. Bioassays were conducted under laboratory conditions. Preliminary tests showed the necessity of using differently sized Y-tubes and flow rates for the two wasp species. For *P. pilosa* and for *C. s. marchali*, the Y-tube was 4 and 1.5 cm in diameter and each lateral arm was 14 and 9 cm long, respectively; for both species, the central arm was 8 cm long. Humidified air was purified with activated charcoal and blown into each lateral arm (75 ml/min and 200 ml/min per arm, respectively). Air was extracted from the central arm (95 ml/min and 380 ml/min, respectively). Humidified air was blown through two Nalophan® bags connected to the arms of the Y-tube. In one arm the bag was empty, and in the other the bag contained a mix of figs from several trees (of the same *Ficus* species). The total number of figs depended on their diameter, which differed among species. Our tests used four figs of *F. hispida*, six figs of *F. racemosa*, and 20 figs of *F. tinctoria*. For *F. tinctoria* and *F. hispida*, we used only male figs. Discrimination by pollinators and NPFWs between male and female figs of dioecious species will be treated in a subsequent paper. In order to eliminate visual cues, a piece of opaque nylon fabric was arranged on the top of each branch. The Y-tube and the nylon were changed and cleaned with acetone and heated in an oven at 120 °C before each trial. To avoid a directional bias, the positions of control and odour source were reversed between the two arms for each consecutive trial. In addition, control runs were performed with humidified air only in both arms of the Y-tube. In order to compare wasp choice frequencies between the treatments, we alternated the different bioassays and controls. For the bioassays, a total of 32 individuals of *P. pilosa* and 32–34 of *C. s. marchali* were tested per odour source for 10 min. The behavioural choice made by each individually tested wasp was recorded by distinguishing three modalities: choice for fig odour, choice for control, or no choice. We considered that wasps made no choice when they stayed motionless for 3 min in the departure section and/or the central arm before the bifurcation of the olfactometer.

Newly emerging adults of *C. s. marchali* and *P. pilosa* were collected from mature figs taken randomly from different individual male trees. Individuals of *P. pilosa* were kept and fed with honey and water between 1–3 days before the tests. Because of their very short lifespan (about 3 h with no feeding at the adult stage), individuals of *C. s. marchali* were tested immediately after their emergence

from the fig. Each day, a maximum of 10 individuals of *C. s. marchali* and *P. pilosa* were tested per odour source. All wasps tested were naïve to receptive fig odours. For the three species studied, pre-receptive figs from several trees per species had been placed in a nylon bag until they reached receptivity to prevent pollination (only males, for functionally dioecious species). Receptive figs were collected randomly from these different individual trees.

For all comparisons, the response of wasps was statistically analysed using $\chi^2$-tests with Yates correction (when observed values in at least one cell of the contingency table were less than 5). First, we tested if a preference of wasps for one of the arms (left vs. right) occurred in the controls. Then, to determine if wasps were attracted by fig odours, for each series of tests, data were arranged in a $2 \times 2$ contingency table. We compared the proportion of wasps that chose the right olfactometer arm or the left, when the tested fig odour was on the left vs. on the right (excluding the no choice response). For each wasp species, we first determined which one of the test odours was most attractive. We then examined if there was a difference of attraction between the two other less attractive odours by comparing the proportions of wasps that chose each odour.

## Results

### Inter-specific variation in the chemical message emitted by receptive figs

For the three species together, we detected a total of 68 compounds and tentatively identified 66 of them (Table 1). The number of compounds (mean ± SD) emitted by individuals differed significantly among species: *F. tinctoria*, 15.3 ± 3.8, *F. hispida*, 27.5 ± 9.9, and *F. racemosa*, 11.6 ± 3.1 (analysis of variance: $F_{2,13} = 7.37$, $P = 0.009$). Pairwise comparisons showed that *F. hispida* emitted a significantly greater number of compounds than the other two species. The total amount of scent collected per fig per h also varied significantly among species: *F. tinctoria*, 0.26 ± 0.04 ng, *F. hispida*, 1.99 ± 1.35 ng, and *F. racemosa*, 1.52 ± 0.87 ng (analysis of variance: $F_{2,10} = 5.67$, $P = 0.03$), and Tukey tests on pairwise comparisons showed significant differences between all pairs of species. Each species blend comprised 12–27 compounds with relative abundances greater than 1%, the total abundance of these compounds representing 87–96% of the total blend (Table 1). Most of the compounds emitted by the three species were terpenes. In quantitative terms, monoterpenes were the most important compounds present in the scent of *F. racemosa*. In comparison, *F. tinctoria* and *F. hispida* emitted higher amounts of sesquiterpenes.

For the different *Ficus* species, the average Bray-Curtis distances of chemical profiles between two individuals of the same species (mean ± SD: *F. tinctoria*, 0.61 ± 0.23, *F. hispida*, 0.59 ± 0.12, and *F. racemosa* 0.22 ± 0.13) were significantly lower than distances between two individuals of different species, both across all the species as well as in pairwise species comparisons (for all tests: distance≥0.74 and P≤0.008; Table 2), suggesting that the three species can be separated by the profile of the volatile compounds they emit (Figure 1). According to the Monte Carlo test 19 of the 68 compounds are good indicators of species volatile profiles (see Table 3).

### Insect behavioural tests

Control tests showed no directional trends in the olfactometer in odourless conditions for either of the wasp species: *C. s. marchali* ($\chi^2 = 0$, d.f. = 1, P = 1; n = 40) and *P. pilosa* ($\chi^2 = 0.3$, d.f. = 1, P = 0.58; n = 15). Results of the tests are presented in Figure 2. Both the pollinator, *C. s. marchali*, and the parasite, *P. pilosa*, were attracted by volatile compounds emitted by receptive male figs of *F. hispida*, their host species (Yates-corrected $\chi^2 = 24.60$, d.f. = 1, P<0.001; n = 32, and $\chi^2 = 4.50$, d.f. = 1, P = 0.03; n = 30, respectively). Neither receptive male figs of *F. tinctoria* nor receptive figs of *F. racemosa* were significantly attractive for *C. s. marchali* ($\chi^2 = 0.13$, d.f. = 1, P = 0.72; n = 32, and $\chi^2 = 2.03$, d.f. = 1, P = 0.15; n = 32, respectively) or for *P. pilosa* ($\chi^2 = 0$, d.f. = 1, P = 1; n = 32, and $\chi^2 = 0.27$, d.f. = 1, P = 0.60; n = 31, respectively). For both wasp species, there was no significant variation in the proportion of individuals attracted to the odour of *F. tinctoria* and *F. racemosa* (for *C. s. marchali*: $\chi^2 = 1.58$, d.f. = 1, P = 0.21; n = 64, and for *P. pilosa*: $\chi^2 = 0.14$, d.f. = 1, P = 0.70; n = 63) and no difference between wasp species (for *F. racemosa*: $\chi^2 = 1.38$, d.f. = 1, P = 0.54; n = 63, and for *F. tinctoria*: $\chi^2 = 0.06$, d.f. = 1, P = 0.80; n = 63). Concerning the attraction by *F. hispida*, *C. s. marchali* was more often attracted by volatile chemicals than was *P. pilosa* (Yates-corrected $\chi^2 = 7.30$, d.f. = 1, P = 0.008; n = 32). Finally, a significantly greater proportion of pollinators than of parasites ($\chi^2 = 8.89$, d.f. = 1, P = 0.005) did not make a choice in our tests.

## Discussion

Combining bioassays and chemical analyses, the present study demonstrates the role of volatile compounds as signals that enable both the pollinator of *F. hispida*, *C. s. marchali*, and a parasite of the mutualism, *P. pilosa*, to locate their host among other sympatric fig species. These results suggest that the specificity of the chemical message emitted by receptive figs and recognition of this message by the fig-wasps could constitute a barrier to host shift, for both pollinator and NPFWs. Host specificity of pollinator

**Table 1** Occurrence and relative amount of tentatively identified volatiles emitted by receptive figs of three *Ficus* species. Compounds detected in each species are divided into three groups based on general biosynthetic origin (Knudsen et al., 2006) and within all the groups in five classes. Within each class, compounds are listed in order of their retention index (RI obs), values of which were compared with published data [Adams, 2001 (RI A)] and MassFinder 3 library (RI MF). We present mean ± SD of the percentage of each compound in the total bouquet, plus the number of trees in which the compound was detected, for each of the three *Ficus* species (O, occurrence)

| Compounds | RI obs | RI A | RI MF | *Ficus tinctoria* ($n = 3$) O | % | *Ficus hispida* ($n = 6$) O | % | *Ficus racemosa* ($n = 5$) O | % |
|---|---|---|---|---|---|---|---|---|---|
| **Fatty acid derivatives** | | | | | | | | | |
| Ethyl butyrate | 804 | 802 | | | | 3 | 1.81 ± 3.36 | | |
| Butyl acetate | 811 | 807 | | | | 2 | 0.59 ± 0.97 | | |
| (*Z*)-3-Hexenol[1] | 853 | 850 | | | | 2 | 0.29 ± 0.43 | | |
| 2-Heptanone | 891 | 889 | | 2 | 1.97 ± 1.79 | | | | |
| 2-Heptanol | 896 | 894 | | 2 | 1.13 ± 1.14 | | | | |
| (*Z*)-3-Hexenyl acetate | 1 006 | 1 004 | | 2 | 0.81 ± 0.71 | 3 | 1.77 ± 2.62 | | |
| Hexyl 2-methylbutyrate | 1 236 | 1 233 | | | | 2 | 0.19 ± 0.27 | | |
| Tridecane[1] | 1 300 | 1 300 | | | | 3 | 1.49 ± 2.28 | 3 | 0.38 ± 0.37 |
| Tetradecane[1] | 1 400 | 1 400 | | | | 3 | 1.03 ± 1.71 | | |
| *Mean of total percent* | | | | | 3.91 | | 7.17 | | 0.38 |
| **Terpenes** | | | | | | | | | |
| **Monoterpenes** | | | | | | | | | |
| α-Pinene[1] | 932 | 932 | | 2 | 0.46 ± 0.41 | 4 | 1.93 ± 2.11 | 4 | 0.82 ± 0.84 |
| Sabinene | 973 | 969 | | 2 | 0.76 ± 0.67 | 3 | 5.73 ± 4.39 | 5 | 2.74 ± 3.24 |
| β-Pinene | 977 | 974 | | 2 | 1.01 ± 0.97 | 3 | 2.63 ± 3.52 | 2 | 1.21 ± 2.14 |
| Myrcene[1] | 990 | 988 | | | | 3 | 0.98 ± 1.48 | 4 | 0.76 ± 0.55 |
| p-Cymene[1] | 1 026 | 1 020 | | | | 2 | 0.36 ± 0.66 | | |
| Limonene[1] | 1 029 | 1 024 | | 2 | 1.78 ± 1.55 | 5 | 3.28 ± 3.67 | | |
| 1,8-Cineole | 1 032 | 1 026 | | | | 4 | 2.54 ± 3.09 | 1 | 0.21 ± 0.49 |
| (*Z*)-β-Ocimene[1] | 1 037 | 1 032 | | | | 2 | 0.35 ± 0.53 | 5 | 7.18 ± 3.98 |
| (*E*)-β-Ocimene[1] | 1 047 | 1 044 | | 3 | 24.37 ± 30.18 | 5 | 3.42 ± 2.90 | 5 | 72.38 ± 15.36 |
| γ-Terpinene[1] | 1 060 | 1 054 | | | | | | 2 | 0.51 ± 0.93 |
| cis-Linalool oxide (furanoid)[1] | 1 069 | 1 067 | | | | 1 | 0.19 ± 0.44 | | |
| trans-Linalool oxide (furanoid)[1] | 1 091 | 1 084 | | | | 1 | 0.11 ± 0.22 | | |
| Linalool[1] | 1 102 | 1 095 | | | | 6 | 13.13 ± 10.32 | 5 | 7.10 ± 4.89 |
| Perillene | 1 105 | 1 102 | | 3 | 2.15 ± 0.88 | 5 | 2.47 ± 2.93 | 2 | 1.19 ± 2.41 |
| *Mean of total percent* | | | | | 30.53 | | 37.12 | | 94.10 |
| **Sesquiterpenes** | | | | | | | | | |
| α-Cubebene[1] | 1 347 | 1 345 | 1 355 | 2 | 1.74 ± 1.88 | | | | |
| Clovene | 1 365 | | 1 365 | | | 1 | 0.24 ± 0.55 | | |
| 1-epi-α-Pinguisene | 1 369 | | 1 367 | | | 2 | 0.67 ± 1.37 | | |
| α-Copaene[1] | 1 375 | 1 374 | 1 379 | 2 | 8.93 ± 8.51 | 2 | 0.70 ± 1.23 | | |
| Daucene | 1 378 | 1 380 | 1 380 | | | 2 | 0.95 ± 1.53 | | |
| β-Panasensene | 1 379 | 1 381 | 1 385 | | | 1 | 1.24 ± 2.99 | | |
| α-Isocomene | 1 384 | 1 387 | 1 389 | | | 2 | 1.14 ± 1.98 | | |
| β-Cubebene | 1 388 | 1 387 | 1 390 | 2 | 1.26 ± 1.23 | | | | |
| β-Elemene[1] | 1 389 | 1 389 | 1 389 | | | 4 | 2.29 ± 2.13 | | |
| β-Isocomene | 1 403 | 1 407 | 1 411 | | | 2 | 0.68 ± 1.09 | | |
| α-Cedrene | 1 411 | 1 410 | 1 410 | | | 1 | 1.08 ± 2.59 | | |
| α-Gurjunene | 1 412 | 1 409 | 1 413 | | | 1 | 0.55 ± 1.31 | | |
| (Z)-α-Bergamotene[1] | 1 413 | 1 411 | 1 411 | | | 3 | 1.37 ± 1.63 | 3 | 0.17 ± 0.20 |
| β-Caryophyllene[1] | 1 419 | 1 417 | 1 421 | 2 | 5.51 ± 4.81 | 6 | 4.89 ± 3.89 | | |
| (E)-α-bergamotene[1] | 1 433 | 1 432 | 1 434 | 2 | 0.55 ± 0.48 | 4 | 3.07 ± 3.02 | | |
| Aromadendrene | 1 440 | 1 439 | 1 443 | | | 3 | 0.46 ± 0.63 | | |

**Table 1** *Continued*

| Compounds | RI obs | RI A | RI MF | *Ficus tinctoria* ($n = 3$) O | % | *Ficus hispida* ($n = 6$) O | % | *Ficus racemosa* ($n = 5$) O | % |
|---|---|---|---|---|---|---|---|---|---|
| (*E*)-β-Farnesene[1] | 1 453 | 1 454 | 1 446 | 1 | 0.31 ± 0.53 | 6 | 16.22 ± 8.49 | | |
| α-Neoclovene | 1 456 | 1 452 | 1 456 | | | 1 | 0.60 ± 1.42 | | |
| α-Humulene[1] | 1 455 | 1 452 | 1 455 | 2 | 0.98 ± 0.99 | 4 | 1.83 ± 1.88 | | |
| Alloaromadendrene[1] | 1 463 | 1 458 | 1 462 | 2 | 1.10 ± 0.96 | 3 | 0.62 ± 0.83 | | |
| Unknown 1[2] | 1 464 | | | | | | | 4 | 1.93 ± 1.66 |
| Selina-4,7-diene | 1 467 | | 1 469 | | | 1 | 0.20 ± 0.45 | | |
| Ischwarane | 1 465 | 1 465 | 1 468 | | | 1 | 0.44 ± 1.02 | | |
| β-Neoclovene | 1 473 | | 1 475 | | | 2 | 0.33 ± 0.50 | | |
| γ-Muurolene | 1 474 | 1 478 | 1 474 | | | 3 | 0.63 ± 0.71 | | |
| α-Curcumene | 1 480 | 1 479 | 1 473 | | | 1 | 0.12 ± 0.22 | | |
| Germacrene D[1] | 1 481 | 1 484 | 1 479 | 2 | 13.38 ± 16.23 | 2 | 0.39 ± 0.60 | | |
| (*Z,E*)-α-Farnesene | 1 483 | | 1 480 | | | 1 | 0.39 ± 0.90 | | |
| γ-Selinene | 1 484 | | 1 484 | | | 1 | 0.14 ± 0.30 | | |
| β-Selinene | 1 488 | 1 489 | 1 486 | | | 2 | 1.53 ± 3.40 | | |
| δ-Selinene | 1 490 | 1 492 | 1 490 | | | 1 | 0.13 ± 0.25 | | |
| Bicyclogermacrene | 1 495 | 1 500 | 1 494 | | | 2 | 0.39 ± 0.57 | | |
| α-Selinene | 1 500 | 1 498 | 1 494 | | | 2 | 0.61 ± 1.04 | | |
| β-Bisabolene | 1 502 | 1 505 | 1 503 | | | 1 | 0.47 ± 0.86 | | |
| Germacrene A | 1 503 | 1 508 | 1 503 | | | 5 | 3.06 ± 3.14 | | |
| (*E,E*)-α-Farnesene[1] | 1 504 | 1 505 | 1 498 | 3 | 17.48 ± 9.92 | 6 | 2.66 ± 0.86 | 3 | 0.80 ± 0.93 |
| β-Sesquiphellandrene | 1 517 | 1 521 | 1 516 | | | 6 | 3.16 ± 1.36 | | |
| δ-Cadinene[1] | 1 520 | 1 522 | 1 520 | 1 | 0.55 ± 0.95 | 2 | 0.27 ± 0.43 | | |
| 7-epi-α-Selinene | 1 516 | 1 520 | 1 519 | | | 2 | 0.32 ± 0.48 | | |
| Unknown 2[2] | 1 525 | 1 512 | 1 512 | | | 2 | 0.20 ± 0.26 | | |
| α-Dendrolasine | 1 563 | 1 570 | | 2 | 2.48 ± 2.99 | | | | |
| *Mean of total percent* | | | | | 54.28 | | 54.04 | | 2.90 |
| **Shikimic derivates** | | | | | | | | | |
| **Nitrogen-containing compounds** | | | | | | | | | |
| Benzylnitrile | 1 142 | | | | | | | 5 | 1.07 ± 0.63 |
| Indole[1] | 1 293 | 1 290 | | | | 3 | 1.67 ± 3.16 | | |
| *Mean of total percent* | | | | | 0 | | 1.67 | | 1.07 |
| **Non-nitrogen-containing compounds** | | | | | | | | | |
| Methyl salicylate[1] | 1 195 | 1 190 | | 3 | 11.28 ± 9.55 | | | 3 | 0.47 ± 0.62 |
| (*Z*)-3-Hexenyl benzoate | 1 562 | 1 565 | | | | | | 3 | 1.08 ± 1.63 |
| *Mean of total percent* | | | | | 11.28 | | 0 | | 1.55 |

[1]Compounds with the identity confirmed by comparison of MS and retention time of authentic standards.
[2]The mass spectra (m/z) of the unidentified compounds are: unknown 1: 134,91,189,119,79,119; unknown 2: 121,136,204,161,91,93.

attraction has already been shown for other fig-fig wasp systems (Grison-Pigé et al., 2002b; Yokoyama, 2003). However in tritrophic interactions, studies on the ability of parasitoids to discriminate among specific plant volatile blends are still rare (see, for example, Mumm et al., 2005), and have never previously been performed on NPFWs. The attraction of two chalcidoid wasps belonging to different families by the same chemical message suggests a convergence in the sensorial channel resulting from the association with the same host. Nevertheless, in our bioassays, the pollinating fig wasp showed a stronger (innate) reaction to the olfactory compounds emitted by figs than did the parasite. The difference in response could be explained by differences between the pollinator and the parasite in lifespan, and thus in opportunities for learning. The response of pollinating wasps, which live only a few hours (Kjellberg et al., 2005), may have been shaped by selection to 'rush' to receptive figs (Patel & Hossaert-McKey,

**Table 2** Summary of Bray-Curtis distances ($0 \leq d \leq 1$) calculated for volatile profiles of samples from three *Ficus* species. Mean ± SD of this distance across all species as well as multiple pairwise comparisons are presented. We also present the results of the multi-response permutation procedures (MRPP), the chance-corrected within-group (T) agreement (A) and the probability that variation among groups is expected by chance (P). $A_{max} = 1$ if all items are identical within groups, A = 0 if heterogeneity within groups equals expectation by chance, and A<0 if heterogeneity within groups is larger than expected by chance

| | Bray-Curtis distance | T | A | P |
|---|---|---|---|---|
| Across species | 0.82 ± 0.15 | –6.27 | 0.35 | 0.00008 |
| Pairwise comparisons | | | | |
| *Ficus hispida/Ficus tinctoria* | 0.85 ± 0.07 | –3.98 | 0.17 | 0.003 |
| *F. hispida/Ficus racemosa* | 0.74 ± 0.25 | –6.22 | 0.37 | 0.0008 |
| *F. racemosa/F. tinctoria* | 0.85 ± 0.07 | –3.22 | 0.31 | 0.008 |

2000), expressed as a strong innate response to a specific chemical signal. In contrast, NPFWs are longer-lived (2–3 weeks, Kjellberg et al., 2005) and oviposit in several different figs over the course of their life. Selection on them may have favoured the acquisition of experience in their selection of hosts for oviposition and the ability to learn to react more strongly to appropriate chemical messages, as shown for other parasitic wasps (Mumm et al., 2005). In addition to long-distance signals, as for other parasitic wasps, NPFWs might also use another chemical message for contact stimulation, probably non-volatile surface compounds, allowing the location of suitable flowers inside the fig.

Distinct qualitative and quantitative differences in the chemical signatures of receptive figs do occur among the three sympatric species (Figure 1). Interestingly, as for other *Ficus* species (Grison-Pigé et al., 2002a), the volatile signature emitted by the *Ficus* species studied was composed of 1–3 dominant compounds accompanied by several minor compounds, all belonging to four different chemical classes: monoterpenes, sesquiterpenes, fatty acid derivatives (alkanes, aliphatic esters, and alcohols), and

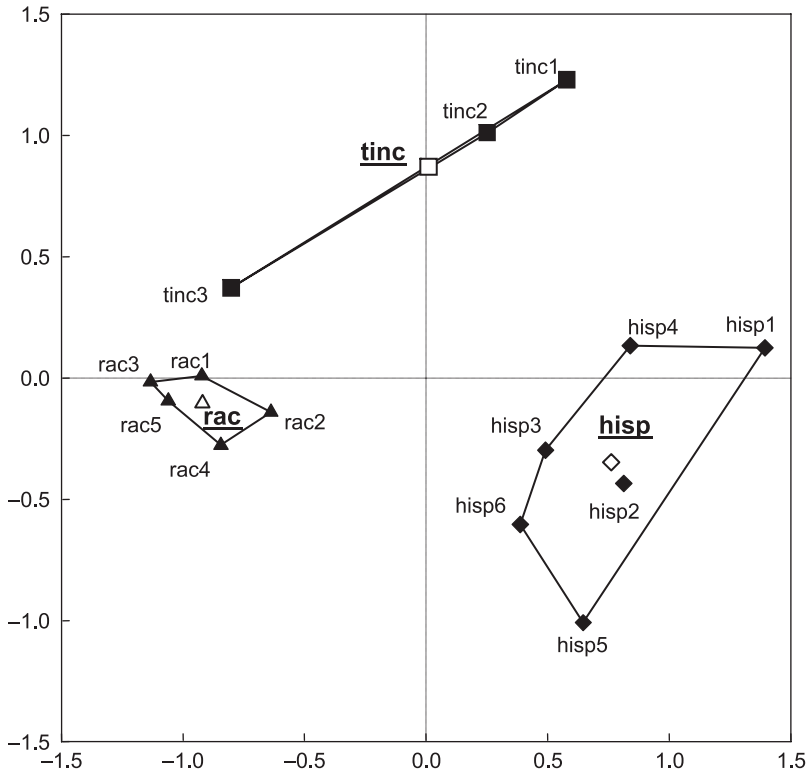


**Figure 1** Non-metric multidimensional scaling ordination (NMDS) of chemical composition of the three *Ficus* species based on Bray-Curtis distance, rotated by principal component, so that the variance of points is maximized on the first dimension, with centroids for species (represented by open symbols, label underlined): rac, *Ficus racemosa*; hisp, *Ficus hispida*; tinc, *Ficus tinctoria*. Stress = 0.053.

**Table 3** Results of Monte Carlo tests of observed indicator value (IV) for each compound of three *Ficus* species found to have a significant IV among the 68 identified compounds. Mean ± SD of the IV from the randomizations are given, as well as the P-values for the null hypothesis of no difference between groups. The P-value is based on the proportion of randomized trials with indicator value equal to or exceeding the observed indicator value. We also indicate the species for which the volatile profile is characterized by the presence of the indicator compound. $IV_{max} = 100$ if the compound is found only in one species and if it occurs in all samples of that species

| Compound indicators | Observed IV | IV from randomized groups | P | IV significant species |
|---|---|---|---|---|
| 2-Heptanone | 66.7 | 23.4 ± 12.75 | 0.034 | *F. tinctoria* |
| 2-Heptanol | 66.7 | 23.7 ± 12.67 | 0.034 | *F. tinctoria* |
| 1,8-Cineole | 66.7 | 30.7 ± 15.72 | 0.031 | *F. hispida* |
| (*Z*)-β-Ocimene | 95.7 | 37.3 ± 14.15 | 0.002 | *F. racemosa* |
| (*E*)-β-Ocimene | 72.5 | 47.1 ± 9.52 | 0.011 | *F. racemosa* |
| Linalool | 64.2 | 42.8 ± 10.58 | 0.041 | *F. hispida* |
| Benzylnitrile | 100 | 32.6 ± 15.37 | 0.003 | *F. racemosa* |
| Methyl salicylate | 95.9 | 38.8 ± 16.20 | 0.008 | *F. tinctoria* |
| α-Cubebene | 66.7 | 23.0 ± 12.40 | 0.031 | *F. tinctoria* |
| α-Copaene | 62.0 | 30.8 ± 15.14 | 0.039 | *F. tinctoria* |
| β-Cubebene | 66.7 | 22.8 ± 12.40 | 0.031 | *F. tinctoria* |
| β-Elemene | 66.7 | 22.9 ± 14.65 | 0.032 | *F. hispida* |
| (*E*)-β-Farnesene | 98.1 | 36.6 ± 13.21 | 0.001 | *F. hispida* |
| Unknown 1 | 80.0 | 29.8 ± 14.90 | 0.016 | *F. racemosa* |
| Germacrene D | 64.9 | 33.4 ± 15.99 | 0.042 | *F. tinctoria* |
| (*E*,*E*)-α-Farnesene | 83.7 | 50.6 ± 11.99 | 0.001 | *F. tinctoria* |
| Germacrene A | 83.3 | 32.2 ± 14.58 | 0.013 | *F. hispida* |
| β-Sesquiphellandrene | 100 | 34.2 ± 13.04 | 0.001 | *F. hispida* |
| Dendrolasine | 66.7 | 24.4 ± 12.67 | 0.034 | *F. tinctoria* |

shikimic acid derivatives. In the present study, some compound indicators are present in all individuals of only one of the species studied [methyl salicylate in *F. tinctoria*, (*E*)-β-farnesene, and β-sesquiphellandrene in *F. hispida*, and benzylnitrile and (*Z*)-β-ocimene in *F. racemosa* (Tables 1 and 3)]. Other compounds, such as [*E*,*E*]-α-farnesene and (*E*)-β-ocimene, are present in all the individuals of at least two species, but their relative proportions in the floral bouquet are different among species. Consequently, they could all be good indicators of some fig species.

In specific plant–insect pollination interactions, we expect that the insect uses as a signal the compounds that are emitted by the host plants. For example, Ayasse et al. (2003) showed that only few compounds emitted by *Ophrys speculum* Link are attractive for its pollinator, the scoliid wasp *Campsoscolia ciliata* Fabr., and that these compounds rarely occur in other plants. However, usually not only the identity of compounds, but also their relative proportions in the floral bouquet play an important role in pollinator attraction and scent specificity. Gibernau (1997) showed that a mixture of three major volatile compounds emitted by figs of *F. carica*, namely, linalool, linalyl oxides, and benzyl alcohol, in certain proportions, was necessary and sufficient to attract its specific pollinator *Blastophaga psenes* (L.). One step further in our understanding of the specificity of the attraction will be to clarify if the pollinator and the parasite use the same chemical compounds as signal within the blend produced by figs, which can be accomplished by combining GC and electro-antennogram recording.

Several studies have revealed that NPFWs could have a negative effect on reproductive success of their host figs (Kerdelhué & Rasplus, 1996; Cook & Rasplus, 2003), as in the case of *P. pilosa* (Peng et al., 2005). Parasites of a mutualism and their host species are predicted to engage in co-evolutionary arms races. Thus, the production of any scent that the parasite can use as a cue to locate its host at the appropriate stage of development might be counter-selected. Maintenance of such counter-selection might depend on a delicate balance of costs and benefits (Dicke et al., 2003). Because the fig cannot reproduce without its obligatory pollinator, the message responsible for partner encounter cannot be changed without a high risk of losing the pollinator. To our knowledge, even though many mutualistic systems are exploited by parasites of the mutualism (Pellmyr et al., 1996; Maloof & Inouye, 2000; Schatz et al., 2006), no studies have determined which signals are used by these parasites to find their host.

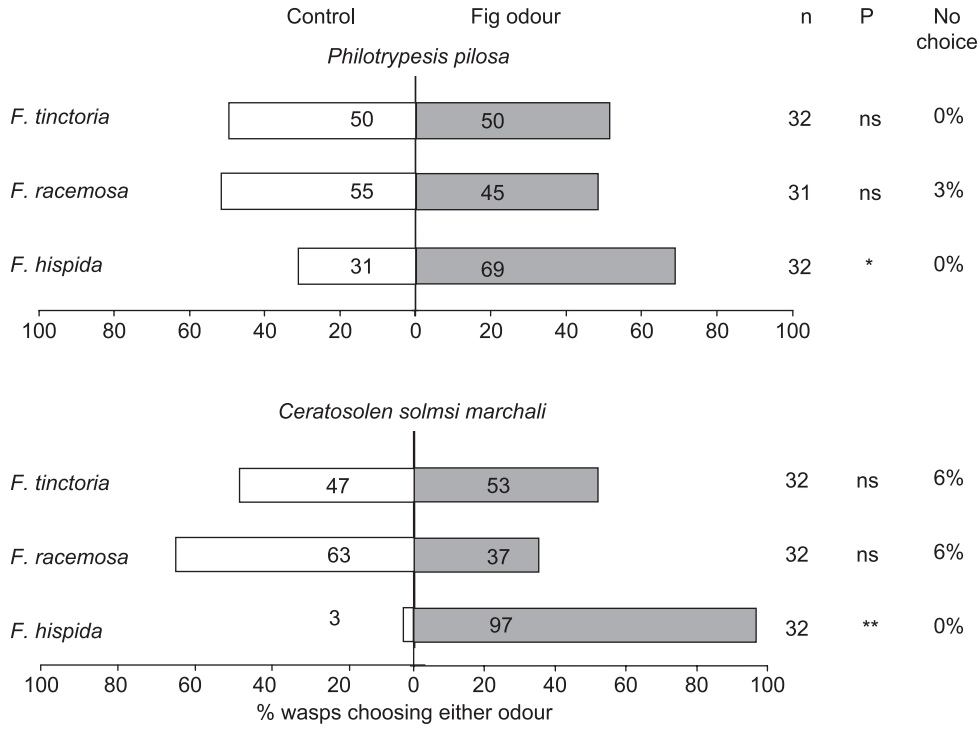


**Figure 2** Responses of *Ceratosolen solmsi marchali* and *Philotrypesis pilosa* when confronted with odours of receptive figs of three sympatric *Ficus* species in Y-tube experiments (ns, non-significant, *P<0.05; **P<0.001).

Therefore, because of the evolutionary stability of the message, we assume that specific compounds responsible for the encounter of mutualist partners could also be used by these parasites as signals to recognize their own host.

It is assumed that one pollinator species is associated with one *Ficus* species. However, several recent studies provide evidence that breakdowns of specificity are relatively common and that host shifts between *Ficus* species of the same section probably happened during the phylogenetic history of *Ficus* pollinators (Kerdelhué et al., 1999; Machado et al., 2005; Jousselin et al., 2008). The specificity of the interaction between NPFWs and *Ficus* species is still a matter of debate (Cook & Rasplus, 2003). Most genera parasitize only a subset of sections of the genus *Ficus*, and as far as we know most species are specialized on a single *Ficus* species. However, host shifts might be easier for externally ovipositing NPFWs, because they are not subject to the physical matching required to pass through the ostiole, which varies greatly in form among species. Nevertheless, NPFWs must be dependent on fig size (and especially on fig wall thickness), on the timing of oviposition of the pollinator (Kerdelhué & Rasplus, 1996), and on their synchronized development time with the pollinator. These points suggest a strong advantage to specificity for NPFWs.

In summary, our results demonstrate that species specificity of the chemical message emitted by figs and recognition of this message by fig wasps can promote specificity in the relationship between figs and their pollinating wasps, as well as their NPFWs. The specificity of this chemical mediation could reduce the colonization of new hosts. However, because breakdowns of specificity may occur within a section of the genus *Ficus* for both pollinators and parasites, comparisons of blend variation among and within different sections of the genus *Ficus* and of the olfactory response of pollinators and NPFWs are now required to examine if chemical mediation can facilitate or prevent host shift of fig wasps to closely related *Ficus* species. Such studies will provide insights into phylogenetic constraints in the emission of volatile compounds among species in the genus *Ficus*.

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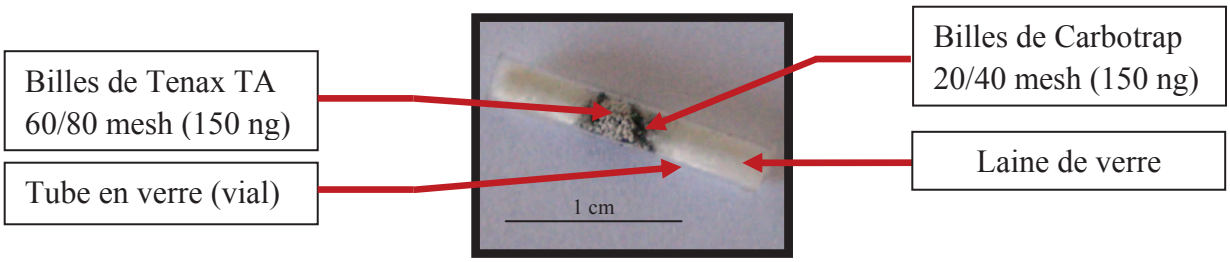
## ANNEXE 2: La méthode du *Chromatoprobe Head-space.*

***Fabrication des filtres :***

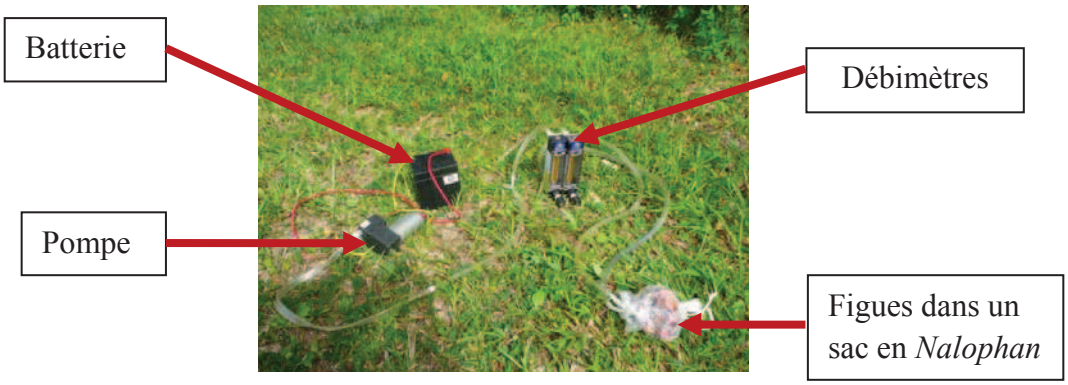
Des tubes de verre de 2 mm de diamètre sont meulés afin d'être percés de part en part. Deux différentes phases adsorbantes (150 ng de Tenax TA 60/80 mesh et 150 ng de Carbotrap 20/40 mesh) sont insérées dans les tubes, maintenus par des bouchons de laine de verre aux deux extrémités. Les filtres ainsi préparés sont alors conditionnés par passage à l'étuve durant deux heures et sous flux d'hélium (100 ml. $min^{-1}$). Ensuite, ils sont « piqués » avec 1 µl d'une solution de standards internes (nonane+dodécane), dilués dans du dicholorométhane, en concentrations connues (109,6 $ng.\mu l^{-1}$ de C9 et 110,4 $ng.\mu l^{-1}$ de C12). La dernière étape de fabrication consiste à retirer l'excédent de solution par un flux contrôlé d'hélium (5 $ml.min^{-1}$) pendant 1 minute.




**Extraction des COVs :**

Les figues sont prélevées sur arbre et placées dans un sac en plastique inerte (le polyéthène téréphtalate=Nalophan®). Deux filtres (l'un pour analyse MS, le second pour l'analyse FID) sont ajoutés dans le sac, et ce dernier est refermé. Durant 30 minutes, les C.O.V. s'accumulent à l'intérieur du sac. L'extraction s'effectue, ensuite, pendant 5 minutes, à débit contrôlé (200 $ml.min^{-1}$), grâce à une pompe aspirante. Pendant les extractions, un échantillon témoin est réalisé en utilisant un sac vide.

Les COVs ainsi adsorbés sur les filtres seront désorbés par thermodésorption lors de l'analyse en GC-MS, ce qui permet de récupérer la totalité de l'échantillon injecté, contrairement à une élution par solvant (comme dans la méthode classique du *dynamic head-space*). Au retour du terrain, les filtres sont conservés à -20°C jusqu'à l'analyse en GC-MS.



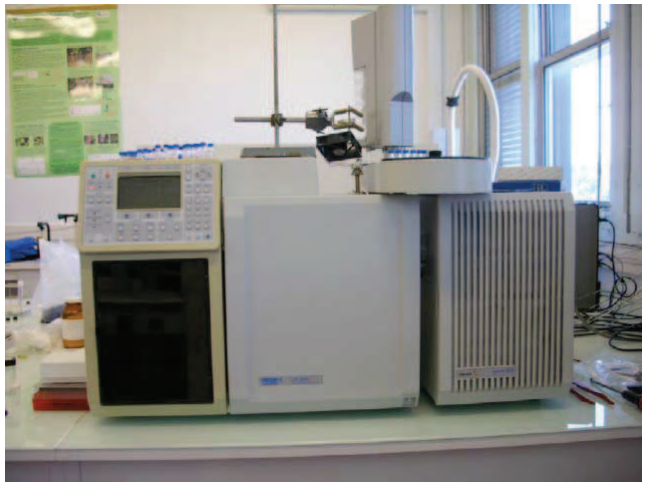
**Analyse en GC-MS :**
Après extraction, les filtres sont injectés dans un spectromètre de masse (*Varian Saturn 2000*) couplé à un chromatographe en phase gazeuse (*Varian CP 3800*) doté d'un détecteur FID (Flamm Ionization Detection).

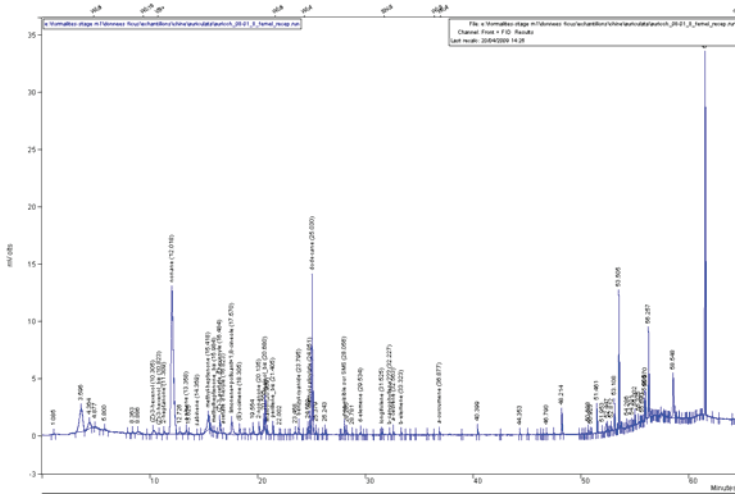
Le gaz vecteur utilisé est l'hélium, et les colonnes répondent aux caractéristiques suivantes :

- Type : CP-sil 8 CB-MS low bleed;
- Longueur : 30m;
- Diamètre interne : 0,25mm;
- Epaisseur du film : 0,25 µm.

La programmation thermique est la suivante :

- 40°C pendant 3min
- 40-100°C : 3,3°.min$^{-1}$
- 100-140°C : 2,9°.min$^{-1}$
- 140-180°C : 2,7°.min$^{-1}$
- 180-250°C : 10°.min$^{-1}$
- maintien à 250°C pendant 8 min.



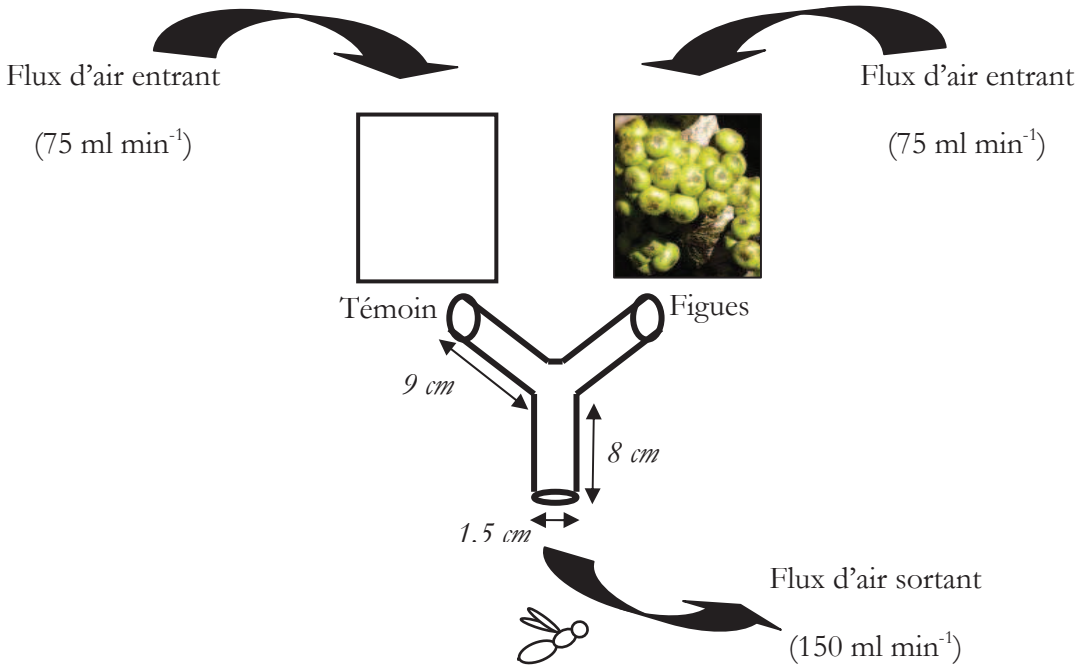


## ANNEXE 3: Tests comportementaux

**Protocole employé pour l'étude des réponses comportementales des pollinisateurs aux signaux olfactifs (olfactomètres à 2 voies)** *(d'après Proffit, 2007)*.

Nous avons utilisé des tubes en verre en Y dont les dimensions figurent sur le schéma. Deux flux d'air humidifié, purifié par un filtre à charbon, et de même débit, passent par un sac en plastique inerte (polyéthylène téréphtalate [Nalophan®]). L'un des flux passe par un sac vide et est utilisé comme témoin. Le second flux passe par un sac contenant les figues dont l'odeur est testée. La circulation d'air est assurée par une pompe qui aspire l'air à l'entrée du tube en Y. Les insectes (naïfs ; un insecte à la fois) sont placés à l'entrée du tube et leur comportement est relevé pendant 10 min. Trois types de comportements ont été notés :

- Choix de la branche chargée d'odeurs
- Choix de la branche témoin
- Non-choix (les individus testés restent dans la branche d'entrée)




Des tests avec des sacs vides connectés aux deux branches de l'olfactomètre sont également effectués, afin de mettre en évidence un éventuel biais dans la préférence des insectes pour l'une ou l'autre des branches de l'olfactomètre.

**Protocole employé pour l'étude des réponses comportementales des chauves-souris aux signaux olfactifs**

Ces expériences se sont déroulées au sein du jardin botanique XTBG, dans la région du Yunnan, en Chine.

Les chauves-souris (*Rousettus leschenaulti,* Desmarest) étaient capturées par filets, et gardées pendant au moins 48h pour habituation à la cage avant expérimentation. Elles étaient nourries *ad libitum*, sauf dans les heures précédant les expériences. Les individus testés étaient des adultes, non-gestantes et non-allaitantes dans le cas des femelles.

La cage d'expérience était située à l'extérieur, avait une longueur de 10m, une largeur de 5m, et une hauteur de 2,5m. Cette cage a été subdivisée en 4 sections équivalentes, et chaque chauve-souris testée était placée seule dans l'une de ces sections.

Les expériences avaient lieu de nuit, sous lumière rouge.

Les figues testées étaient enfermées dans des tissus opaques, afin d'éviter toute discrimination visuelle par les chauves-souris, et accrochées à la cage. Deux types de figues étaient testés simultanément (mâles VS femelles ou ornitochore VS chiropterochore). Les réactions comportementales des chauves-souris étaient alors observées (soit en direct, soit par support vidéo) pendant 45 min, et étaient catégorisées en : absence de comportement, vol, *circling*, contact, morsure.





**Protocole employé pour l'étude des réponses comportementales des lémuriens aux signaux olfactifs**

Ces expériences se sont déroulées au MNHN, sur le campus de Brunoy.

Les lémuriens (*Microcebus murinus*) testés sont des animaux nés en captivité. Ils étaient placés individuellement dans les cages au moins 48h, pour habituation, avant expérimentation. Aucun changement n'a eu lieu quant à leur nourriture. Les individus testés étaient des adultes, non-gestantes et non-allaitantes dans le cas des femelles.

Les cages utilisées étaient des cubes d'un mètre de côté.

Les expériences avaient lieu dans le noir, sous lumière rouge.

Il était présenté à chaque animal, simultanément et pendant 45 min, 3 *eppendorfs* contenant un papier filtre imprégné d'odeur de figue, d'odeur de banane ou de solvant (témoin). Les comportements étaient observés en direct.




**RESUME: Parfums de figues : *Approche évolutive de la communication entre une plante, ses insectes pollinisateurs et ses vertébrés disperseurs***

Pour l'ensemble des organismes vivants, la communication (intra ou interspécifique) est le véhicule de l'information, un élément central dans l'interaction entre individus. Elle a ainsi des impacts primordiaux sur les traits d'histoire de vie des espèces, et est donc façonnée par la sélection naturelle. Au cours de cette thèse, nous nous sommes intéressés à l'évolution de la médiation chimique, c'est-à-dire à la transmission d'informations par voie chimique entre deux individus, dans les interactions plantes/animaux. Nous avons choisi le modèle figuier et ses partenaires mutualistes, de la guêpe pollinisatrice aux vertébrés disperseurs de graines. La médiation chimique (ici émission de bouquets volatils constitués de terpènes et de dérivés benzéniques et d'acides gras) permet aux figuiers d'assurer leur reproduction, par attraction des pollinisateurs et des disperseurs de graines. Il existe environ 800 espèces de figuiers à travers le monde, à distribution principalement tropicale. Les figuiers, fructifiant toute l'année, sont souvent qualifiés d'espèce clef de voute des écosystèmes tropicaux, car ils assurent ainsi le maintien des populations de frugivores dans les forêts tropicales dans les périodes où très peu d'espèces d'arbres fructifient. L'interaction figuier/disperseur est plutôt généraliste, alors que le mutualisme figuier/pollinisateur, un exemple de '*nursery pollination*', est hautement spécifique et obligatoire. Au cours de cette thèse, nous avons mis en évidence que les contraintes phylogénétiques et géographiques exercées sur la médiation chimique étaient négligeables, alors que les pressions de sélection que représentent les mutualistes sont nettement plus importantes dans l'évolution de cette médiation. Ainsi, sexe et stade phénologique du figuier et identité du partenaire mutualiste sont des informations qui peuvent être portées par la médiation chimique. Notre approche, globale et multidisciplinaire, des interactions entre les figuiers et leurs partenaires mutualistes aux deux phases de dispersions des gènes de la plante, permet une meilleure compréhension de l'évolution de la communication, et montre que les pressions façonnant ce message façonnent l'interaction elle-même.

**Mots clefs** : communication interspécifique, médiation chimique, composés volatils, mutualisme, pollinisation, dispersion des graines, *Ficus*, Agaonidés, lémuriens, chauves-souris

**ABSTRACT: *« Parfums de figues » : An evolutionary approach to communication between a plant, its pollinating insects and its vertebrate seed dispersers***

For all living organisms, communication (intra-or interspecific) is the vehicle of information, an element central to interactions among individuals. It thus has fundamental impacts on life history traits of species, and is shaped by natural selection. This thesis is concerned with the chemical mediation of communication, i.e., the transmission of information by chemical signals, in the context of animal-plant interactions. We chose as a model system the interactions between figs and their mutualist partners, including both pollinating fig wasps and vertebrate seed dispersers. Chemical mediation (in this case the emission of bouquets of volatile compounds comprising terpenes, benzenoids and fatty acid derivatives) enables figs to ensure their reproduction by attracting pollinators and seed dispersers. There exist about 800 species of *Ficus* worldwide, principally in tropical regions. Figs, which fruit throughout the year, are often considered keystone species of tropical ecosystems, because their year-round fruiting maintains populations of seed-dispersing frugivores during fruit-poor 'lean seasons'. Interactions between fig species and seed-dispersing animals are 'generalist' (i.e., broad food species ranges), while fig/pollinator mutualisms, examples of 'nursery pollination' systems, are highly species-specific and obligatory. In this thesis, we showed that phylogenetic and geographic constraints acting on chemical mediation are negligible, whereas selection pressures grounded in pollination and seed-dispersal mutualisms play a strong role in shaping the evolution of chemical mediation. Thus, chemical mediation carries information such as the sex and developmental stage of the fig tree, as well as the identity of the mutualistic partner. Our comprehensive, multidisciplinary approach to interactions between figs and their mutualist partners in the two phases of dispersal of the plant's genes leads to an improved understanding of the evolution of communication, and showed that the selective pressures shaping the message are also those shaping the interaction itself.

**Keywords** : interspecific communication, chemical mediation, mutualism, volatile compounds, pollination, seed dispersal, *Ficus*, Agaonidae, lemurs, bats