Post pollination events in a sexually deceptive orchid (*Ophrys fusca* Link): a transcriptional and metabolic approach

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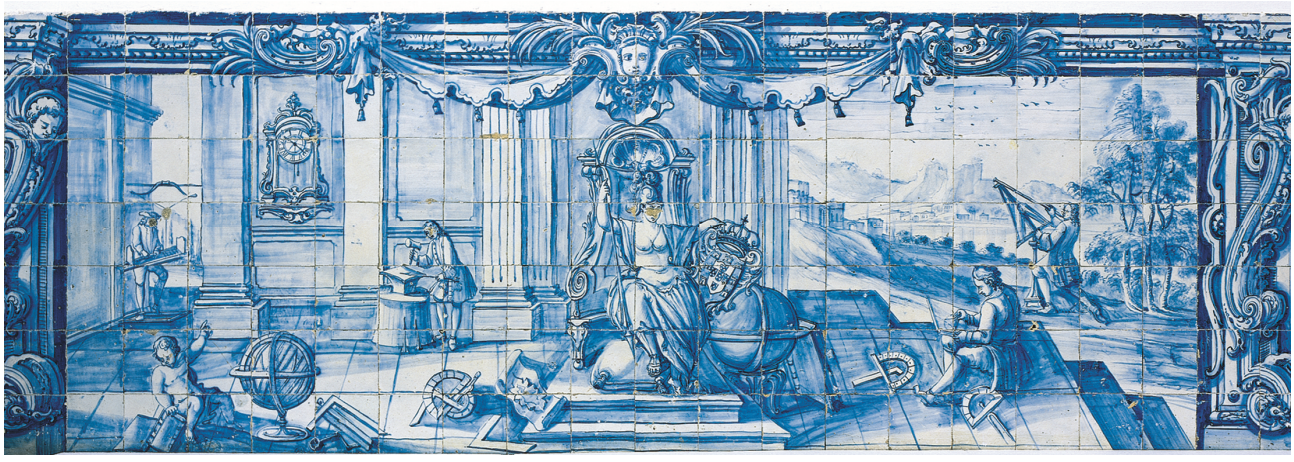

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TÍTULO:
MECANISMOS DE PÓS-POLINIZAÇÃO EM *OPHRYS FUSCA* LINK (ORCHIDACEAE): ABORDAGENS TRANSCRITÓMICA E METABOLÓMICA

RESUMO

A presente dissertação pretende contribuir para o aumento do conhecimento numa área específica da polinização em orquídeas, designada como polinização deceptiva (*sexual deception*) usando a espécie *Ophrys fusca* como modelo. Tendo por base 100 labelos de 100 plantas diferentes, colhidas no seu habitat natural, recorreu-se a técnicas de transcritómica e metabolómica com o objectivo de (1) analisar a expressão génica por microarrays de cDNA após a polinização; (2) proceder à caracterização metabólica por cromatografia gasosa e espectrometria de massa (GC-MS). Os resultados obtidos permitiram contribuir para a compreensão dos mecanismos de polinização por *sexual deception*, nomeadamente no que respeita às características do labelo (ex. pigmentação, emissão de compostos), dos processos de senescência ou da biologia floral das orquídeas. A construção de um chip de cDNA para *O. fusca* permitirá realizar hibridações com outras espécies de *Ophrys*, possibilitando a determinação do grau de conservação dos mecanismos genéticos na polinização por *sexual deception*.

PALAVRAS-CHAVE: polinização deceptiva, pseudocópula, *Ophrys*, expressão génica, senescência das pétalas, metabolismo secundário, n- alcanos, n- alcenos, orquídeas.

TITLE:

POST POLLINATION EVENTS IN A SEXUALLY DECEPTIVE ORCHID (*OPHRYS FUSCA* LINK): A TRANSCRIPTIONAL AND A METABOLIC APPROACH.

ABSTRACT

This work aims at contributing to the knowledge on orchid pollination biology, through the study of the peculiar pollination mechanism of *Ophrys fusca* by sexual deception. In this mechanism, *Ophrys* labellum mimics the female sex pheromones thereby deceiving male pollinators that attempt to copulate with the orchid labellum. Labellum transcriptome analysis by a custom-made cDNA microarrays allowed to verify gene expression modulation of post pollination changes. Processes involved in labellum morphology, petal senescence and pollination biology were adressed. A metabolic profiling by gas-chromatography mass- spectrometry was focused on compounds involved in *Ophrys*-pollinator crosstalk, in order to determine their dynamics after pollination. By means of both transcriptional and metabolic analysis, the work here presented gives an important contribution towards the understanding of orchid pollination biology by deceit. The custom-made cDNA chip may be useful for performing cross-species hybridization to track differences on transcripts modulation thereby disclosing the genetic basis underlying sexual deception.

KEYWORDS: sexual deception, pollination, *Ophrys*, microarrays, petal senescence, secondary metabolism, *n*- alkanes, *n*- alkenes, orchids.

RESUMO

A família das orquídeas (Orchidaceae) inclui mais de 22 000 espécies, representando cerca de 10% das plantas com flor. A sua extraordinária diversidade floral reflecte a importância das relações planta-polinizador na evolução das orquídeas, sendo as diferentes estratégias de polinização consideradas como uma das razões para a diversificação e especiação na família. Os mecanismos de polinização em orquídeas sempre intrigaram os cientistas, incluindo Darwin. Uma das estratégias mais fascinantes na biologia destas plantas é a capacidade de polinização deceptiva, ocorrendo em cerca de 1/3 das espécies. A presença de uma pétala modificada, o labelo, tendo como função principal atrair insectos polinizadores, é igualmente uma das características mais distintivas das orquídeas. Os mecanismos de polinização deceptiva mais comuns incluem a imitação de flores que apresentam néctar (*food deception*), ocorrendo em 38 géneros; e a imitação de insectos-fêmea (*sexual deception*), abrangendo 18 géneros. O género *Ophrys* sempre foi considerado um modelo para estudo de polinização deceptiva, nomeadamente do caso de flores sexualmente deceptivas (*sexual deception*). As flores deste género emitem substâncias químicas idênticas às feromonas libertadas pelas fêmeas sexualmente receptivas dos insectos polinizadores, bem como apresentam características morfológicas (ex: forma, cor, pilosidade) que mimetizam o corpo dos insectos-fêmea. Os estudos desenvolvidos em polinização de orquídeas têm sido desenvolvidos nas áreas da biologia celular, micromorfologia, genética populacional, análise química e na determinação funcional de determinados genes, bem como em estudos bioquímicos e fisiológicos. Contudo, para a compreensão global de um processo são necessárias técnicas que permitam obter dados a larga escala. Este trabalho pretende constituir um contributo para o conhecimento dos mecanismos regulados pela polinização em orquídeas. Para tal, uma espécie de orquídea selvagem abundante em Portugal, *Ophrys fusca* Link, foi usada como modelo de estudo do mecanismo de polinização deceptiva (*sexual deception*). Para atingir o presente objectivo, foram aplicadas duas técnicas diferentes: a análise da expressão génica por microarrays de cDNA e a caracterização metabólica por cromatografia gasosa e espectrometria de massa (GC-MS). O labelo foi seleccionado como foco do estudo, dada a sua importância na emissão de compostos importantes na comunicação com o insecto polinizador e no processo geral de polinização em orquídeas. A amostragem foi efectuada em 100 labelos de 100 plantas diferentes no seu habitat natural, e seleccionaram-se dois tempos de estudo: 2 dias após a polinização (DAP) e 4 DAP.

A análise do labelo por microrrays de cDNA permitiu verificar a modulação da expressão génica após a polinização. Com este estudo de larga escala conseguiu-se verificar que aos 2 DAP, o evento de polinização é reconhecido como uma resposta a um stress e aos 4 DAP, detectaram-se genes que indicam a mobilização de nutrientes bem como uma nova síntese proteica, necessária para a progressão específica da senescência do labelo. A polinização despoleta processos de proteólise, mobilização de nutrientes como o fosfato, carbono e azoto, e desactiva mecanismos energeticamente dispendiosos, como a fotossíntese e fotorespiração bem como as principais vias metabólicas que permitem manter a vitalidade do labelo. Os transcritos identificados revelam processos importantes do metabolismo secundário envolvidos em características do labelo (ex. pigmentação, emissão de compostos), em proteólise a larga escala (ex. proteases cisteínicas) e dirigida (ex. fosfatases e quinases), stress e defesa, além de vias associadas à mobilização de nutrientes. Inicialmente, a expressão génica de diversos transcritos descritos em situações de stress e de patogenicidade (ex. GST, proteínas Lea5, metalotioneínas tipos 2 e 3, quitinases, proteínas PR, proteases cisteínicas, RNases) indicam que a modulação da transcrição é regulada por vias não específicas de reconhecimento do evento de polinização, semelhantes a uma situação de stresse abiótico e/ou biótico. Contudo, aos 4 DAP, verificou-se a transcrição de genes associados à síntese proteica, indicando a activação de um novo processo de tradução de proteínas específicas que irão dirigir o labelo para a morte celular irreversível.

A análise do perfil metabólico dos extractos dos labelos foi focada em compostos da cutícula, especificamente alcanos e alcenos, descritos como responsáveis por despoletar o comportamento de pseudocópula dos machos polinizadores. Os resultados demonstram que, após a polinização, a quantidade total dos compostos não diminui, estando de acordo com resultados anteriores observados em *Ophrys sphegodes*. Esta observação poderá dever-se à função que estes compostos desempenham, nomeadamente como parte integrante das camadas das ceras prevenindo a desidratação.

A análise do labelo após a polinização por microarrays permitiu a identificação de transcritos, nomeadamente duas sequências de stearoil ACP desaturase (SAD), envolvidos nas vias biossintéticas dos compostos da cutícula, importantes na interacção *Ophrys*-polinizador. A subexpressão destes transcritos em conjugação com a manutenção da produção do odor após a polinização indica que a correlação entre os níveis de expressão dos genes com os seus produtos de síntese não pode ser directamente efectuada. O estudo do labelo após a polinização por técnicas de proteómica irá permitirá a detecção das enzimas bem como alterações pós tradução

importantes na regulação das proteínas. Esta abordagem irá possibilitar a compreensão da regulação das proteínas após o evento de polinização.

O presente estudo permitiu obter uma visão geral no labelo dos mecanismos regulados pela polinização, contribuindo para a compreensão da polinização por *sexual deception* recorrendo a técnicas de Ó'micas. A análise do labelo através de técnicas de transcritómica e metabolómica após a polinização permitiu dar um importante contributo para a compreensão dos processos de senescência, características do labelo (ex. pigmentação, emissão de compostos), bem como da biologia floral das orquídeas. Além disso, a construção de um chip de cDNA construído especificamente para a orquídea em estudo irá permitir a realização de hibridações com outras espécies do mesmo género, possibilitando o estudo da conservação dos mecanismos genéticos na regulação dos eventos pós-polinização de orquídeas com flores sexualmente deceptivas.

ABSTRACT

Orchidaceae family includes more than 22,000 species of plants, representing around 10 % of all flowering plants. The extraordinary floral diversity in orchids reflects the importance of plant-pollinator associations in their evolution, and pollination biology is regarded as a driving force in orchid diversification and speciation. Pollination biology in Orchidaceae has long intrigued evolutionary biologists, and interest in orchid pollination dates back to Darwin. The most fascinating in orchid biology is pollination by deception, occurring in approximately 1/3 of the species, being food (38 genera) and sexual (18 genera) deception the most common types. Sexual deception mechanism was first described in the European *Ophrys* genus by Pouyanne in 1917, and in this mechanism, *Ophrys* orchids mimic their pollinators' mating signals, and are pollinated by male insects during mating attempts. Studies on orchid pollination have mainly focused on cell biology, population genetics, micromorphology, chemical analysis and gene-function studies, as well as biochemical and physiological studies on flowers. A general approach towards the understanding on orchid pollination biology, as well as in the events following pollination, by means of high throughtput techniques is lacking. The study here presented intends at contributing to the knowledge on post pollination-regulated mechanisms in the sexual deceptive orchid *Ophrys fusca* Link, a common bee orchid in the Mediterranean, natural occurring in Portugal. To accomplish such goal, two different approaches were assigned: a transcriptional analysis and a metabolic profiling. Transcriptomics and metabolomics were both used to gather insights on the post pollination changes occurring in *Ophrys fusca* labellum. To access pollination-enhanced events, two time points were considered for analysis: 2 and 4 days after pollination (DAP).

Labellum transcriptional analysis allowed probing gene expression modulation of post pollination changes. The first response to pollination appears to be a stress response (2DAP) and later at 4DAP, nutrient mobilization occurs and *de novo* protein synthesis is induced for senescence progression. Pollination sets off proteolysis, remobilization of nutrients such as phosphate, carbon and nitrogen from labellum and deactivates energy-consuming processes (e.g. photosynthesis, photorespiration) and major metabolic pathways related to labellum upholding. Transcripts identified by microarray analysis reveal pivotal processes associated with secondary metabolism responsible for labellum traits (e.g. pigmentation, compounds emission involved in pollination), proteolysis, stress and defence, and remobilization of nutrients associated with pollination induced-senescence. Labellum transcriptional regulation seems to be mediated by non-specific stress-related pathways, disclosed by the expression of several stress- and pathogen-related transcripts (GST, antimicrobial snakin proteins,

Lea5 protein, metallothioneins types 2 and 3, chitinases, PR protein, Cys proteases, RNases), until the newly protein synthesis is achieved for senescence progression.

Metabolic profiling in labella extracts was focused on cuticular compounds (alkanes and alkenes), known to trigger the pseudocopulatory behaviour of male pollinators. Results show that post pollination machinery does not rely on an abrupt decrease of odour production, which is in agreement with previous reports on other *Ophrys* species, probably due to compounds function as part of the desiccation-preventing wax layers. Through labellum gene expression analysis, transcripts related to biosynthetic pathways of cuticular compounds, involved in *Ophrys* pollinator attraction, were identified: stearoyl ACP desaturases (SAD). Down regulation of these transcripts along with maintenance of odour production may indicate that correlation between RNA level and its by-products cannot be directly made. Thus, a labellum post pollination proteomics approach will allow tracking enzymes responsible for alkenes' production, thereby giving a more comprehensive walkthrough of their regulation on pollination event. Such observations could adjoin some awareness on the genetic basis of pollinator attraction.

By combining both transcriptional and metabolic profiling analysis to study post pollination events in a sexually deceptive orchid, the work here presented gives an important contribution for the understanding of this peculiar pollination system.

ABBREVIATIONS

aa-dUTP-Aminoallyl-deoxyuridine triphosphate
ADP- Adenosine diphosphate
aRNA- Antisense RNA
ATP- Adenosine triphosphate
BLAST- Basic local alignment sequence tool
bp- base pairs
cDNA- Complementary DNA
CE- capillary electrophoresis
cfu- colony-forming unit
CoA- coenzyme A
cRNA- complementary RNA
Cy3- Cyanine 3
Cy5- Cyanine 5
DAP- days after pollination
dCTP- Deoxycytidine triphosphate
ddH_2O- double distilled water
DEPC- Diethyl pyrocarbonate
DIG- Digoxigenin
DMSO- Dimethyl sulfoxide
DNA- Deoxyribonucleic acid
DNase- deoxyribonuclease
dNTP- Deoxyribonucleotide triphosphate
dscDNA- double stranded complementary DNA
DTT- Dithiothreitol
dTTP- Deoxythymidine triphosphate
dUTP- deoxyuridine triphosphate
EC- Enzyme commission number
EGTA- Ethylene glycol tetraacetic acid
EST- Expression sequence tag
FA- fatty acids
FC- Fold change
FDR- False discovery rate
Fe-S- iron-sulphur
FRET- Fluorescence resonance energy transfer
GC-MS- Gas- chromatography mass-spectrometry
GEO- Gene Expression Omnibus
GEPAS- Gene Expression Pattern Analysis Suite
GO- Gene ontology
HPLC- high- performance liquid chromatography
iFRET- Induced fluorescence resonance energy transfer
IVT- in vitro transcription
kb- kilobase
LB - Luria-Bertani
LOWESS- locally weighted scatterplot smoothing
LSD- Fisher's least significant difference
Ma- million years ago
MAD- mean absolute deviation
MIAME- Minimum Information About a Microarray Experiment
MIPS- Munich Information Center for Protein Sequences
MIQE- Minimum Information for Publication
mRNA- Messenger RNA
MS- Mass spectrometry
NADP- Nicotinamide adenine dinucleotide phosphate
NADPH- reduced $NADP^+$
NCBI- National Centre for Biotechnology Information
nt- nucleotide
of Quantitative Real-Time PCR Experiments
PCA- principal component analysis
PCD- programmed cell death
PCR- Polymerase chain reaction
Pi- phosphate inorganic
PSI- Photosystem I
PSII- Photosystem II

PTFE- Oolytetrafluoroethylene
PUFA- polyunsaturated fatty acids
PVP-40- Polyvinylpyrrolidone molecular weight 40,000
qPCR- Quantitative real-time PCR
RankProd- Rank products method
RNA- Ribonucleic acid
Rnase- Ribonuclease
rNTP- Ribonucleotide triphosphate
rRNA- Ribosomal RNA
RT- reverse transcription
SAG- senescence-associated gene
SDS- Sodium dodecyl sulfate
SEM- standard error of the mean
SNP- Single nucleotide polymorphism
SSC- Saline sodium citrate
sscDNA- single-stranded complementary cDNA
STDEV- standard deviation
TBE- Tris/Borate/EDTA
UniRef- UniProt Reference Clusters
UV- Ultraviolet
nm- Nanometre
ºC- Celsius degrees

Measurement Units

µg- Microgram
µL- Microliter
µm- Micrometres
A230- Absorbance at 230 nm
A260- Absorbance at 260 nm
A280- Absorbance at 280 nm
atm- atmosphere
g- Gram
M- Molar
mg- Milligram
min- Minute
mJ- MilliJoule
mL- Millilitre
mm- Millimetres
mM- Millimolar
mZ- mass-to-charge ratio
rpm- Rotation per minute
s- Second
S- Svedberg unit
U- Unit
v- volume
w- Weight
xg- centrifugal acceleration relative to Earth's gravity
ŋg- Nanogram
ρM- Picomolar
ρmol- Picomole

"In my examination of Orchids, hardly any fact has struck me so much as the endless diversities of structure- the prodigality of resources- for gaining the very same end, namely, the fertilization of one flower by pollen from another plant. This fact is to a large extent intelligible on the principle of natural selection."

Charles Darwin

"On the Various Contrivances by which British and Foreign Orchids are Fertilised by Insects", 1866

"And the day came when the risk to remain tight in a bud was more painful than the risk it took to blossom."

Anaïs Nin

"Living on Purpose: Straight Answers to Universal Questions", 2000

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Chapter 1

GENERAL INTRODUCTION TO ORCHID POLLINATION: *OPHRYS FUSCA* A SEXUALLY DECEPTIVE FLOWER

1.1. Family Orchidaceae

Orchidaceae Adans (1763) is a morphologically diverse and widespread family of monocots, with estimated 880 genera and more than 25 000 species (Swarts and Dixon, 2009; WCSP, 2010). After Asteraceae, Orchidaceae is the second-largest family of flowering plants, and one of the most recent species-rich plant family undergoing a major evolutionary radiation (Bateman *et al.*, 2003). Orchids are distributed over all continents, except Antarctida, being particularly numerous and diverse as epiphytes in wet tropics (Fay and Chase, 2009). Orchidaceae is divided into five subfamilies: Apostasioideae, which embraces the most primitive orchids (e.g. *Apostasia*, Fig. 1.1**A**); Cypripedioideae (e.g. *Cypripedium*, Fig. 1.1**B**); Epidendroideae, the largest subfamily (e.g. *Dendrobium*, *Phalaenopsis*, *Cymbidium*- Fig. 1.1**C**); Vanilloideae (e.g. *Vanilla*, Fig. 1.1**D**) and Orchidoideae (e.g. *Orchis*, *Chiloglottis*- Fig. 1.1**E** and *Ophrys*- Fig. 1.1**F**) (Dressler, 1981; Cameron *et al.*, 1999).

Figure 1.1- Family Orchidaceae diversity: (**A**) *Apostasia wallichii*, (**B**) *Cypripedium acaule,* (**C**) *Cymbidium* William Weaver 'Atlantis', (**D**) *Vanilla planifolia*, (**E**) *Chiloglottis formicifera* and (**F**) *Ophrys fusca*.

In the last 10 years, a rapid advance towards the understanding of orchid relationships has been largely addressed mainly by the contribution of molecular phylogenetic studies (Chase *et al*., 1994; Cameron *et al*., 1999; Freudenstein *et al.*, 2004). Establishing relationships within Orchidaceae family has been a challenge. The most recent phylogenetic study (Freudenstein *et al.*, 2004) reveals relationships within the family (Fig. 1.2) highlighting the subfamily Orchidoideae diversity, in which *Ophrys* is included.

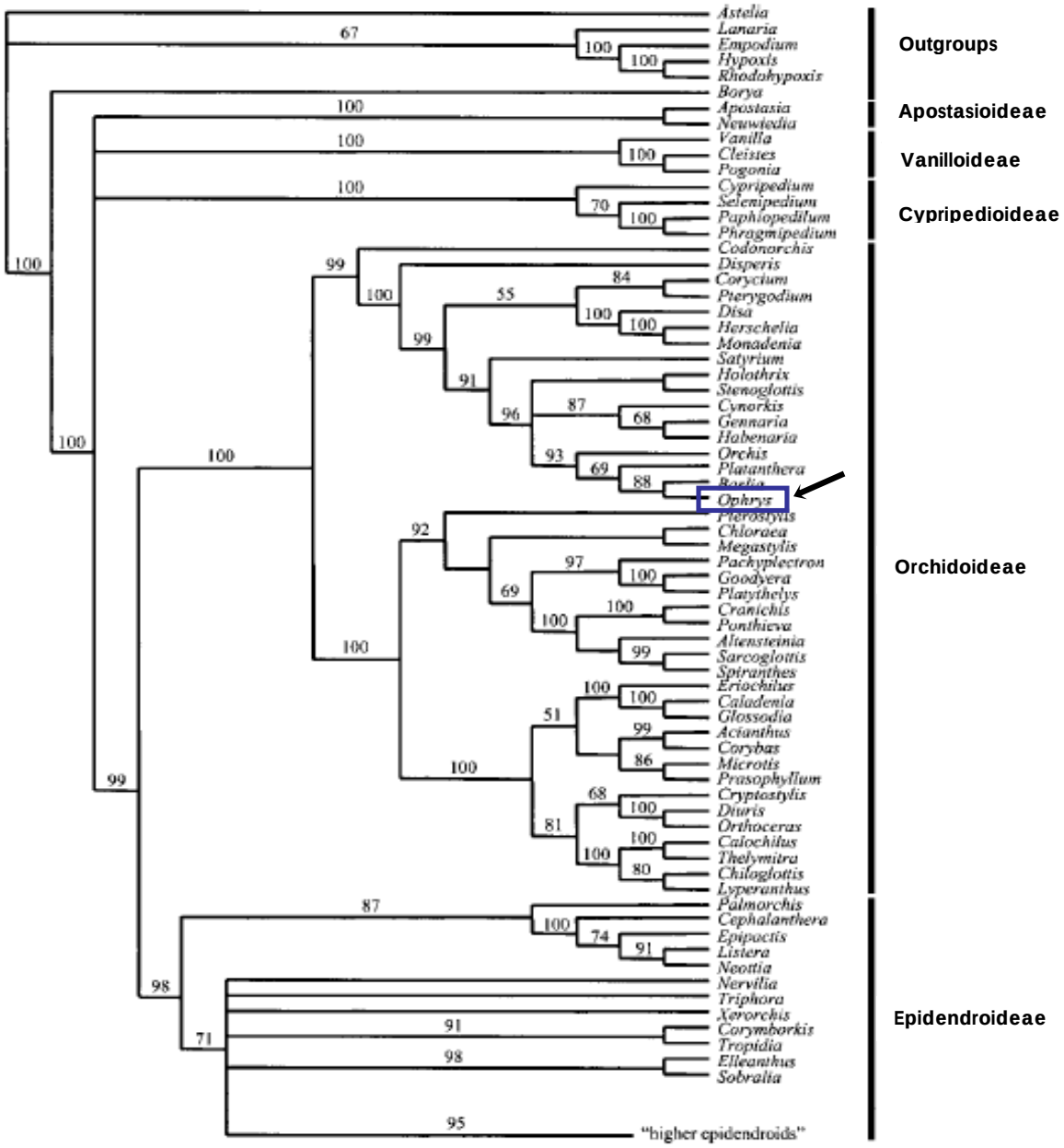

Figure 1.2- Phylogenetic relationships in Orchidaceae. Jackknife support percentages are shown (.50%). Adapted (Freudenstein *et al.*, 2004).

Only recently with the description of the first reliable fossil in 2007 (Ramirez *et al.*, 2007) and two new orchid fossils in 2009 (Conran *et al.*, 2009), a direct calibration of orchid phylogeny pointed out for a common ancestor in the Late Cretaceous (~77

million years ago, Ma) (Gustafsson *et al.*, 2010), these data revolutionizing the understanding of orchids phylogeny. It was estimated that the crown age of the five subfamilies may in general be younger (~1-8 Ma) than supposed before (Gustafsson *et al.*, 2010). This finding encompasses a high evolutionary rate reflecting its successful fitness and adaptation during a short time period.

1.2. *Ophrys* L.

1.2.1. Distribution and habitat

Ophrys occurs mainly around the Mediterranean Basin (Fig. 1.3). Additionally, distribution includes parts of North of Africa (Morocco, Algeria, Libya and Tunisia), Cyprus, Middle East, Caucasus and Anatolia, as well as parts of the Near Orient (Caspian Sea and Persian Gulf) (Soliva *et al.*, 2001; Pedersen and Faurholdt, 2007).

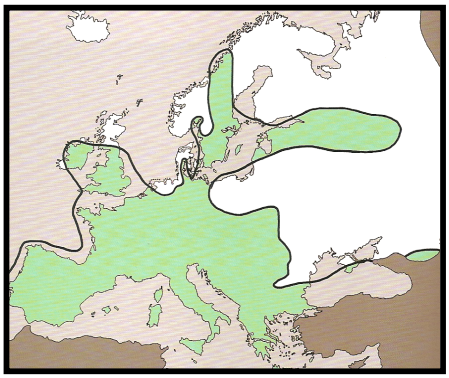

Figure 1.3- Distribution of *Ophrys* in Europe. Adapted (Pedersen and Faurholdt, 2007).

Based on the diversity of species and the frequent occurrence of hybrids, Nelson (1962) considered that the center of *Ophrys* origin is located in the eastern Mediterranean region. In fact, *Ophrys* species are mainly adapted to the diversity of Mediterranean Basin habitats. Mediterranean Basin (Fig. 1.4) is recognised as a biodiversity hotspot (Myers *et al.*, 2000) and therefore is considered a spotlight for conservation.

Figure 1.4- Mediterranean Basin as a Biodiversity Hotspot. In (Center for applied Biodiversity Science, Conventional International).

In this area, flora diversity is outstanding with 15,000 to 25,000 species, 60% of which are unique (Myers *et al.*, 2000). Orchids often arise in species rich-habitats that are predominantly affected by anthropogenic activities, such as marshes and calcareous grasslands (Cozzolino *et al.*, 2003). The dry to semi-dry calcareous grasslands habitat (6210- Appendix I for further information- BFN, 2006), which is widely distributed around Europe, enclose important populations of orchid species in which *Ophrys* species are included (Fig. 1.5). This habitat is considered a priority for conservation by the European Commission Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora, which generally aims to assure the long-term survival of Europe's most valuable and threatened species and habitats.

Figure 1.5- *Ophrys* habitats (**A**) In Greece. Adapted (Pedersen and Faurholdt, 2007). (**B**) In Portugal. Picture by FMonteiro.

1.2.2. Taxonomy

Ophrys was first described by Carl von Linné (1753). Based on morphological characters (Dressler, 1993; Bernardos *et al.*, 2005- Fig. 1.6**A**) and on molecular methods (Pridgeon *et al.*, 1997; Cameron *et al.*, 1999; Soliva *et al.*, 2001- Fig. 1.6**B**), the Euro-Mediterranean genus *Ophrys* is regarded as a monophyletic group, placed in the subfamily Orchidoideae, tribe Orchideae, subtribe Orchidinae. The monophyly of the genus contrasts with its species relationships, generally poorly resolved as a result of controversial and morphologically-based classifications. Determining the species boundaries within the genus will have profound consequences for conservation.

Efforts on systematics and taxonomy have been made to clarify the species number and identity in *Ophrys*, but no consensus has arisen among specialists. Regarding Iberian Peninsula, the number of recognized *Ophrys* species varies among authors, mainly due to different taxonomic treatments. While Delforge (2005) recognizes

118 orchid species, Amich and co-workers (2007) considered 122 orchid species, which accounts for 23 % of endemic species present in the Iberian Peninsula.

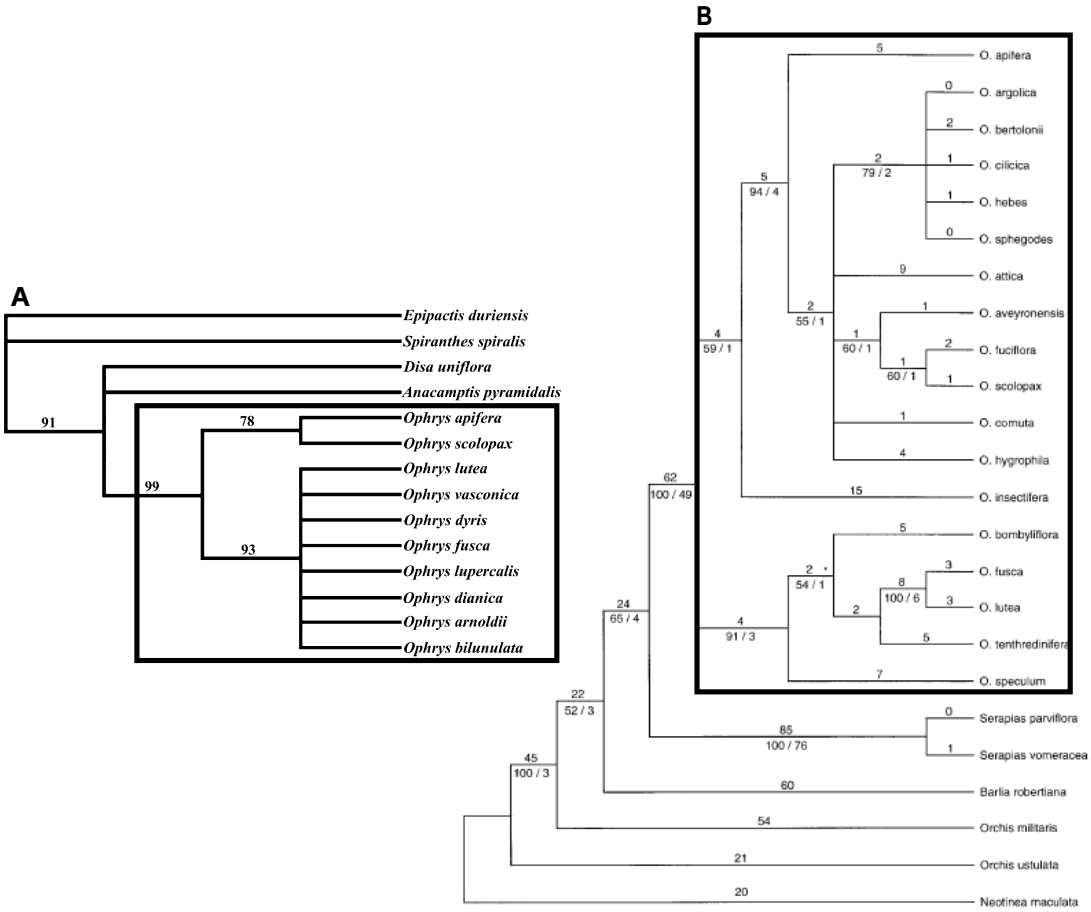

Figure 1.6- *Ophrys* species phylogenetic relationships, based on morphological characters (**A**, adapted (Bernardos *et al.*, 2005)) and on molecular methods (**B**, adapted (Soliva *et al.*, 2001)). Black box highlights *Ophrys* monophyly. Bootstrap with values > 50% are shown in branches.

Studies in sexually deceptive orchids such as *Ophrys*, present a challenge due to taxonomic boundaries at species-level which is particularly difficult from morphology alone (Mant *et al.*, 2005a). This is demonstrated by the degree of synonyms found in *Ophrys* species (Delforge, 2005), without mentioning the large number of assumed hybrids. Delforge (2006), based on the morphological diversity, prompted to recognize 251 species in the widely used Mediterranean orchid Flora, while Pedersen and Faurholdt (2007) only recognized 19 species, 65 subspecies and five stabilized hybrid aggregates. Godfery (1928) proposed a different division into two sections, *Pseudophrys* and *Ophrys*, based on labellum micromorphology (i.e. morphological features of the stigmatic cavity, structure of the labellum and speculum configuration, for more detail see Devillers and Devillers-Terschuren, 1994), and on pseucopulation

type (abdominal or cephalic). The section *Pseudophrys* consists on the O. *fusca*-O. *lutea- O. iricolor- O. omegaifera* lineage. In this lineage, the pollinating insects remove pollinia with the abdomen, a process called abdominal pseudocopulation (Fig. 1.7**A** and 1.7**B**). When pollinia are attached to the pollinators' heads, as it occurs in the lineages belonging to O*phrys* section (which includes species such as *O. speculum* and *O. sphegodes*), the process is known as cephalic pseudocopulation (Fig. 1.7**C** and 1.7**D**),). The different locations of pollinia on the insect body generate an efficient barrier to gene flow between members of these two sections.

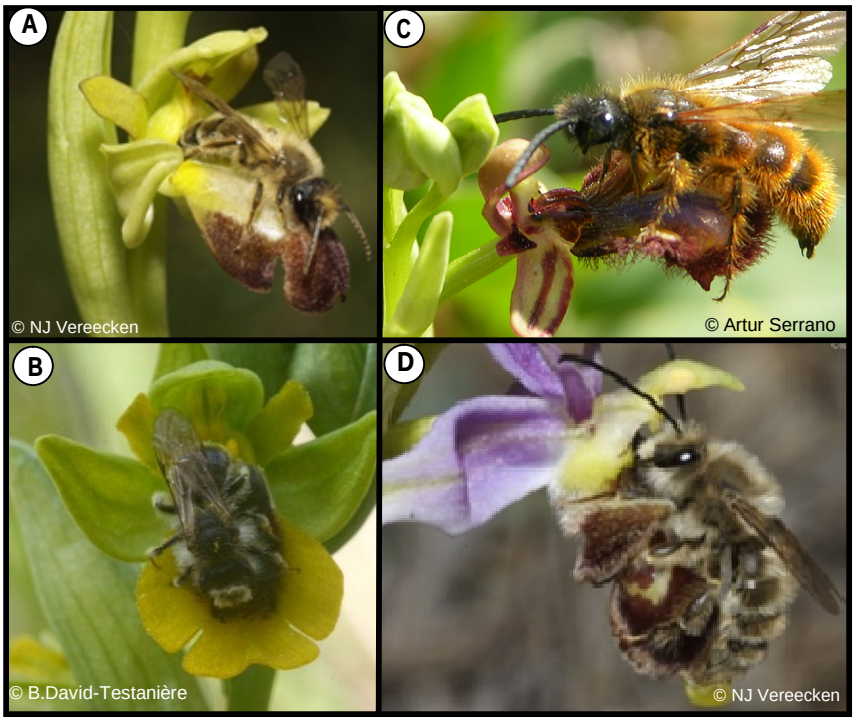

Figure 1.7- Abdominal pseudocopulation of (**A**) *Ophrys lupercalis* and (**B**) *Ophrys lutea* by A*ndrena* sp. males. Cephalic pseudocopulation in (C) mirror orchid *Ophrys speculum* pollinated by *Campsoscolia ciliata* and (D) *Eucera elongatula* male on *Ophrys scolopax*.

Despite the obvious morphological differences, low interspecific genetic differentiation at both ITS (Internal Transcribed Spacer) and trnL-F loci hindered a completely resolved phylogeny of *Ophrys*. In fact, *Ophrys* species are interfertile, and a few or any post-zygotic reproductive barriers exist between species (Cozzolino and Scopece, 2008). Despite low interspecific taxonomic resolution has been obtained from recent molecular studies, several phylogenetic reconstructions based on plastidial and nuclear ribosomal markers independently showed that section *Pseudophrys* is monophyletic (Soliva *et al.*, 2001; Bateman *et al.*, 2003; Devey *et al.*, 2008).

Methods for identifying *Ophrys* species rely mainly on morphological traits and its specific pollinator species. This limitation makes *Ophrys* a difficult group of plants to

conduct evolutionary studies. Véla and co-workers (2007) successfully separated closely related *Ophrys* species by analyzing chemical signatures obtained from Gas-chromatography studies of labellum extracts (i.e. chemotaxonomy). *Ophrys* has been highly and controversially split based on morphological characters but more efficient molecular and morphometric tools are needed for delimiting species and determining their relationships (Bateman *et al.*, 2003).

1.2.3. *Ophrys fusca* **Link**

Ophrys fusca Link (J.Bot. (Schrader) 2(4): 324; 1799), known as the dark bee orchid, is a species native to the Mediterranean. In Europe a great number of narrow geographic species and subspecies exist and the pronounced morphological diversity in Mediterranean basin area is especially remarkable in the *O. fusca* complex (Pedersen and Faurholdt, 2007). A great microdiversity of floral details characterizing relatively homogeneous swarms, separated or not in space or time, may happen in *O. fusca* complex, as reported recently (Amich *et al.*, 2009). *Ophrys fusca* was originally used to describe only one of the whole Mediterranean Basin species. Yet, detailed investigations revealed that in fact *O. fusca* described at least 29 species, which are poorly distinguished by morphology (Delforge, 2005; Stökl *et al.*, 2005). Within *O. fusca*, morphological variation mainly occurs in five groups recognized as subspecies by Pedersen and Faurholdt (2007): *O. fusca* subsp. *fusca*; *O. fusca* subsp. *iricolor* (Desf.) K. Richt.*; O. fusca* subsp. *blitopertha* (Paulus and Gack) Faurholdt and Pedersen*; O. fusca* subsp. c*inereophila* (Paulus and Gack) Faurholdt *and O. fusca* subsp. *pallida* (Raf.) Camus. For Portugal, only subspecies *fusca* (Fig. 1.8**A**) and *iricolor* (Fig. 1.8**B**) are described (Pedersen and Faurholdt, 2007).

Ophrys fusca subsp. *fusca* flowers from January to June, in most areas with a peak in March-April. The main differences for distinguishing subsp. *fusca* from subsp. *iricolor* relies on lip morphology: the first has a lip slightly to strongly curved, with or without a narrow yellow margin, whereas the second has a lip wine-red underneath mirror shining and sharply delimited (following Pedersen and Faurholdt, 2007).

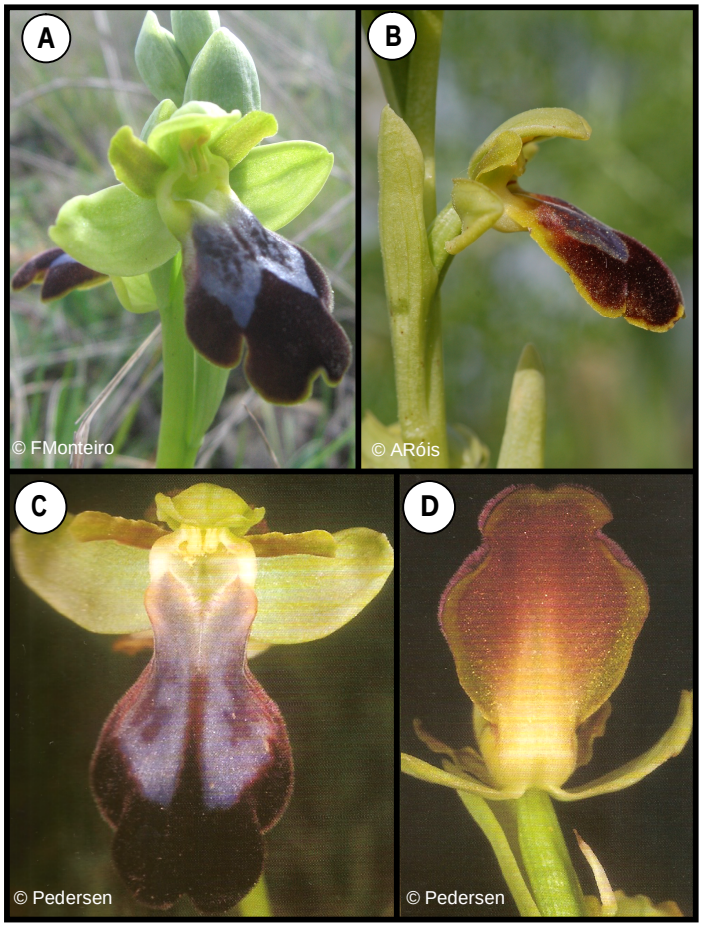

Figure 1.8 - *Ophrys fusca* subspecies reported for Portugal: *fusca* (**A**,**B**) and *iricolor* (**C**,**D**).

1.3. Orchid reproductive biology: focus on flower morphology

The most spectacular evolution in orchids is revealed by their peculiar reproductive biology. In most flowering plants, such as the model plants *Arabidopsis* and rice, the ovary matures during flower development and contains ovules, fully developed prior to pollination, that become ready for fertilization, generally occurring soon after pollination (Yu and Goh, 2001; Tsai *et al.*, 2008). In orchids, ovules post pollination development and maturation, early development and maturation of pollen grains, packaged as pollinia (pollen grains bound together by viscin threads in masses for effective pollination) and the release of thousands or millions of immature embryos (globular stage) in mature capsules, are features characteristic of the remarkable fitness of orchid diversity and adaptation (Raghavan and Goh, 1994; Nadeau *et al.*, 1996; Yu and Goh, 2001). These various strategies unique to orchids contributed to the success of the family. Other innovation assisting adaptation to pollinators is flower morphology which has been attributed as the main feature for adaptive radiation in

Orchidaceae (Gavrilets and Losos, 2009). Orchid flowers have a zygomorphic structure, including three types of perianth organs: three outer tepals (T1–T3; often termed sepals) in the first floral whorl, and two lateral inner tepals (t1, t2; petals) as well as a median inner tepal (t3) called the lip (or labellum) in the second floral whorl (Fig. 1.9, reviewed in Mondragón-Palomino and Theiβen, 2007, 2009).

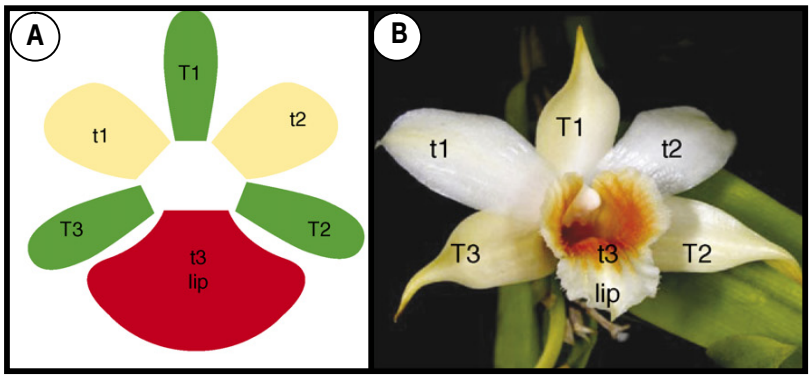

Figure 1.9- Orchid perianth structure. (**A**) Scheme on the typical structure of an orchid flower perianth and its correspondence on a *Dendrobium cariniferum* flower (**B**). Adapted (Mondragón-Palomino and Theiβen, 2007).

The labellum is a morphologically complex median inner perianth segment (petal), that in most orchids acts as the primary visual attractant and landing platform for pollinators, most commonly insects (Bateman and Rudall, 2006).

An interesting feature of the development of most orchid flowers is resupination (180ºC developmental rotation in floral orientation). Resupination (from the Latin *resupinus*, which means facing upward) is the phenomenon describing the orientation of zygomorphic flowers during development so that the median petal (lip) obtains the lowermost position in the mature flower (Ames, 1938; Fischer *et al.*, 2007). Resupination is generally assumed to expose the lip as a landing platform and nectar guide, by emphasizing colours and patterns, to attract pollinators and facilitate pollination (Ernst and Arditti, 1994; Mondragón-Palomino and Theiβen, 2009). Also, recently it was shown that resupination occurred after the flower became zygomorphyfic (Mondragón-Palomino and Theiβen, 2009), and this assumption is well exemplified by Apostasioideae, the most primitive orchid subfamily, in which resupination occurs in the zygomorphic genus *Neuwiedia* but not in the actinomorphic *Apostasia* (Kocyan and Endress, 2001). There are several main characteristics that a flower must have to be called an orchid, such as: a zygomorphic flower, a gynostemium or column, a rostellum, pollinia, and labellum (lip). The male (stamens with pollen bearing anther) and the female parts (pistil consisting of an ovary and stigma) are united into a single structure called column or gynostemium, which is the centre of the flower (Ballings, 2006). The

rostellum (Fig.1.10, side and front views) consists of a tissue projection near the end of the gynostemium which separates the stigma from the pollinia (single pollinium), thereby preventing self-pollination (Withner *et al.*, 1974; Cozzolino and Widmer, 2005).

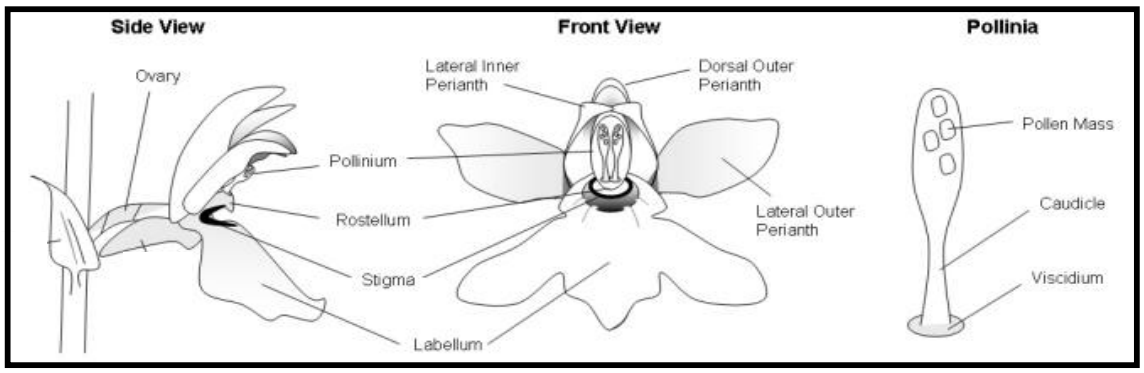

Figure 1.10- General overview on Orchidaceae flower morphology. Adapted (Lang, 1980).

The anther which has two pollen sacs (thecae) is situated at the top of the column. Pollen may be released as single grains, like in most other plants, in the subfamilies Apostasioideae, Cypripedioideae and Vanilloideae; whereas in the other subfamilies, which comprise the great majority of orchids, the anther carries two pollinia (Ballings, 2006). A pollinium is a waxy mass of pollen grains held together by the glue-like alkaloid viscin. Each pollinium is connected to a filament which can take the form of a caudicle (terrestrial orchids, e.g. *Dactylorhiza* or *Ophrys*) or a stipe (in most epiphytic orchids, e.g. *Vanda*). Caudicles or stipes holds the pollinia to the viscidium (adhesive discs= retinacles, Martin, 2005), a sticky pad which sticks the pollinia to the pollinators body (Fig. 1.10, pollinia).

Orchid species have evolved mainly through the development of pollinator specificity as opposite to the incompatibility mechanisms occurring in other plants (Jersáková *et al.*, 2006). Such specificity is achieved by a combination of floral scent and morphological characteristics (such as flower pilosity and/or colour).

1.3.1 *Ophrys* flower morphology

In all *Ophrys* species, the labellum specially functions to draw pollinator's attention, despite differing in shape, colour, scent and texture from the other parts of the flower. Also, tepals may display different colours (e.g. green- *O.fusca fusca*; pink- *O.tenthredinifera*). Gynostemium of *Ophrys* species have the same basic structure: the anther is situated above the stigma (Fig.1.11). Each anther cells contain a pollinarium, consisting of pollinium, a caudicle and a viscidium. In turn, each viscidium is enclosed in a separate bursicle (Fig. 1.11), which is a modification of the rostellum forming a purse-

like structure (Claessens and Kleynen, 2002; Martin, 2005). Also, caudicles of most *Ophrys* species are inflexible in the anther cells, awaiting the insects which will transport them to another flower (Claessens and Kleynen, 2002). Exceptions have been shown for *O.apifera* (see Orchid's pollination biology section) and *O. helenae* (Paulus, 2006).

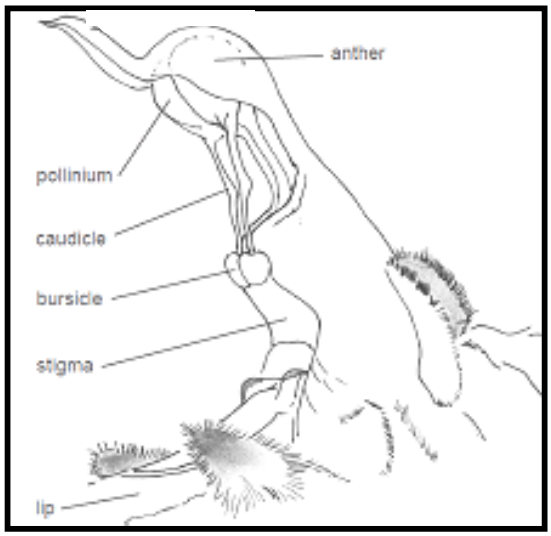

Figure 1.11- Gynostemium side view on *Ophrys apifera*. Adapted (Claessens and Kleynen, 2002).

1.4. Pollination syndromes: definition and consequences

Plant-pollinator interactions consist on a form of mutualism in which each member benefits (Labandeira *et al.*, 1994; Fenster *et al.*, 2004). Pollination syndromes can be considered as morphologically convergent adaptive trends exhibited by floral features (Fenster *et al.*, 2004; Hoballah *et al.*, 2007). Passive pollination syndromes include anemopily (wind) and hydrophily (water) pollination, while zoophily (animal) is considered an active syndrome. In general, pollination by insects and other animals is more efficient than wind-dependent pollination. Melittophily (bee pollination) is the most important pollination type (Gullan and Cranston, 2000). In the bee-mediated pollination the following events are characteristic: flower only opens when bees are active and the pollen collecting apparatus is ready and specific for each plant-pollinator association (Fenster *et al.*, 2004). However, in some cases, the pollinator has specialized features that make it especially suitable for pollinating a particular plant, which is a common trait encountered in orchids pollination. Such specialized traits in plants and in pollinators are considered as a process of coevolution (Kareiva, 1999; Hodges and Whittall, 2008). Instead, some authors account for the occurrence of a unilateral evolution in orchids, without influencing pollinator's evolution, rather than a coevolution (Schiestl, 2005; Jersáková *et al.*, 2006 and references therein). This unilateral

evolution is based on the following assumptions: orchids appeared when most key pollinator groups had already evolved and established complex mutualisms with flowering plants (Labandeira *et al*., 1994; Jermy, 1999). Also, orchids are often deceptive (see below) and therefore unlikely to influence the evolution of pollinator traits (Jersáková *et al.*, 2006). Occasionally, such specialization is so high that the plant depends on a single pollinator species (e.g. *Ophrys*). This particular characteristic of pollinator constancy, which may be responsible for the isolation of small populations, is especially prevalent in Orchidaceae (Gullan and Cranston, 2000; Coyne and Orr, 2004).

1.4.1. Orchid's pollination biology

The highly specialised mechanisms by which orchids are pollinated have been the subject of many studies (Nilsson, 1988; Johnson *et al*., 1998; Schiestl *et al*., 2003; Vereecken and Schiestl, 2008; Micheneau *et al.*, 2009), initiated by Darwin (1862) observations in the book: *The Various Contrivances by which Orchids are Pollinated by Insects*, one of the most well-known study. Since Darwin, orchid pollination complexity and diversity have been an intriguing matter for biologists. Orchids have developed highly specialized pollination systems and the chance of being pollinated is often scarce. This is the reason why flowers usually remain receptive for very long periods and why most orchids deliver pollen in a single mass, so that each time pollination succeeds thousands of ovules can be fertilized (Paling, 2007). Most orchids depend on insects for cross-pollination since their pollen lies in sticky masses preventing pollen dispersal by wind.

In general, the mechanism of orchid pollination relies on the attraction of the insect through visual (flower colour and/or shape), chemical (scent emission) and/or rewardable (nectar) cues displayed by the flower. Usually, the insect perceives a nutritional offer as a reward, although in some cases (e.g. genera *Ophrys*, *Chiloglottis*) it may be a sexual mate (Grant, 1994). When the insect lands on the labellum, it attempts to feed on the flowers nectar or to mate with the flower. In orchids that produce pollinia, pollination usually happens as follows: when the pollinator visits the flower, it touches a viscidium, which promptly sticks to its body, generally on the head or abdomen. While leaving the flower, it pulls the pollinium out of the anther, as it is connected to the viscidium by the caudicle or stipe. The caudicle then bends and the pollinium is moved forwards and downwards. After, when the insect visits a second flower of the same species, pollinia are aligned below the rostellum and come into contact with the stigma. As a result, self-pollination is avoided mainly due to the rostellum, which separates the pollinia from the stigma. Indeed, Darwin (1862) previously

suggested that self-pollination was inhibited by physical reconfiguration of pollinia, hypothesis that received recent support (Peter and Johnson, 2006). Yet, some orchids mainly or totally rely on self-pollination, especially in colder regions where pollinators are particularly rare. Several floral features are related to self-pollination, namely: in the absent of pollinators caudicles may dry out and the pollinia fall directly on the stigma (e.g. *Pterygodium vermiferum*, Oliver *et al.*, 2008; *Ophrys apifera*, Fenster and Martén-Rodríguez, 2007, Fig.1.12**A**,**B**); or, the anther may rotate and then enter the stigma cavity of the flower (as in *Holcoglossum amesianum*, Liu *et al.*, 2006, Fig. 1.12**C**).

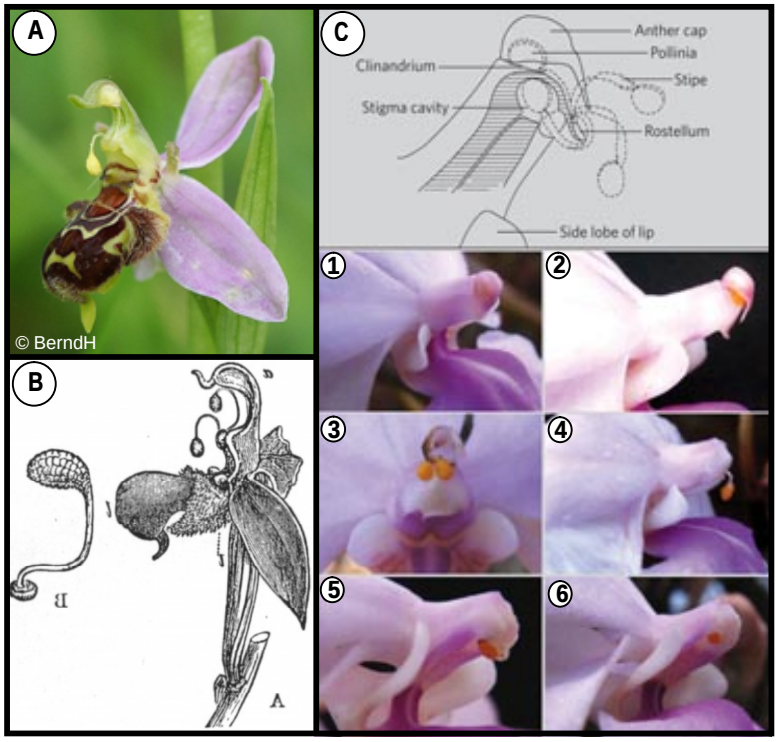

Figure 1.12. Self-pollination mechanisms in *Ophrys apifera* (**A**,**B**) and *Holcoglossum amesianum* (**C**). (**A**)- Self-pollination in *Ophrys apifera*; (**B**)- Drawing by Charles Darwin on *Ophrys apifera* self-pollination, adapted (Darwin, 1862); (**C**)- *Holcoglossum amesianum* 360-degree twist towards self-pollination; steps 1-6 illustrate the arrangement of floral components and movements during the transport of pollen from the anther to the stigma. Adapted (Liu *et al.*, 2006).

Interestingly, the absence of a true rostellum and the impossibility of pollinarium removal at any time of the flower lifespan explain the obligatory auto-pollination mechanism on the *Jumellea stenophylla* orchid species (Micheneau *et al.*, 2008). Asexual reproduction also occurs in some species of the genera *Phalaenopsis*, *Dendrobium* and *Vanda* by producing offshoots in one of the nodes along the stem, known as keiki (Košir *et al.*, 2004). Further, Orchidaceae is known for its enormous diversity of pollination mechanisms and unusual high occurrence of non-rewarding flowers, known as deception mechanisms, compared to other plant families (Jersáková *et al.*, 2006).

1.4.2. Mechanisms of deception in orchids

From 7500 angiosperm species pollinated through a deception mechanism, approximately 6500 are orchids (Renner, 2006). Pollination through deception (i.e. an absence of floral rewards for pollinators) is a widespread phenomenon in angiosperm plants, and in Orchidaceae family is extremely common (Nilsson, 1992; Streinzer *et al*., 2009). Jersáková and co-workers (2006) reviewed extensively the deception mechanisms in orchids, including food and sexual deception, among others. In fact, food (38 genera) and sexual (18 genera) deception are the most common types of pollination in orchids by mimicry, occurring in approximately one-third of the species (Jersáková *et al.*, 2006; Renner, 2006). In food deception, pollination strategy relies on the general resemblance of a non-rewarding species with the nectar-rewarding species, by exploiting the instinctive food-searching ability of pollinators (Cozzolino and Widmer, 2005). A food deception case is illustrated by the Batesian floral mimicry (reviewed in Roy and Widmer, 1999), in which non-rewarding species strictly resembles a specific co-occurring rewarding species (e.g. *Disa nivea*, Anderson *et al.*, 2005; *Eulophia cucullata*, Peter and Johnson, 2008). A recent evidence of Batesian mimicry has been reported in the genus *Eulophia* (Peter and Johnson, 2008), in which orchid is presumably a Batesian mimic of the sympatric species *Sesamum radiatum* (Fig. 1.13).

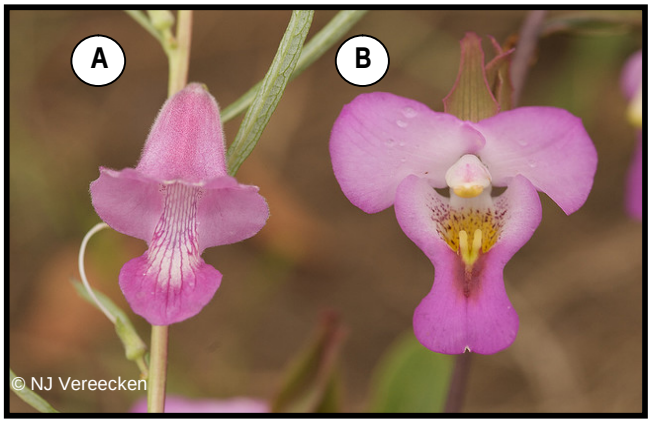

Figure 1.13- Floral detail of *Sesamum radiatum* (Pedaliaceae) (**A**) and *Eulophia cucullata* (Orchidaceae) (**B**) growing in sympatry, as an example of Batesian floral mimicry. Both species grow and bloom intermixed.

Food-deceptive floral mimicry is associated with pollinators that mainly use colour, rather than scent, as their primary foraging cue (Juillet and Scopece, 2010). On the other hand, sexually deceptive orchids attract pollinators primarily through chemical mimicry of female sexual pheromones, thereby deceiving males into attempted mating with orchid labellum (Schiestl *et al.*, 2000; Schiestl, 2005). Flower morphological features, i.e. labellum shape, colour and pilosity that mimic pollinator

female body-shape, are also important for drawing male's attention (Schiestl, 2005; Fig. 1.14**A**,**B**). This mechanism of mimicry in plants has been titled Pouyannian mimicry in honour of M. Pouyanne who first described the phenomenon in genus *Ophrys* (Pouyanne, 1917 and references therein). During copulation attempt, known as pseudocopulation (Fig. 1.14**C**), the pollinia become attached to the male's body and is transferred upon visitation to flowers further visited (Pouyanne, 1917; Kullenberg, 1961; Schiestl, 2005).

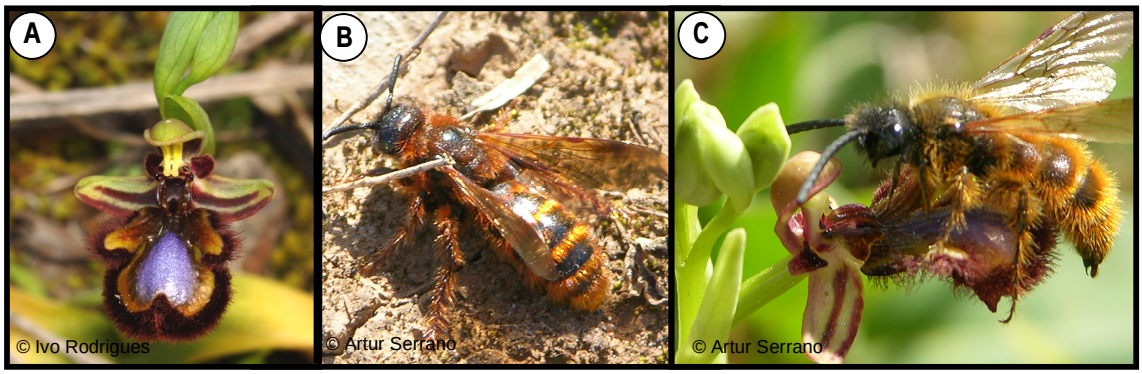

Figure 1.14- Sexual deception in *Ophrys speculum* (mirror bee orchid). (**A**)- Flower detail; (**B**) Pollinator species, *Campsoscolia ciliata*; (**C**) Pseudocopulation event.

Over long distances, sexually deceptive orchids lure pollinators by foraging insect sex pheromones, whereas at close range, visual (e.g. labellum colour and/or shape) and tactile (e.g. labellum pilosity) cues of the female insect are essential (Schiestl, 2004; Gaskett and Herberstein, 2010). A number of terrestrial orchid species which are represented in the European genus *Ophrys* (Schiestl, 2004), South African *Disa* (Johnson *et al.*, 1998) and nine Australian genera of the tribe Diurideae, e.g. *Cryptostylis* (Schiestl *et al.*, 2004; Gaskett and Herberstein, 2010) and *Chiloglottis* (Mant *et al.*, 2005b) reproduce via this deceptive pollination mechanism. Altogether comprise about 400 described species (Schiestl, 2005 and references therein). Recent studies on orchid pollination, particularly in *Ophrys* genus, have focused on the mechanisms by which flowers attract their insect pollinators (Schiestl *et al.*, 2000; Schiestl, 2005; Spaethe *et al.*, 2007; Vereecken and Schiestl, 2008; Stökl *et al.*, 2008a, b; 2009; Vereecken *et al.*, 2010).

1.4.3. Pollination by sexual deception

In *Ophrys*, pollination occurs by means of sexual deception which is unique to Orchidaceae (Nilsson, 1992; Raguso, 2004). In Europe, only the genus *Ophrys* is pollinated by this mechanism. This genus encompass a spectacularly diverse set of species all pollinated by sexual deception, with a few exceptions such as *O. apifera*

and *O. helenae*, which display self-pollination in pollinator's absence (Paulus, 2006). In sexual deception mechanism, patrolling male bees (Andrenidae, Anthophoridae, Colletidae, Megachilidae, and Apidae), predatory and parasitic wasps (Sphecidae and Scoliidae) and occasionally beetles (Scarabaeidae, Kullenberg, 1961; Borg-Karlson, 1990; Paulus and Gack, 1990), attempt to copulate on flowers that mimic mating signals (shape, colour and scent) of receptive female insects (Fig. 1.15; Kullenberg, 1961; Schiestl *et al.*, 1999; Schiestl, 2005).

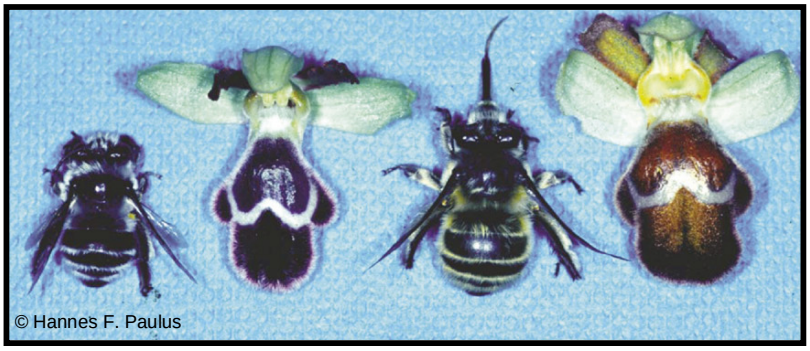

Figure 1.15- Sexual deception in two *Ophrys* species. From left to right, *Anthophora sicheli* female and *Ophrys fleischmannii*; *Anthophora dalmatica* and *Ophrys omegaifera*.

Odours are special floral signals crucially involved in sexual mimicry pollination systems (Borg-Karlson and Tengö, 1986; Borg-Karlson, 1990). Signals mimicked by orchids, in particular the sex pheromones of the female insects, are usually described as species-specific, thus most *Ophrys* species are pollinated by one or a few closely pollinator species (Borg-Karlson, 1990; Schiestl, 2004). To attract males and elicit mating behaviour, these flowers produce active components similar to the sex pheromone of receptive females of the imitated insect species (Streinzer *et al.*, 2009). Chemical analysis revealed that *Ophrys* flowers produce complex species-specific mixtures of more than 100 compounds, mainly saturated and unsaturated hydrocarbons, aldehydes, alcohols, esters, ketones, and terpenoids present in minor amounts (Borg-Karlson, 1990). Floral fragrances contain chemical compounds identical to those of the pheromonal secretions of the respective female insects (Ayasse *et al.*, 2001; Schiestl, 2005; Vereecken and Schiestl, 2008). Due to its high specific attraction mechanism, flowers of sexually deceptive orchids usually do not possess obvious colour signals, thereby avoiding fortuitous attraction of unspecific pollinators (Spaethe *et al.*, 2010). Additionally, Ascensão and co-workers (2005) described in *O.fusca* and *O.lutea* labella a presence of an osmophore (i.e. floral scent glands) at the apical region near the central notch. This is formed by the entire border and the abaxial surface from the distal part of the apical region of the labellum together and consists of a secretory papillate epidermis and two or three subsecretory parenchyma layers. Also, the adaxial

indumentum of the labellum was considered to provide important tactile and visual stimulation to the pollinator species (Ascensão *et al.*, 2005).

Since the sex pheromone compounds emitted by the flower attract only males of the target species, pollen transfer is highly efficient and pollen lost is low (Spaethe *et al.*, 2010). The finding that *O. sphegodes* flowers, namely the labellum, produce the same compounds (namely, 14 compounds comprising mainly alkanes and alkenes) as the female sex pheromone of its specific pollinator species, *Andrena nigroaenea*, has open a major breakthrough on plant-insect association studies (Schiestl *et al.*, 1997, 1999, 2000; Schiestl and Ayasse, 2001). *Ophrys* genus can be faced as an attractive and suitable model to study evolutionary processes underlying plant- insect association.

1.4.5. *Ophrys fusca* as a case study

Species from *Ophrys fusca*-group are commonly pollinated by *Andrena* bees (Schiestl and Ayasse, 2002; Stölk *et al.*, 2008a, b, 2009). *Ophrys* species are interfertile and reproductive isolation is achieved by pre-pollination mechanisms, i.e. attraction of one single pollinator species reproductively isolated from other sympatrically occurring species. Floral scent variation ultimately will act as a mechanism of reproductive isolation (Schiestl and Schlüter, 2009). Flowers of *Ophrys sphegodes* and *O. fusca* are known to be both pollinated by *Andrena nigroaenea* males (Schiestl *et al.*, 2000; Schiestl and Ayasse, 2002). The pollinia are deposited on the head of the male *O. sphegodes* pollinator bee (cephalic pseudocopulation), whereas *O. fusca* pollinator males reverse into the flower and receive pollinia on the tip of the abdomen. As a result, an effective isolation mechanism between species is achieved and loss of pollinia to heterospecific orchids is minimized (Schiestl *et al.*, 2000). Studies regarding *O. fusca* pollination are scarce; yet, its pollinator species are described as being: males of the bee *Andrena nigroaenea* (Schiestl and Ayasse 2002) and *Colletes cunicularius* (*Ophrys fusca* in southern Spain, Peakall and Schiestl, 2004). In Portugal, an effective identification on *O. fusca* pollinator species is lacking. Also, a huge variation between *O. fusca* populations is easily observed, which ultimately difficult determination of its pollinator species. Several studies performed in *O. sphegodes* allowed a comprehensive view on sexual deception mechanism (Schiestl *et al.*, 1997, 2000; Schiestl and Ayasse, 2001). Schiestl and Ayasse (2001) related the increase of farnesyl hexanoate production in pollinated flowers as being responsible for guiding pollinator visitation to unpollinated flowers of the inflorescence. Consequently, it can be supposed that this phenomenon may occur in other *Ophrys* species as well. Several *Ophrys* species seem to produce different compounds or differences in relative proportions of the same compounds. For instance, in *O. fusca* and *O. bilunulata*,

Schiestl and Ayasse (2002) showed that only slight differences in the relative proportions of both alkanes and alkenes triggered the species-specific attraction of pollinators, with almost all the compounds being produced by both species.

1.5. Pollination: a molecular perspective

1.5.1. Developmental events regulated by pollination

It is well established that compatible pollination activates a series of post pollination developmental events which contributes to reproduction and ovary growth, pigmentation changes and petal senescence (O'Neill, 1997). The developmental events enhanced by pollination prepare the flower for fertilization and embryogenesis, while promoting floral organs wilting that have completed their function in pollen dispersal and reception (Zhang and O'Neill, 1993). Developmental processes associated with this transition include senescence of the perianth, pigmentation changes, ovary maturation, ovule differentiation, and female gametophyte development (O'Neill, 1997). In most orchid flowers, pollination (insertion of a pollinium, rather than pollinia removal) initiates rapid senescence of orchid flowers thereby reducing their commercial value (Ketsa and Rugkong, 1999; Abdala-Roberts *et al.*, 2007; Attri *et al.*, 2007; 2008). Mechanisms underlying petal senescence, particularly in orchids, are still enigmatic.

1.5.2. Petal senescence: a controlled subset of events

Petals provide an excellent model to study senescence since they have a finite lifespan and once flower is pollinated or is no longer receptive for pollination purposes, maintenance of this organ is energy expensive (Jones *et al.*, 2005). Senescence is considered a type of programmed cell-death (PCD), since it involves structural, biochemical and molecular changes similar to the characteristic PCD traits (Tripathi and Tuteja, 2007). According to Engelberg-Kulka and co-workers (2006), PCD is defined by the death of a cell or cells mediated by an intracellular program. Senescence involves several physiological changes such as loss of water from the senescing tissue, leakage of ions, transport of metabolites to different tissues; and biochemical changes, such as generation of Reactive Oxygen Species (ROS), increase in membrane fluidity and peroxidation, hydrolysis of proteins, nucleic acids, lipids and carbohydrates (Tripathi and Tuteja, 2007). Previous reports in petal senescence, have revealed a set of genes up regulated during senescence, generally considered to be stress related, including:

metallothioneins, abscisic acid (ABA)-responsive genes (Breeze *et al.,* 2004) and glutathione *S*-transferases (Meyer *et al.*, 1991; Price *et al.*, 2008). Besides, significant number of senescence up regulated genes is also pathogenesis-related (Hanfrey *et al.,* 1996; Thomas *et al*., 2003). In senescing plant tissues, cysteine proteases, the closest functional homologue to caspases- animal cysteine proteases- are commonly found (Buchanan-Wollaston, 1997; Wagstaff *et al.*, 2002). Membrane damage in senescing tissues occurs essentially by oxidative stress invoked during the process (Attri *et al.*, 2008). Membrane integrity loss, increases in neutral lipids, sterol to phospholipid ratio, and in the saturation:unsaturation index of fatty acids are also consequences of the senescence event (Thompson *et al.*, 1982). In addition, remobilization of nutrients from senescing flower tissues occurs and is believed to contribute either to ovary or to new flowers development (Thomas *et al.*, 2003; Hoeberichts *et al.*, 2007; Mea *et al.*, 2007). Natural and/or induced senescence in leaves is a very well studied process (Lim *et al.*, 2003; Gepstein, 2004), generally characterized by a decline in photosynthetic capacity, chlorophyll degradation and leaf yellowing (van Doorn, 2001; Mishina *et al.*, 2007). In addition to down regulation of photosynthesis related genes, leaf senescence is characterized by an amplified expression of a multitude of genes (Table 1.1) that are often referred as SAGs, i.e. senescence-associated genes (Buchanan-Wollaston, 1997; Guo *et al.*, 2004). These SAGs can be grouped in several functional categories and are involved in several pathways (Table 1.1).

Table 1.1. Major functional categories of SAGs (senescence-associated genes) expressed in *Arabidopsis thaliana* leaf senescence transcriptome. Adapted (Gepstein, 2004; Guo *et al.*, 2004).

Functional category	Most frequent genes
Macromolecule degradation (breakdown of lipids, proteins, nucleic acids)	Cysteine proteases, ubiquitin-related genes, RING finger proteins, nucleases, lipases/acylhydrolases, phospholipases and proteinases families.
Nutrient recycling (transport of purines, pyrimides, sugars and ions)	Oligopeptide transporters, purine and pyrimidine transporters, and ABC transporters
Defense and cell rescue mechanisms (Abiotic and biotic stresses, oxidative stress)	Metallothioneins, glutathione *S*-transferase, glutathione peroxidase.
Transcriptional regulation	Zinc finger proteins and transcription factors of the WRKY, NAC, AP2, MYB, HB families
Signal transduction (protein phosphorylation/ dephosphorylation)	Receptor-like and calcium-dependent protein kinases, phosphatases and phospholipases.

NAC, no apical meristem (NAM); AP, Apetala proteins.

During flower senescence, macromolecules are degraded and organelles are dismantled in order to engage nutrients remobilization to developing tissues (Chapin and Jones, 2007; Bai *et al.*, 2010; Müller *et al.*, 2010). This process allows plant to recover carbon, nitrogen, and phosphorus from the petals before corolla abscission (Price *et al.*, 2008). The last stages of senescence involve loss of nucleic acids (DNA and RNA), proteins and organelles, which is achieved by activation of several nucleases, proteases and cell wall modifiers (Tripathi and Tuteja, 2007). The degradation of macromolecules (namely proteins, nucleic acids, and lipids), decline in photosynthesis, remobilization of nutrients and the dismantling of cellular organelles are common features concerning both leaf and petal senescence events (Lim *et al.*, 2003; Price *et al.*, 2008).

Studies of both leaf and flower senescence have focused almost exclusively on the identification of senescence-associated genes (reviewed in Buchanan-Wollaston *et al.*, 2003; van Doorn and Woltering, 2008). These studies suggested that initiation and progression of senescence program requires new gene transcription and pointed out to an additional control of senescence at the post transcriptional level (Thomas *et al.*, 2003; Bai *et al.*, 2010). Signalling and transcriptional networks are regulators of when and where senescence starts. Execution, control and integration of constitutive processes require activation/inactivation of cascades which are strongly post transcriptionally regulated. Recent studies using a proteomic (e.g. two-dimensional gel electrophoresis, 2-DE) approach showed that proteins with putative function in large-scale degradation of macromolecules and organelles are activated after pollination, therefore highlighting their post transcriptional role in *Petunia hybrida* flower senescence (Bai *et al.*, 2010). It is therefore conceivable that a complex network of both transcriptional and post transcriptional regulation is involved in flower senescence, as suggested for other cellular processes such as cell cycle (Rogers, 2006).

1.5.3. Pollination studies in orchids

In flowering plants, pollination sets off a cascade of developmental events, including perianth senescence, changes in pigmentation and ovule differentiation, in which ethylene plays a regulatory role (O'Neill *et al.*, 1993; O'Neill and Nadeau, 1997; Taiz and Zieger, 1998; Ketsa *et al.*, 2001). Besides, pollination also affects floral scent emission, as previously reported for snapdragon and petunia (Negre *et al.*, 2003), and in orchids (e.g. *Ophrys sphegodes*, Schiestl *et al.,* 1997). Pollination is often accompanied by an increase on ethylene levels, a major signal for petal senescence, especially in long-lived flowers like orchids (Rogers, 2006), which are generally classified as ethylene-sensitive (van Doorn, 2001). In short-lived flowers, senescence can be controlled

independently from pollination and modulated by hormones (e.g. brassinosteroids, positive regulators; cytokinins, negative regulators of senescence) (Rogers, 2006; Lim *et al.*, 2007). The physiological and molecular mechanisms of pollination induced senescence have been the subject of several studies in orchids (e.g. *Phalaenopsis*, Porat *et al.*, 1994; *Cymbidium*, Heyes and Johnston, 1998; *Dendrobium* and *Phalaenopsis*, Iwaya-Inoue *et al.*, 2005). Those studies have been mainly focused on the determination of ethylene sensitivity and on regulation of ethylene biosynthetic genes (1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO)) (Nadeau *et al.*, 1993; Mita *et al.*, 2006). It is well known the role of ethylene as a major signal for petal senescence (van Doorn, 2001; Jones, 2008), which is characteristic of the ethylene-sensitive flowers, as most orchids are (Rogers, 2006). O'Neill and co-workers (1993) verified that post pollination events in orchid's involved coordinated interorgan regulation of both ACS and ACO expression. Yet, a general approach regarding post pollination regulated mechanisms in orchids is lacking. In carnation (*Dianthus caryophyllus*) and *Phalaenopsis* sp. orchid, pollination enhanced genes encoding for ACO and ACS have been associated to the post pollination burst of ethylene synthesis related to petal senescence (Porat *et al*., 1994; Tang *et al*., 1994; Nadeau and O'Neill, 1995; Woltering *et al*., 1995). Ethylene-inducible transcripts from petals including mRNAs with homology to glutathione *S* transferase (Itzhaki *et al*., 1994), phosphoenolpyruvate mutase (Wang *et al.*, 1993) and cysteine proteinase (Jones *et al*., 1995) were also described.

1.5.4. Ethylene and pollination: a close relation

The phytohormone ethylene is the primary regulator of floral senescence in a wide range of plant genera (Kende, 1993; Macnish *et al.*, 2010). In orchids, as in other ethylene-sensitive flowers, initial response to pollination is an increased ethylene production by the stigma, often followed by increased ethylene synthesis from ovaries and petals (Jones and Woodson, 1997; Llop-Tous *et al.*, 2000). Ethylene produced by different floral organs is responsible for coordinating pollination-associated events such as ovary growth and senescence of perianth (Llop-Tous *et al.*, 2000; Rieu *et al.*, 2003). Petal wilting, colour change and abscission occurring after pollination may be mediated by endogenous ethylene as suggested by experimental data where inhibitors of ethylene synthesis/action prevent the pollination effect on petal wilting in carnation, orchids and petunia (Woltering and Somhorst, 1990; Porat *et al.*, 1994; van Doorn, 2001 and references therein). In contrast, increases on cytokinin levels in petunia delays flower senescence (Lara *et al.*, 2004). In carnation flowers the increased ethylene production associated with petal senescence, regulates PCD by

transcriptional activation of SAGs, which encode for cysteine proteases, RNases and glutamine synthase (Woodson *et al.*, 1992; Marrs, 1996; Della Mea *et al.*, 2007). In carnation (Wang and Woodson, 1991), tomato (Holdsworth *et al.*, 1987; Llop-tous *et al.*, 2000), orchids (Nadeau *et al.*, 1993; Bui and O'Neill, 1998; Mita *et al.*, 2006), mulberry (Pan and Lou, 2008), petunia (Tang and Woodson, 1996) and *Pelargonium* (Wang *et al.*, 1994; Clark *et al.*, 1997; Dervinis *et al.*, 2000) flowers, pollination-induced ethylene production is associated with the expression of ACC synthase (ACS) and ACC oxidase (ACO) genes. According to O'Neill and co-workers (1993), post pollination events in orchids, ranging from perianth senescence to ovule differentiation involve coordinated inter-organ regulation of both ACS and ACO expression, the two key ethylene biosynthetic enzymes. In *Phalaenopsis*, many studies have been performed regarding ethylene sensitivity due to their high commercial value (Nadeau *et al.*, 1993; Porat *et al.*, 1994; Bui and O'Neill, 1998). Enzyme levels of ACS and ACO activities in the stigma, labellum and ovary were initially low, but increased to peak levels at 24 hours after pollination (ACO, Nadeau *et al.*, 1993; ACS, Bui and O'Neill, 1998), and after their activities decreased. It cannot be ruled out a post translational regulation of ACS expression as suggested by recent reports (Wang *et al.*, 2001; Chae *et al.*, 2003; El-Sharkawy *et al.*, 2008).

1.6. Aims and scope of the work

Flowers that demonstrate strict pollination regulation, as orchids, provide excellent model systems to unravel regulation mechanisms that are pollination- elicited. Orchids being, in general, of considerable important for commercial exploitation and immeasurable beauty and mysticism, justify continuous worldwide natural habitat conservation efforts and a better understanding of their pollination mechanisms. Since orchids display unique differences in reproductive biology, as compared to *Arabidopsis*, they offer additional advantages for studying gene function and evolution in pre-pollination and post-pollination stages. Due to its enormous variation colors, orchids are also ideal as a model for studying floral coloration. Some investigations have highlighted genes related to orchid flower pigmentation, most of them encoding enzymes of the flavonoid pathway (Liew *et al.*, 1998; Johnson *et al.*, 1999).

The main objective of this thesis is the study of the peculiar pollination mechanism of *Ophrys fusca* by means of sexual deception, as a contribution for the understanding of orchid pollination biology. Specific objectives of the present study are:

1. To identify post pollination differentially expressed genes and implied cellular and metabolic processes;

2. To disclose the metabolomic profile of pollinated vs unpollinated flowers and detection of compounds putatively involved in pollination;
3. To relate the post pollination transcriptional profile with processes undergoing in a sexual deceptive pollination system.

1.7. Thesis outline

This PhD thesis aims at contributing to the knowledge on orchid pollination biology, through the study of the pollination mechanism of *Ophrys fusca* by sexual deception. To accomplish this objective, a transcriptional profiling study was performed after pollination event. A metabolic profiling approach was also considered to unravel compounds dynamics in *Ophrys* pollination.

This thesis is organized in four chapters. The first chapter comprises a general introduction, focusing on *Ophrys*, highlighting the main aspects regarding orchid pollination biology and outlining molecular mechanisms enhanced by pollination. The second chapter focuses on a transcriptional approach by means of cDNA microarray, where putative functions assigned to genes differentially expressed after pollination is discussed. Chapter three comprises a metabolic profiling through GC-MS analysis by focusing on metabolites putatively involved in plant- insect association, namely in *Ophrys fusca*. Chapter four addresses concluding remarks through an integrative approach.

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Chapter 2

LABELLUM TRANSCRIPTOME AFTER POLLINATION

2.1. Abstract

Microarray technology applied to ecological studies is challenging and the number of field studies with non-model species is increasing. One of the major drawbacks rely on the large environmental variations associated to field experiments, yet by increasing the number of biological replicates and samples to be analysed problems can be surpassed. In orchids, pollination is a highly regulated process. Due to their peculiar reproductive biology, floral morphology, color diversity and economical importance, it is of major interest to unravel mechanisms that are pollination- elicited, thereby contributing to the knowledge on orchid pollination and/or reproductive biology. To achieve this goal, a transcriptional profiling through cDNA microarrays of genes differentially expressed during pollination was performed to give an insight on the labellum changes after pollination in the sexual deceptive orchid *Ophrys fusca*. Labellum transcriptome of in-field growing *O. fusca* plants was analysed, at 2 and 4 days after pollination (2DAP and 4DAP). Three thousand three hundred eighty-four clones were PCR amplified, isolated and printed in duplicate on glass slides. Screening of the arrayed cDNAs was performed by hybridizing cDNAs of labella from pollinated and unpollinated flowers (resulted from amplified RNA), test and control, respectively. Statistical analysis revealed 277 genes differentially expressed, found to correspond to 140 unique sequences at both 2DAP and 4DAP. Transcripts down regulated by pollination belong to secondary metabolism (phenylpropanoids, lipids, and alkaloids) and photosynthetic related genes; whereas up regulated transcripts are involved in RNA degradation, protein synthesis, mobilization of nutrients, stress and defence responses associated to senescence-induced event. Results highlight the existence of extremely regulated mechanisms underlying pollination and senescence, with particular interest for the understanding on orchid pollination biology.

Keywords: cDNA microarrays, *Ophrys fusca*, orchid, pollination, senescence, real-time PCR.

2.2. Introduction

2.2.1. Microarrays: outline and cDNA technology.

A DNA microarray is a multiplex technology that consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features, each containing a specific DNA sequence, known as probe, which can be a short section of a gene or other DNA element (Schulze and Downward, 2001). They can be hybridized to a complementary DNA (cDNA) or cRNA samples, called target (Schulze and Downward, 2001). Probe-target hybridization is usually spotted and quantified by detection of fluorophore- or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target. Microarray technology has become a powerful tool for high-throughput gene expression analysis, due to the huge amount of information retrieved from a single experiment. Applications include quantitative analysis of gene expression (Schena *et al*., 1995), DNA sequencing and gene discovery (Hacia, 1999), disease diagnosis (Huang *et al*., 2001), drug discovery (Marton *et al*., 1998), and toxicogenomic research (Liu *et al*., 2003), among others. Microarray technology has evolved rapidly and has been developed for a variety of applications, namely: proteins, antibodies, tissues-specific, microRNA (miRNA), transfection, single nucleotide polymorphisms (SNPs), tilling, peptides and cDNA. Presently, there are two types of cDNA arrays: those performed on glass slides (microarrays or DNA chips) and those on nylon filters, known as high-density filter arrays (HDFAs) (Rajeevan *et al*., 1999). Solid surface of a glass slide has several advantages when compared to filter arrays, mainly due to efficient covalent attachment of the target DNA to the coated-slide surface that provides stability of the spotted DNA (Zammatteo *et al*., 2000).

There are two different formats of microarray-based technologies depending on the target nucleic acid components, i.e. the oligonucleotide array and the cDNA microarray (Lee *et al.*, 2003). The first one is more applicable when working with species which genomes have previously been sequenced. The 'oligo' type of array consists on oligonucleotide targets, generally less than 25mer in length (Lipshutz *et al*., 1999), generated *in situ* on a solid surface by light directed synthesis (Fodour *et al*., 1991; Hacia *et al*., 1996). The cDNA type of array is more suitable when species with unsequenced genome are concerned. Regarding cDNA microarray construction, several steps are required: experimental design, total RNA extraction, construction of cDNA libraries, clone amplification and purification, slide printing, labelling, hybridization and data analysis (Fig. 2.1). Briefly, target cDNAs are cloned, and PCR amplified. Purified PCR

products are printed onto glass microscope slides with a microarray robot. cDNA targets are synthesized from total RNA or mRNA derived from test and reference/control samples and labelled with distinct fluorescent dyes (Cy3-dUTP, usually for control sample, and Cy5-dUTP, for test sample). Pooled probes are hybridized to the glass slide. Hybridized fluorescent signals are detected with a dual-wavelength laser scanner. Separately scanned images are combined and numerical data is achieved by means of specialized computer software. Normalized ratios of Cy3/Cy5 are calculated for individual target genes (Wu *et al.*, 2001; Lida and Nishimura, 2002).

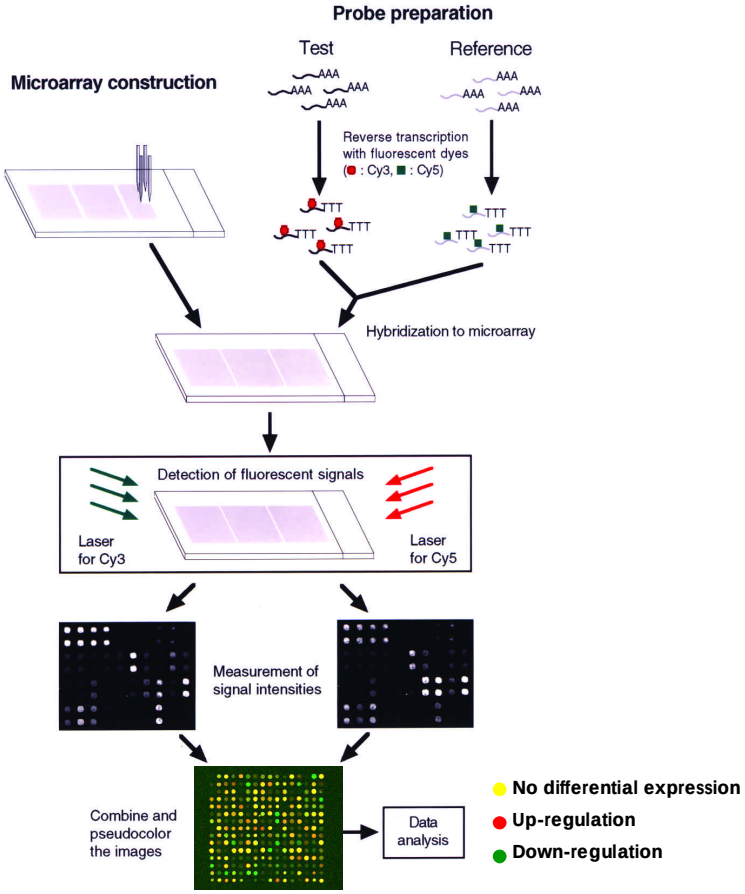

Figure 2.1- Overview of steps in cDNA microarray experiments.
Adapted (Lida and Nishimura, 2002)

Technical variation

Random factors contributing to technical variation include differences among replicated spots within a slide and among slides (Kerr and Churchill 2001; Churchill 2002; Leung and Cavalieri, 2003). Systematic sources of variation include differential dyes incorporation (Churchill, 2002) and multiple print tips (print group effects). Dye bias effects can be controlled by including dye swaps in the experimental design

(Rosenzweig *et al*., 2004), whereas printing effects are controlled by data normalization (Yang *et al*. 2002).

Normalization

Normalization is needed to minimize systematic variations in gene expression levels between two mRNA or total RNA samples, so that biological differences can be undoubtedly distinguished and comparison of expression levels across slides can be performed (Yang *et al.*, 2002; Lee *et al.*, 2008). Imbalances between the red (Cy5) and green (Cy3) dyes may arise from differences between the labelling efficiencies or scanning properties of the two fluorochromes by using different scanner settings (Smyth and Speed, 2003). Microarray data pre-processing contains three phases: quality control, within-slide normalization, and between-slide normalization. Within-slide normalization is used to correct the dye intensity errors introduced across one microarray slide (Leung and Cavalieri, 2003; Berger *et al.*, 2004). One scatterplot-based normalization technique that is particularly suitable for balancing the intensities is called locally weighted scatterplot smoothing (LOWESS) and its original application was for smoothing scatterplots in a weighted least-squares fashion (Berger *et al*., 2004). Replicate slides greatly help the between-slide variability analysis and help in addressing formal statistical considerations when drawing biological conclusions (Berger *et al.*, 2004). The print-tip LOWESS balances systematic differences introduced by the printing tips, which can be due to improper printing; and adjusts the differences of red and green labelling, caused by variations other than biological (i.e. differential labelling incorporation, autofluorescence) (Yang *et al.*, 2002; Do and Choi, 2006).

Despite emergence of new methods for cDNA microarrays normalization, the print-tip LOWESS provides a well-tested and widely used normalization method which gives good results on a variety of arrays (Sebastiana *et al.*, 2009; Jeong *et al.*, 2010; Nishitani *et al.*, 2010).

Identification of differentially expressed genes in microarray data

One of the main objectives in microarray analysis relies on the identification of differentially expressed genes between two experimental conditions. This task may be difficult due to the huge number of genes, simultaneously, under analysis. A sorted list of *t*-statistics will indicate highly expressed genes but an estimate of the false discovery rate (FDR) is needed in order to address a cut-off such that significantly differentially expressed genes can be identified (Šášik *et al.*, 2004). The false discovery rate is

assessed using *q*-values[1], the probability of a statistically insignificant gene appears in fact significant as the observed gene. The false discovery rate is measured using a p-value, which represents the expected proportion of Type I errors among the rejected hypotheses. Thus, the proportion of false discoveries can be controlled by defining a threshold, e.g. 99 % or 95 % of confidence level. Tools are available for calculation of *p*-values from expression data, including SAM (significance analysis of microarrays) (Tusher *et al.*, 2001) and QVALUE (Storey and Tibshirani, 2003). Further, Breitling and colleagues (2004) developed the Rank Product (RP) test, which provides a powerful new statistics test for defining differentially expressed genes in microarray experiments. In contrast to previous techniques, such as SAM, RP showed to be independent of the gene-specific measurement variance, which is particularly important when there is only a small number of replicates, as it frequently happens in the majority of biological studies. Because of its non-parametric nature, it requires only a few well-defined assumptions about the data, such as: relevant expression changes affect only a minority of genes, measurements are independent between replicate arrays, most changes are independent of each other and measurement variance is equal for all genes (Breitling *et al.*, 2004). This method also allows a permutation-based estimation that provides a very suitable way for determining a p-value (Breitling *et al.*, 2004).

2.2.2. Microarrays: labelling technologies and overcome RNA limited amounts

In traditional microarray analysis, the target molecules are labelled with fluorochromes during reverse transcription from mRNA or total RNA to cDNA. The hybridized arrays are analysed by the excitation of the fluorophore on the surface with a specific laser wavelength. The availability of several fluorochromes, such as the most widely used Cy3 and Cy5, allows for two-colour microarray analysis between a sample and a reference on one array. However, the cost of this technology is high which restricts its use in many experiments. The microarrays generally exploit the advantages of nonradioactive fluorescent detection methods, either with direct detection and quantitation of signal or with two-colour fluorescence and competitive hybridization between samples from different sources or different conditions (Schena *et al.*, 1995; Schena, 1996). Microarray technology has greatly facilitated gene expression profiling at the mRNA level by allowing analysis of thousands of transcripts in parallel. However, one limitation of the method is the requirement for relatively large amounts of RNA. Major labelling technologies are based on direct or indirect labelling of the targets,

[1] The *q*-value is defined to be the FDR analogue of the *p*-value.

when large amounts of starting material is available, or rely on signal amplification methods and RNA amplification, for less amounts of starting material.

2.2.2.1. Direct labelling

Briefly, the direct labelling method incorporates dUTP fluorescently labelled with dye fluorochromes during reverse transcription of RNA (Hedge *et al.*, 2000). Direct labelling engages incorporation of a dNTP fluorescently labelled with bulky dye adducts (Cy3 or Cy5) during the reverse transcription process. It has the advantage of being a quick and easy method to perform, although there are evidences that it might introduce sequence-specific artefacts as for background in image chip acquisition (Do and Choi, 2007). The direct cDNA labelling method makes use of fluorescently modified deoxynucleotides such as Cy5-labeled dCTP or Cy3-labeled dCTP, which are incorporated during first-strand cDNA synthesis from an RNA template, using a reverse transcriptase (Fig. 2.2). The main advantage of the direct labelling method relies on its simplicity. Probe molecules directly labelled overcome the need for a post-labelling reaction, which is required in the indirect labelling method. However, Cy5-labeled dCTP is less efficiently incorporated during the labelling reaction than Cy3-labeled dCTP. This imbalanced incorporation of Cy5 and Cy3 dyes can be improved by using the indirect labelling method described below (Do and Choi, 2007).

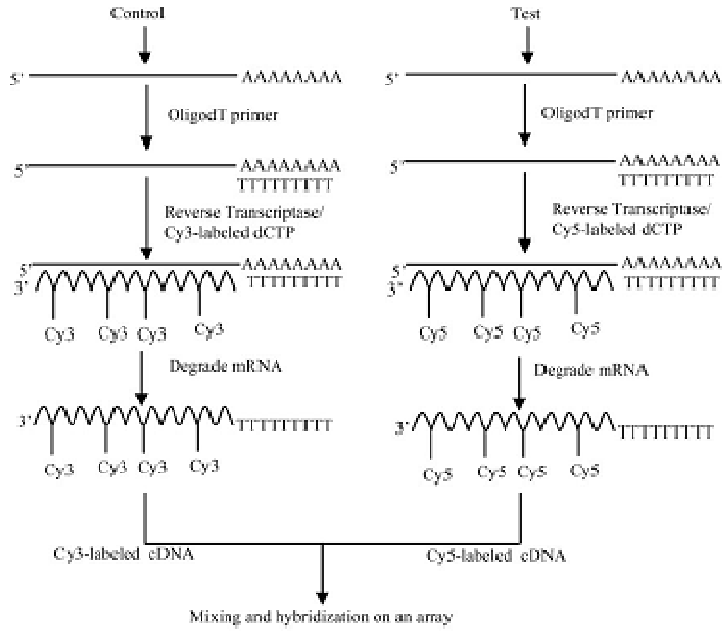

Figure 2.2- Direct fluorescent-dye incorporation scheme for microarray experiments. Adapted (Do and Choi, 2007)

2.2.2.2. Indirect labelling

In the indirect cDNA labelling method, an aminoallyl-modified nucleotide such as aa-dUTP is incorporated during reverse transcription reaction (Fig. 2.3).

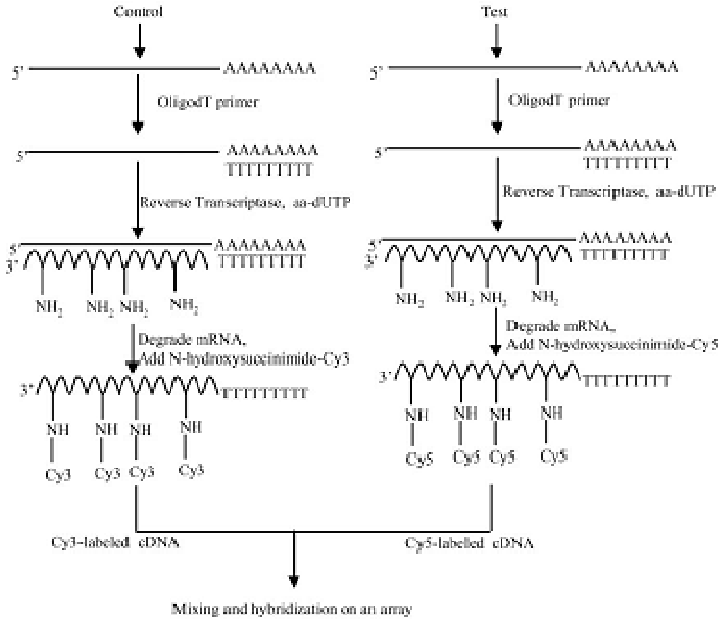

Figure 2.3 - Indirect fluorescent-dye incorporation scheme for microarray experiments. Adapted (Do and Choi, 2007)

The reactive amine of the aa-dUTP can be integrated by a variety of RNA-dependent and DNA-dependent DNA polymerases. The aa-dUTP molecule is incorporated much more easily into DNA or mRNA than the bulky fluorescent bases, which allows more uniform labelling to be carried out (Wang, 2005). Indirect labelling outperforms direct labelling by maximizing the signal intensity through increases in incorporation of the fluorochrome and by increasing the possibility of equal labelling with both CyDyes. However, the indirect labelling method requires an additional post-labelling step which, therefore, extends the time required to perform the procedure. Hence, post-labelling method is less prone to producing artefacts caused by the size of CyDye nucleotides (i.e. chain termination, proximity quenching, and sequence-specific bias) and also produces higher yields and longer cDNAs, which is beneficial when array targets have been derived from the 5'-ends of cDNAs.

Both direct and indirect labelling methods depend on the incorporation efficiency of fluorescence-labelled dUTP or dCTP, which can be an important source of systematic error in two-colour microarrays (Tseng *et al.*, 2001; Dombkowski *et al.*, 2004).

2.2.2.3. Overcome of RNA limiting amounts

In order to obtain consistent hybridizations and adequate signals, labelling protocols such as the direct and indirect methods generally require huge amounts of starting material. This requirement cannot be fulfilled in many situations, e.g. clinical, cell culture and plant studies. Two main strategies have been developed to achieve strong signals from minute amounts of RNA: signal amplification methods and RNA amplification (Wang *et al.*, 2003; Do and Choi, 2007).

2.2.2.3.1. Signal amplification methods

The basic principle of signal amplification methods is that one molecule can produce a signal equivalent to 10-100 unamplified molecules. This labelling scheme is very important when only a small amount of total RNA (1–5 µg) is available. Several signal amplification techniques have been developed, including: rolling circle amplification (RCA) (Lizardi *et al.*, 1998), tyramide signal amplification (TSA) (Karsten *et al.*, 2002); dendrimer (3DNA) technology developed by Genisphere® (Manduchi *et al.*, 2002; Yu *et al.*, 2002); and branched DNA (*b*DNA), a dendrimer-based strategy, among others (reviewed in Park *et al.*, 2006). Signal amplification seems to be suitable to achieve a highly sensitive assay format. However, its widespread application is substantially limited due to its associated costs (Park *et al.*, 2006). Genisphere's signal amplification technology, which is based on the 3-dimensional nucleic acid (3DNA) dendrimer, has been considered a very promising labelling approach (Stears *et al.*, 2000) due to its labelling efficiency when using a limited starting template (Badiee *et al.*, 2003). When comparing with direct and indirect labelling methods, which are dependent on the incorporation efficiency of modified dUTPs, the dendrimer labelling method is entirely dependent on nucleic acid hybridization kinetics (Nilsen *et al.*, 1997), and thus is recommended when amount of RNA available is limited. The main disadvantage is its high cost. Alternative methods for microarray detection have been investigated, such as chemiluminescent detection (Rajeevan *et al*., 1999) and gold nanoparticles based-technology (Shipway *et al*., 2000; Wang *et al*., 2002).

2.2.2.3.2. RNA amplification

To overcome the need of large amounts of RNA for hybridization purposes, increasingly sophisticated methods for RNA amplification from small biological samples have been developed. There are several methods to amplify RNA, including: the

arithmetic transcription methods (Gelder *et al.*, 1990), PCR based exponential amplification or a combination of both arithmetic and exponential amplification (Puská *et al.*, 2002). Most methods are established through the T7-based antisense RNA (aRNA) amplification technique, first described by Gelder and co-workers (1990). The so-called "Eberwine Procedure" or more recently *in vitro* Transcription (IVT) is based on cDNA synthesis and template-directed *in vitro* transcription reaction (Fig. 2.4).

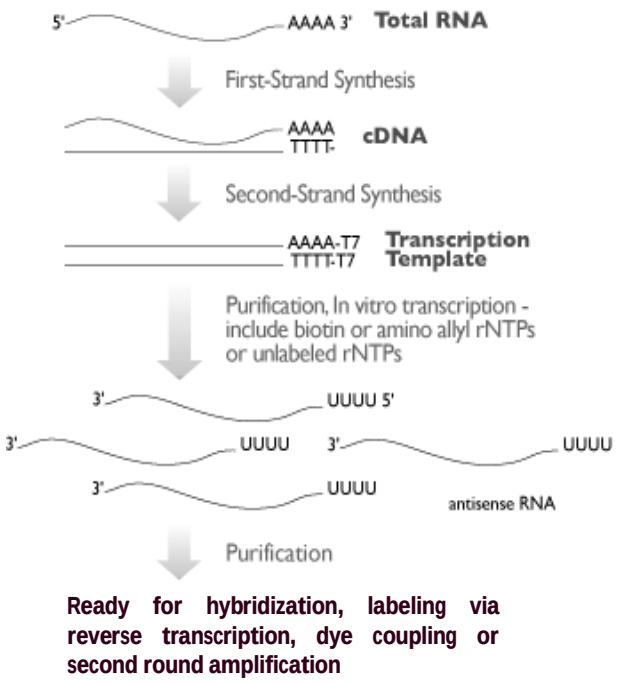

Figure 2.4- antisense RNA (aRNA) amplification based in Eberwine procedure or in vitro transcription (IVT). (Source: http://www.ambion.com/jp/figs/f00469.gif~)

The T7 RNA polymerase promoter is incorporated into the cDNA molecule by priming cDNA synthesis with a synthetic oligonucleotide containing the T7 promoter sequence. The single stranded cDNA (sscDNA) is converted into double stranded cDNA (dscDNA) by the DNA polymerase. After the synthesis of dscDNA, T7 RNA polymerase is added and antisense RNA (aRNA) is transcribed from the cDNA template. The labelling of aRNA without the reverse transcription reaction is possible by the addition of aa-UTP during or after *in vitro* transcription (Fig. 2.4). Each round of the Eberwine procedure produces about a 100-fold amplification of the starting material, and three rounds yield a one-million-fold amplification of the mRNA input (Baugh *et al.*, 2001). There are alternative RNA amplification approaches to the IVT procedure, although its applications are restricted (Ginsberg and Che, 2002; Xiang *et al.*, 2003). The most common method of RNA amplification is based on *in vitro Transcription* (IVT), as described previously. RNA amplification from small amounts of RNA by IVT has several

drawbacks. When reducing the starting amount of RNA, reduction of the reproducibility of data can often occur. Amplification process can increase technical biases due to fidelity loss but, since the biases tend to be systematic, accurate expression data can be obtained. When comparing expression changes between samples, it is imperative that all samples have to be similarly amplified (Day *et al.*, 2007). Taking into account the biases introduced in aRNA amplification, the total RNA labelling method appears as the best method when RNA sample is high enough. In the case of a low RNA sample, the aRNA labelling is the advised and the more reliable method (Li *et al*., 2004).

2.2.3. cDNA microarrays: advantages and limitations

cDNA microarrays can provide genome-scale information on gene expression patterns. In plant science, the majority of microarray studies involve the identification and characterization of genes responding to different types of stresses (salt, drought, cold stress, wounding, insect feeding, pathogen infection) (Aharoni and Vorst, 2001; Endo *et al*., 2002; Figueiredo *et al.*, 2008; Sebastiana *et al.*, 2009). Data from cDNA microarrays only provide information on the relative expression of the genes.

In a microarray experiment, spot intensity is expected to be related to the amount of mRNA and, consequently, to the amount of protein. However mRNA levels are not always directly related to protein levels (e.g. Bai *et al.*, 2010). The regulation of mRNA levels is only one aspect of biological control. Protein levels are also controlled at several post transcriptional steps, along with post translational modifications that regulate protein activities (Schulze and Downward, 2001; Gracey and Cossins, 2003). Microarray studies give insights on the transcriptional regulation but, extrapolation to protein levels should be carefully done. Another limitation of cDNA microarrays relies on the cross-hybridization of different transcripts from genes belonging to the same gene family, which influences expression levels (Murphy, 2002). Once statistically significant expression ratios are established it is important to confirm data obtained, by using alternative methods for gene expression validation. Currently, the techniques most frequently used are quantitative real-time PCR (qPCR), northern blotting, ribonuclease protection assay or *in situ* hybridization (Beně and Muckenthaler, 2003).

2.2.4. Microarrays analysis in natural populations

In ecology studies, the use of microarray technology has increased rapidly over the past few years. Studies have been performed in model species such as thale cress *Arabidopsis thaliana* (The Arabidopsis Initiative, 2000), baker's yeast *Saccharomyces cerevisiae* (Goffeau *et al.*, 2006) and fruit fly *Drosophila melanogaster* (Adams *et al.*,

2000), which are well suited to laboratory experiments and have fully sequenced genomes (Kammenga *et al*., 2007). Microarrays have been applied to the understanding of genetic mechanisms regarding species interactions, adaptations and outcomes of evolutionary processes. However there are few drawbacks: model species have a relatively simple life cycle and generally are opportunistic generalists, which limits their potential for ecological research (Kammenga *et al*., 2007). Microarray technology has great potential in providing a different and more efficient approach for identifying genes related to ecological and evolutionary success in non-model organisms (Ranz and Machado, 2006). Therefore, the number of field studies with non-model species is increasing. Since in-field experiments are prone to high environmental variation, in these studies, microarrays have been focused on differential gene expression associated to large effects, as in behavioural transitions in bees (Whitfield *et al*., 2003). A clear assumption of ecological microarray studies relies on the evolutionary pressure to which expression levels are subjected to. Thus, differences in expression levels among taxa are mostly or even certainly due to adaptation to different environments (Kammenga *et al.*, 2007). According to Whitehead and Crawford (2006), variation in gene expression within and among species is largely determined by natural selection. When working with natural populations it is important to account the sensitivity of gene expression levels to environmental variations. However, the effect of these variations can be overcome through the use of sufficient biological replicates obtained from in-field material to achieve statistical significance (Whitehead and Crawford, 2006). Besides the obvious drawbacks encountered when using biological data from natural populations, they often offer an advantage over most common used organisms since they are subject to natural selection, and biologically important changes in gene expression can be identified. According to Kammenga and colleagues (2007), this assumption is based on the postulate that variation in the expression of a gene which has evolved by selective pressures is biologically important since natural selection can only act on variation that causes phenotypic changes affecting the longevity, reproductive fitness or probability of survival (Oleksiak *et al.*, 2001).

2.2.5. Quantitative real-time PCR (qPCR)

Real-time polymerase chain reaction (qPCR) provides quantitative data analysis in molecular medicine, biotechnology, microbiology and molecular diagnostics and has become the method of choice for quantifying gene expression (Mocellin *et al*., 2003). Essentially, real-time PCR amplifies a specific target sequence in a sample whilst it monitors the amplification progress using fluorescent technology (Valasek and Repa,

2005). Quantitative real-time PCR amplifies the cDNA using PCR technique, detects and quantifies amplification products in real time (Nolan *et al.*, 2006). qPCR has become the technique of choice in gene expression analysis, namely in the confirmation of data obtained from microarray studies (Bustin *et al.*, 2005). Due to lack of consensus on how to perform and best interpret qPCR experiments, Bustin *et al.*(2009) proposed a set of guidelines that describe he minimum information necessary for evaluating qPCR data, named as MIQE (Minimum Information for Publication o Quantitative Real-Time PCR Experiments). In these guidelines, experimental variations (e.g. amount of starting material, RNA extraction and reverse transcription efficiencies) are evaluated when gene expression is measured in different samples, namely to guarantee technical reliability (Bustin *et al.*, 2005; Silver *et al*., 2006; Bustin *et al.*, 2009). Intersample variation between biological and technical replicates can interfere with data analysis thus normalization to one or more reference genes is crucial (VanGuilder *et al*., 2008). Accuracy of qPCR relies on the normalization performed with reference genes, based on the assumptions that their expression is: (*i*) similar among all samples in a given tissue under study; (*ii*) resistant to experimental conditions; and (*iii*) undergoes all steps of the qPCR with the same kinetics as the target gene (Silver *et al*., 2006; VanGuilder *et al*., 2008). Reference genes such as 18S rRNA, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), cyclophilin, α and β-tubulin and β-actin are commonly used as they are ubiquitously expressed in cells and tissues (VanGuilder *et al*., 2008). Although the use of reference genes is commonly accepted as the most appropriate normalization strategy (Huggett *et al.*, 2005), their utility must be experimentally validated for particular tissues or cell types and specific experimental designs (Bustin *et al.*, 2009). Real-time PCR reactions are carried out in a thermocycler that allows measurement of a fluorescent detector molecule, which decreases post-processing steps and minimizes experimental error (Nolan *et al.*, 2006; VanGuilder *et al*., 2008). Frequently used fluorescence based technologies, include: (i) probe sequences that fluoresce upon hydrolysis (TaqMan®, Applied Biosystems) or hybridization (LightCycler®, Roche), (ii) fluorescent hairpins or (iii) intercalating dyes (e.g. SYBR® Green).

SYBR Green I (SG), the most widely used intercalating dye in real-time PCR applications, is included in many commercially available kits at undisclosed concentrations (Giglio *et al*., 2003; Nolan *et al.*, 2006). Major advantages in using a SYBR Green I approach include: nonspecifity binding of SG to DNA; melt curve analysis required for confirming the generation of a unique/specific amplicon (Bustin *et al*., 2005) and relatively inexpensive when compared to other detection chemistries [e.g. FRET, iFRET, scorpions, molecular beacons, Taqman (Giglio *et al*., 2003).

Real-time PCR requires the design of specific oligonucleotides for each gene to be analysed. Therefore, a widely applied strategy is to identify potentially interesting

genes with microarray experiments and confirm those candidates qPCR analysis (Klok *et al.*, 2002; Gachon *et al.*, 2006; Figueiredo *et al.*, 2008; Fortes *et al.*, 2008; Sebastiana *et al.*, 2009). Indeed, the reliability of plant microarray experiments may sometimes be questioned, since plants display a high number of multigenic families (a distinctive feature of plant genomes as opposed to animals) and cross-hybridization between cDNA representative members of gene families on cDNA-based chips may lead to false interpretations (Gachon *et al.*, 2004).

Thus, qPCR has become the method of choice for high-throughput and accurate expression profiling of selected genes in gene expression analysis, due to its sensitivity, reproducibility and large dynamic range (Spurgeon *et al.*, 2008). Moreover, real-time PCR has become a routine and robust approach for measuring the expression of genes of interest, validating microarray experiments, and monitoring biomarkers (Figueiredo *et al.*, 2008; Fortes *et al.*, 2008; Sebastiana *et al.*, 2009; Fenart *et al.*, 2010).

2.3. Material and methods

2.3.1. Experimental design

Studies regarding pollination processes and dynamics in general and, particularly in *Ophrys*, are scarce. *O. sphegodes* pollination dynamics was first studied by Schiestl and co-workers (1997) and later by Schiestl and Ayasse (2001), with different time points tested (2-4 days after pollination and 1 or 2-4 days, respectively). Time points used for this study in *O. fusca* were 2 and 4 days after pollination (DAP) and were based on chemical dynamics reported earlier for *O. sphegodes* (Schiestl *et al.*, 1997; Schiestl and Ayasse, 2001). *O. fusca* labellum was selected for analysis due to its role on eliciting copulatory behaviour on male pollinators, namely by releasing compounds similar to those emitted by the sex pheromones of virgin females of the pollinator species and by tactile (i.e. trichomes) and visual (i.e. color, shape) cues (Schiestl *et al.*, 2000; Ascensão *et al.*, 2005; Schiestl, 2005). *Ophrys fusca* subsp. *fusca* (designated as *Ophrys fusca* thereafter) plants were selected at anthesis stage and sampled from a Portuguese natural occurring population. Hundred plants were selected in the field at anthesis stage (i.e. fully opened flowers, with pollinia and with roughly the same developmental stage). The experiment design achieved was as follows: 25 lips (corresponding to 5 biological replicates with 5 labella from 5 different individual plants each) were collected from unpollinated flowers with 2 days exposure and other 25 lips after 4 days exposure; 25 lips of pollinated flowers were collected 2 days after pollination and 25 lips 4 days after pollination (Sampling scheme is illustrated in Fig. 2.5).

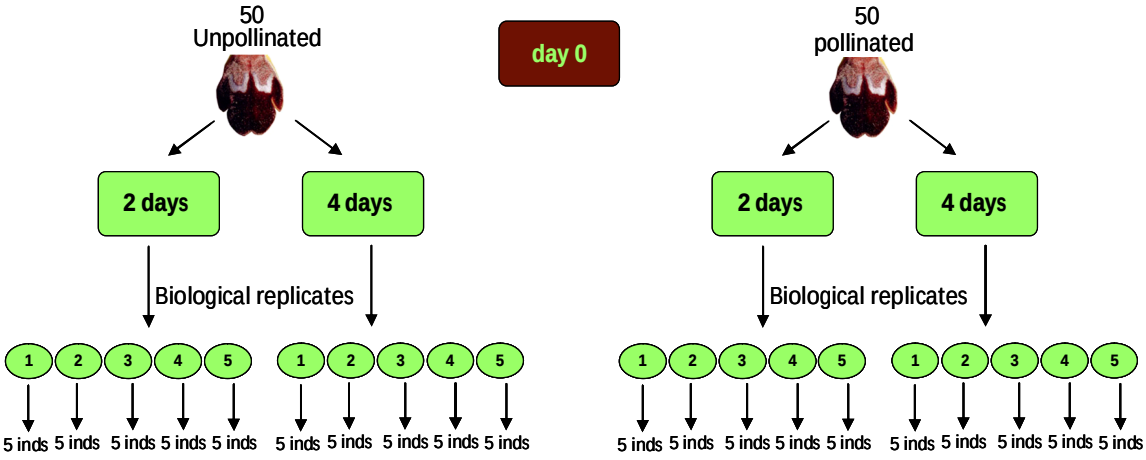

Figure 2.5- Experimental design scheme. **inds**- five different individuals

Plants were covered with a white and inert net disposable (mesh size, 2.0 mm x 2.0 mm) for preventing pollinator's visits either in pollinated or unpollinated flowers (Fig. 2.6). Cross- pollination was performed manually with a sterile plastic stick.

Figure 2.6- *Ophrys fusca* plants covered by the net disposables in the field. (**A**) Overview of the plants in the population under study; (**B**) Detail of one net disposable.

2.3.1.1. Labella collection for cDNA microarray studies

Two assays were performed in the field in order to guarantee sufficient material for transcriptomics approach. Labella were cutted at the edge of the stigmatic surface with a sterile scalpel and placed in 15 mL sterile tubes. Lips were immediately emerged in liquid nitrogen and maintained in dried ice (avoiding RNA degradation) until storage at -80 ºC.

2.3.2. Total RNA extraction

Total RNA from *Ophrys fusca* labella was extracted following the Hot Borate method (Wan and Wilkins, 1994) with minor modifications. This method was designed for isolating RNA of recalcitrant plant tissues with high levels of phenols, secondary metabolites and/or polysaccharides that influence RNA extraction. Briefly, 1 g of grinded plant material were added to 10 mL of extraction buffer (0.2 M sodium tetraborate, 30 mM EGTA, 1 % (w/v) SDS, 1 % (w/v) deoxycholic acid, 10 mM DTT, 1 % (w/v) Nonidet P-40, 2 % (w/v) PVP-40), previously heated to 90 ºC. After Proteinase K (0.3 mg mL^{-1}) (Invitrogen, Paisley, UK) addition and homogenization by vortexing, samples were incubated for 90 mins at 42 ºC and 150 rpm (KS 4000i control, IKA®). For protein precipitation, 160 mM KCl was added and samples were kept on ice for 1 hour. After

centrifugation at 9500 xg during 30 mins at 4 ºC (3K18, Sigma Laboratory Centrifuges), supernatant was collected and precipitated overnight with 2 M LiCl. The pellet was collected by a 30 mins centrifugation at 12000 xg (4 ºC), then washed three times with cooled 2 M LiCl and supernatant recovered by centrifugation for 15 mins at 12000 xg (4ºC) between each washing step. Pellet was resuspended in 10 mM Tris-HCl pH 7.5 After a 10 mins centrifugation at 12000 xg, supernatant was collected and polysaccharides were selectively precipitated with 0.2 M KAc pH 5.5 on ice for 30 mins. Supernatant was recovered by a 10 mins centrifugation at 12000 xg and 4 ºC. For RNA precipitation 2.5 x volumes of 100 % ethanol were added and samples were kept -80 ºC for 2 h. Later on, samples were then centrifuged for 30 mins at 9800 xg (4 ºC) and pellet washed with 4 mL of cooled 70 % ethanol. Supernatant was recovered by centrifugation at 9800 xg for 10 mins at 4 ºC and dried in the fume hood. Once dried, pellet was resuspended in 300 µL of ddH_2O depc and total RNA was precipitated overnight at -20 ºC with 0.1 x volume NaAc 3 M pH 6.0 and 2.5 x volumes of 100 % EtOH. Following, precipitated RNA was recovered by a 30 mins centrifugation at 14000 xg (4 ºC) and then washed with 70 % ethanol. Finally, Total RNA was dried and resuspended with depc ddH_2O. All solutions used were treated with DEPC (Sigma), overnight under agitation and then autoclaved for 30 mins (120 ºC, 1 atm). RNA quality was accessed by gel electrophoresis 1.2 % (w/v) with ethidium bromide (10 mg mL^{-1}, Sigma) and also by values of A260/A280 within 1.8-2.0 (Sambrook *et al*., 1989) and of A260/A230 above 2 (Wilkins and Smart, 1996). RNA concentration was determined using a UV-visible spectrophotometer (UV-1603, Shimadzu), according to the equation [total RNA] = 40 x (A260/A320) x dilution factor/ 1000, and also by using Nanodrop® ND-1000 (Thermo Fisher Scientific, USA). Total RNA was stored at -80 ºC until further use.

2.3.3. mRNA purification

Messenger RNA (mRNA) was purified from 150 µg and 100 µg of total RNA labella from unpollinated and pollinated flowers, respectively, using the FastTrack® MAG mRNA Isolation Kit (Invitrogen, Paisley, UK), according to the manufacturer instructions. Briefly, about 500 µL of preheated Binding buffer B6 at 65 ºC was added to RNA samples and equal volume of RNase-free water. After, 100 µL of FastTrack® MAG Beads were immediately added to samples. Through a 10 mins centrifugation at room temperature at 16000 xg, ribosomal RNA was removed by discarding supernatant. Messenger RNA (mRNA) was washed four times with 500 µL Wash Buffer W7. Both mRNAs purified were resuspended in 20 µL ddH_2O depc and analyzed by 1.2 % (w/v) agarose gel electrophoresis with ethidium bromide (10 mg/mL) to access mRNA quality.

2.3.4. Construction of cDNA libraries following Gateway® technology

Messenger RNA of labella from unpollinated and pollinated *Ophrys fusca* flowers was used to create two cDNA libraries: one library from unpollinated flower lips (including 2 and 4 days' time points) and another library with lips from pollinated ones (2 and 4 days' time points). Complementary DNA libraries were constructed using CloneMiner™ cDNA library Construction Kit (Invitrogen, Paisley, UK), according to manufacturer instructions (Karnaukhova *et al.*, 2003). This technique combines the performance of Superscript™ II reverse transcriptase with the Gateway® technology, without the use of traditional restriction enzyme cloning methods. Due to limitations in plant material, approximately 3 µg of mRNA was used for performing unpollinated cDNA library, while for pollinated library construction 2 µg were used.

2.3.5. Library characterization

Redundancy in each library was evaluated by randomly sequencing 24 and 23 cDNA entry clones from unpollinated and pollinated libraries, respectively, with M13 forward primer (5'-TGCAACATTTTGCTGCC-3') of the CloneMiner ™ cDNA library Construction Kit (Invitrogen, Paisley, UK). Briefly, bacteria containing the selected clone were grown overnight in liquid LB media supplemented with kanamycine (50 µg mL-1) at 37 ºC and 200 rpm (KS 4000i control, IKA®). Plasmid DNA was extracted according Birnboim and Doly (1979) and resuspended in 20 µL deionised water. Contaminant RNA was removed through an incubation step at 37 ºC for 30 mins, with 2 µL of RNase (10 mg mL^{-1}). Plasmid DNA quantity and quality was accessed in a 1 % (w/v) agarose gel by using λ DNA (Fermentas) of known concentration.

Average insert size and percentage of recombinants for each library were determined through digestion with *Bsp*1407I (Fermentas). Digested clones were analyzed by 1 % (w/v) TBE agarose gel electrophoresis with ethidium bromide (10 mg/mL) to access clone insert size and percentage of recombinant clones. After, plasmid DNA was sequenced as described below.

2.3.6. Sequencing and sequence analysis

About 100-200 ηg of plasmid DNA were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit components (Applied Biosystems, Foster City, USA), according to manufacturer instructions. Briefly, in a 10 µL reaction mixture the following components were added: 1 µL de ABI mix (Applied Biosystems), 4 µL de 2.5 X

Sequencing Buffer (200 mM Tris-HCl, 5 mM $MgCl_2$, pH 9.0; Applied Biosystems), 0.1 µM of M13 forward primer (5'-TGCAACATTTTGCTGCC-3') and 0.6 µL of DMSO (Sigma-Aldrich). The annealing temperature of the sequencing cycle was adapted to M13 forward optimal primer temperature (55 ºC) and an extra hot start step was performed. For the purification of the PCR products, eighty microliter of 60 % ethanol was added to each sample in order to precipitate the DNA followed by centrifugation for 30 mins at 16000 xg. The pellet was washed with 70 % ethanol, dried at room temperature and resuspended in 30 µL formamide (Applied Biosystems). DNA was denatured at 94 ºC for 3 mins and placed on ice for 10 mins. Sequencing was performed on an ABI Prism 310 Genetic Analyser (Applied Biosystems, USA) as described by Sanger *et al.* (1997). Assemblage of ESTs into contigs, representing unigenes, was performed on SeqMan II 5.0 software (DNAStar) (90 % similarity over 40 nt length). Annotation of both contigs and singletons were assigned by using BLASTx (Altschul *et al.*, 1997) on NCBI non redundant proteins and UniRef50 (Suzek *et al.*, 2007) annotated protein databases, setting an *E*-value of 10^{-5} as threshold.

2.3.7. cDNA Microarray construction

2.3.7.1. Clone selection and purification

Library clones were randomly selected by PCR screening on both cDNA libraries. cDNAs clones were PCR amplified in 100 µL-volume reactions in 96-well reaction plates (Bioplastics, The Netherlands). Each reaction contained PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 2 mM $MgCl_2$, 0.13 mM dNTPs, 10 pmol of each M13 forward (5'-GTAAAACGACGGCCAG-3') and M13 reverse (5'-CAGGAAACAGCTATGAC-3') primers and 2 U Taq DNA polymerase (5 U μL^{-1}). Five microliters of bacterial suspension were used as template and lysis was carried out at 98ºC for 5 mins. Clone amplification was performed in a thermocycler (2720 Thermal Cycler, Applied Biosystems) using the following program: initial denaturation for 3 mins at 96 ºC, followed by 25 cycles of 30 s at 96 ºC, 30 s at 56 ºC, 3 mins at 72 ºC and a final extension step at 72 ºC for 7 mins. The amplicon quality, size and quantity were determined by electrophoresis (1.2 % agarose TBE gel) by using a DNA molecular marker (MtrnL: 1050 bp, 750 bp, 350 bp). A total of 3384 cDNA clones (1692 clones from each cDNA library) were selected based on the presence of a unique band with more than 500 bp. Bacterial stocks were kept at -80 ºC after adding up freezing media (60 % glycerol + 40 % LB). In order to promote an efficient cDNA adhesion to the microarray glass slides in the printing step, non-incorporated nucleotides and primers were removed by vacuum filtration on Multiscreen® $PCR_{\mu}96$ Plates (Millipore, Bedford, MA, USA) according to manufacturer's

instructions. Purified PCR products were resuspended in 40 µL of deionised water, dissolved by shaking for 30 mins at 100 rpm (Thermomixer Compact, Eppendorf), and transferred to V bottom printing plates (Greiner- Corning, NY, USA). Plates were kept at -20 ºC until further use.

2.3.7.2. Glass slides preparation

Two different surface coated slides were tested: homemade poly-L-lysine and commercially available UltraGAPS™ (Corning, USA) with amino silane surface. Glass slides (Industrial quality, Germany) were coated with poly-L-lysine. Briefly, slides were kept on washing solution (200 g NaOH pellets, 1200 mL ethanol 100 %) overnight with agitation. The slides were washed five times on filtered (0,45 µm filter, Millipore) deionised water and kept on a poly-L-lysine (70 mL poly-L-lysine, 70 mL PBS in 560 mL water solution) for 30 mins. Slides were washed again with filtered deionised water and dried by centrifugation at 100 xg for 10 mins at room temperature (3K18, Sigma Laboratory Centrifuges). Coated slides were kept on a dry and light-protected environment until further use.

2.3.7.3. Printing parameters

Microarray printing was performed with a VersArray ChipWriter Compact System (Biorad) with 12 printing pins (Telechem International, Sunnyvale, CA, USA). The following parameters were established: number of printing plates (36), number of clones to be printed (3384), number of replicated spots (2), distance between spots (300 µm) and number of slides (24). The printing scheme was 12 subgrids, each one with 24 columns and 24 lines, repeated twice. Printing conditions, such as temperature and relative humidity, were tested.

2.3.7.4. Microarray printing

The printing plates containing the cDNA clones were air dried and cDNA was resuspended on 10 µL of printing buffer (50 % DMSO (Sigma-Aldrich) and 0.4 x SSC (Invitrogen)) under agitation at 150 rpm for 90 min at room temperature (KS 4000i control, IKA®). Negative controls were added to the plates, namely printing buffer and a yeast gene, Yap1[2]. Additionally, positive controls were added to the printing plates: cDNAs sequenced from the two libraries (positive controls for hybridization process).

[2] Yap1 is a yeast transcriptional activator required for oxidative stress tolerance.

Prior to microarray printing, plates were shaken for one hour at 100 rpm at room temperature (KS 4000i control, IKA®). After optimization of printing parameters, 24 coated slides were cleaned using a pressurised air- duster (1,1,1,2-tetrafluoroethane, Dust-Pro, Sigma). Temperature and relative humidity inside the printing chamber varied between 21.4 ºC and 22.0 ºC and between 48 % and 53 %, respectively. After printing, slides were kept inside the printing chamber overnight, so that spots could be fully dried. Binding of DNA to the coated surface of slides (cross-linking) was achieved by keeping the slides for 5 mins at 80 ºC and 3 mins under exposure to 70 mJ of U.V. light. Slides were set on a dry and light-protected environment, until further use.

2.3.7.5. Gel star staining

Gel star staining was used to evaluate printing quality. GelStar® nucleic acid gel stain can be used for sensitive fluorescent detection of double-stranded (ds) and single-stranded (ss) DNAs, oligonucleotides and RNA in gels. Slides were kept in a 1/10000 TBE diluted Gel Star® solution (FMC, Rockland, ME) for 6 mins, under agitation (KS 4000i control, IKA®), followed by three washes of 15 mins each on 1x TBE pH 8.0 and one washing step by immersion in deionised water. Slides were dried by centrifugation (3K18, Sigma Laboratory Centrifuges) at 260 xg for 5 mins, at room temperature for UltraGAPS™ Coated Slides (Corning, USA) or 260 xg for 10 mins for Poly-L-lysine slides (homemade slides). Image was acquired at 532 nm (Cy3 channel) using a VersArray ChipReader® vs 3.1. Build 1.68 (BioRad, USA) scanner.

2.3.8. RNA labelling and microarray hybridization

2.3.8.1. RNA amplification

RNA amplification was achieved for all biological replicates of unpollinated and pollinated samples, for both 2 days and 4 days' time points, by using 1 µg of total RNA with the MessageAmp™ II aRNA Amplification Kit (Ambion, Applied Biosystems), following manufacturer instructions. Briefly, *In vitro* transcription was performed in 20 µL-volume reactions and antisense RNA (aRNA) was synthesized through incubation at 37 ºC for 14 h. After purification, aRNA was eluted in 100 µL of depc water and kept at -80 ºC until further use. RNA concentration and quality was evaluated by using a Nanodrop® ND-1000 (Thermo Fisher Scientific, USA), through values of A260/A280 within 1.8-2.0 (Sambrook *et al*., 1989) as well as values of A260/A230 above 2 (Wilkins and

Smart, 1996). RNA quality was also accessed through gel electrophoresis 1.2 % (w/v) with ethidium bromide (10 mg mL^{-1}, Sigma).

2.3.8.2. Target labelling

Two micrograms of aRNA from each biological replicate was reverse transcribed and labelled following RPN 5660 CyScribe™ cDNA Post Labelling Kit (Amersham, GE Healthcare). Labelled cDNAs were purified with the CyScribe™ GFX™ purification kit (Amersham, GE Healthcare), according to manufacturer instructions. Unpollinated cDNA was labelled with Cy3-dUTP (control) and pollinated cDNA was labelled with Cy5-dUTP (test). Labelled cDNAs were kept at -20 ºC until further use.

2.3.8.3. Pre-hybridization washes (slide blocking)

This step has the purpose of blocking the unused surface of the slide and removing loosely bound probe DNA from coated slides. Slides blocking was performed by keeping the slides in 1 % BSA (fraction V, Sigma), 5 X SSC (Invitrogen, Paisley, UK) and 0.1 % SDS solution for 30 mins at 50 ºC. The blocking solution was previously homogenized for 30 mins at 50 ºC. After incubation in the blocking solution, slides were washed in 0.1 % SDS for 1 min, under low agitation (KS 4000i control, IKA®). Following a washing step with 2 X SSC for 3 mins with agitation, a 0.2 X SSC solution for 3 mins with 100 rpm agitation was carried out. All washing steps were performed at room temperature. The slides were dried by centrifugation at 260 xg during 5 mins at room temperature (3K18, Sigma Laboratory Centrifuge) and with a pressurised air-duster (1,1,1,2-tetrafluoroethane, Dust-Pro, Sigma). All the solutions were prepared with filtered (0.45 µm, Millipore) and with sterile deionised water. Washing steps was performed in glass containers (VWR Scientific).

2.3.8.4. Hybridization conditions

Labelled cDNA (40 µL) was added to the hybridization mixture containing: 1.6 µL of poly-A DNA (10 mg mL^{-1}, Sigma), 1.6 µL salmon sperm DNA (5 mg mL^{-1}, Invitrogen), 1.6 µL of 50 X *Denhardt´*s solution (1 % Ficoll (type 400), 1 % PVP-40, 1 % BSA), 8.45 µL of 20X SSC and 1.69 µL of 10 % SDS. The hybridization mixture was denatured at 97 ºC for 2 mins, and cooled to 60 ºC. One microliter of 10 X DIG blocking buffer (Roche Applied Science, Mannheim, Germany) was added and the mixture was centrifuged at room temperature for 30 s at 16000 xg (Biofuge *pico*, Heraeus) to eliminate air bubbles.

Application of pressurised air- duster (Dust Pro, Sigma) on the slide as well as on the cover-slip was performed for dust particles removal. Hybridization solution was heated at 60 ºC, applied over the array and covered with a cover-slip (60 mm x 24 mm) (Hybri-slips, Sigma). Slides were placed in a hybridization chamber (ArrayIt Hybridization Cassette, Telechem) and 10 µL of H_2O was added in each corner of the chamber before sealing. The hybridization chamber was kept in a water bath at 60 ºC for 17 h inside an incubator (Memmert, Celsius).

A total of 14 hybridizations were achieved. For each time point, seven hybridizations were carried out matching five biological replicates and two technical replicates, i.e. repetition of two different biological replicates with the same labelling chemistry.

2.3.8.5. Post- hybridization washes (background removal)

Following hybridization, slides were put in a 0.5 X SSC and 0.1 % SDS solution for coverslip removal. After, slides were washed with 0.5 X SSC and 0.1 % SDS for 15 mins with agitation at 60 rpm, at room temperature (KS 4000i control, IKA®). Second wash step was performed with a 0.5 X SSC and 0.001 % SDS solution, and kept for 15 mins under agitation at 60 rpm at room temperature. Two more washes in 0.06 X SSC solution were carried out (2 mins and 1 min, respectively) at room temperature with agitation at 60 rpm. Slide washing was performed in the dark, and solutions were prepared with filtered (0.45 µm, Millipore) and sterile deionised water. Finally, slides were dried by centrifugation at 260 xg for 5 mins at room temperature (3K18, Sigma Laboratory Centrifuge) and with a pressurised air- duster (1,1,1,2-tetrafluoroethane, Dust-Pro, Sigma). The dried microarray was stored in a light-free environment and kept at room temperature until scanning. Scanning was carried out as soon as possible since the fluorescent dyes degrade over time.

2.3.9. Image and data collection

Microarray image was acquired with VersArray ChipReader® vs 3.1. Build 1.68 (BioRad, USA) scanner. The slides were scanned simultaneously at 535 nm (Cy3) and 635 nm (Cy5) with a 5 km resolution and 100 % laser power. Excitation light induces fluorescence: Cy3 emits light in the green part of the visible spectrum (573- 613 nm) and Cy5 emits light in the red part of the visible spectrum (672- 712 nm), the emitted light is then detected by the scanner. Other parameters, like laser sensitivity, were manually adjusted to achieve a similar intensity in both channels (evaluated by analysis of the signal intensity histogram). The images acquired were processed with the VersArray

Analyser™ software vs 4.5.1.46 (BioRad, USA). This software creates a grid over the spots and uses an algorithm to identify each spot and to analyse both spot and background intensities. Both images from one microarray slide are opened with an image-analysis software and a grid is created on both. The grid defines the spot area from which the signal is extracted by the image-analysis software. Quality of the spots was accessed and data extracted were flagged and excluded from further analysis if spots fail to fulfil the required conditions: background was calculated as the trimmed mean of pixel intensity on spots local corners; low intensity[3] signal spots (trimmed mean of raw intensity/ trimmed mean of background < 1.4); uneven background[4] (trimmed mean of raw intensity/ standard deviation of background < 2); uneven spots[5] (trimmed mean of raw intensity/ standard deviation of raw intensity < 1) and not validated spots or flags (e.g. saturated spots, spots failed to print) were removed from analysis before normalization. Also, spots with trimmed mean raw intensity in Cy3 and/or Cy5 channel above 50 000 counts considered saturated (Shena, 2002), were flagged and excluded from analysis. The output of the software is a Microsoft Excel® table with the following parameters: spot number; grid; column; line; position (grid:line:column); trimmed mean of the spot intensity in the Cy5 channel; trimmed mean of the background intensity in the Cy5 channel; trimmed mean of the spot intensity in the Cy3 channel; trimmed mean of the background intensity in the Cy3 channel; and flags. Each line of the table corresponds to one of the spots in the microarray.

2.3.10. Statistical analysis

2.3.10.1. Data normalization and processing

Data were normalized with *Gene Expression Profile Analysis Suite* (GEPAS) software version 3.1 (Herrero *et al.*, 2003a) and *Diagnosis and Normalization for Microarray Data* (DNMAD) (Vaquerizas *et al.*, 2004) software [http://gepas.bioinfo.cnio.es]. The print-tip LOWESS method (Yang *et al.* 2002) was used for within-slide normalization considering background subtraction (Appendix II). Normalized data, which were then processed with the *Expression Data Preprocessor* (Herrero *et al.*, 2003b) software, were selected considering: filter missing values with 70 % of minimum percentage of existing values and background correction.

[3] Spots that have signal lower than 1.4 times its local background.
[4] Also known as signal-to-noise ratio. Spots that have dust particles or shifted neighbour spot that might affect signal intensity.
[5] Smeared and doughnut shaped spots.

2.3.10.2. Identification of differentially expressed genes

Differentially expressed genes were statistically identified with the rank product (RP) method (Breitling *et al.*, 2004) runned in Bioconductor using the RanKProd package version 2.20.0 (http://www.bioconductor.org/packages/release/bioc/html/RankProd.html) with 1000 balanced permutations. Genes were considered differentially expressed when presenting a false positive rate (FPR) of 0.05 (5 % of chance in having false positive values) and 1.3 fold change cut off was also set as threshold.

2.3.11. Sequencing and sequence analysis

Clones considered differentially expressed were sequenced and analysed as described previously (see section 2.3.6. Sequencing and sequence analysis). Gene ontology (GO) classification was accomplished by accessing GO annotation files resulting from the obtained UniRef50 accession homologies on Unref50 database. The GO terms were selected at the parental class Biological process (first level). Since several transcripts had no GO term retrieval, mainly due to the low number of unigenes analysed in our study when compared to high throughput 454 sequencing programs (Bettencourt *et al.*, 2010; Parchman *et al.*, 2010), transcripts were additionally grouped into functional categories according to MIPS Functional Catalogue Database classification (Ruepp *et al.*, 2004). The Functional Catalogue (FunCat) is a hierarchically structured, organism independent, flexible and scalable controlled classification system enabling the functional description of proteins from any organism (Ruepp *et al*., 2004).

2.3.12. Quantitative real time PCR (qPCR)

2.3.12.1. Sample preparation

Quantification by real-time PCR was performed using the same RNA samples used in microarray hybridizations (see section 2.3.8.1. RNA amplification). DNAse treatment was done with the Turbo DNA- *free* kit (Ambion, Inc.), following manufacturer's instructions. Synthesis of cDNA from aRNA was performed using RevertAid® H Minus Reverse Transcriptase (Fermentas, Ontario, Canada) according to manufacturer's instructions. Briefly, a mixture of 2µg of aRNA, together with 3 µL of N9 random primers (GE Healthcare) and DEPC-treated deionized water to a total volume of 13 µL, was heated at 70 ºC for 10 mins and, after, immediately chilled on ice. Four microliters of 5 x Reverse Transcriptase Buffer (Fermentas) were added, together with 2 µL of 10 mM dNTPs, 0.5 µL of Ribolock (Fermentas) ribonuclease inhibitor. This mixture

was incubated at 25 ºC for 5 mins, after which 1 µL (200 U) of RevertAid® H Minus Reverse Transcriptase (Fermentas) was added. Reverse transcription was allowed to occur at 25ºC for 10 minus followed by 1 h at 42 ºC and stopped by heating the samples at 70 ºC for 10 mins.

2.3.12.2. Oligonucleotide design

Primer sequences were designed using Primer Express version 3.0 (Applied Biosystems, USA). Quality of the designed oligonucleotides was evaluated using the following software's: Primer Express version 3.0 (Applied Biosystems), PrimerSelect (Lasergene, DNAStar Inc., USA) and DNA Calculator (Sigma-Genosys, Sigma-Aldrich Inc.). Oligonucleotide sequences, annealing and melting temperatures, and amplicon sizes of validated genes are presented in Table 2.1. Reference genes used to normalize the expression of target genes encode for: 40S ribosomal protein S10-like (Acc.No. HO849881); oligopeptidase, putative (Acc.No. HO850106) and 4-α-glucanotransferase (Acc.No. HO849990).

Table 2.1. Oligonucleotide sequence, annealing (Ta) and melting (Tm) temperatures and amplicon size (bp) used for transcript quantification reactions in the StepOne™ Real Time PCR Systems (Applied Biosystems). F- forward; R- reverse.

Target sequence	GenBank ID	Primer sequence	Ta /Tm (ºC)	Length (bp)
40S ribosomal protein S10-like	HO849881	F: TGATGCAGAGCTTCAAGTCG R: TTCGCGGACTTTTTCAGAGT	58/ 81	154
Oligopeptidase, putative	HO850106	F: GTATGCCCTCACGCCAGTTC R:ATAGATAGACATTGGCTGTTCGGATA	58/ 79	110
4-α-glucanotransferase	HO849990	F: GGAGTTGGGATTGATCGGTCTA R: GCATGGTGGAGCAGTCATGA	58/ 79	130
Pathogenesis-related protein 10c	HO849917	F: AATCTCGGCCCCAAACTCCT R: ACTTCCCTTCGGCTCCACC	60/ 83	245
Class III chitinase	HO850071	F: ACCCAAACCTCTCCTCTCTATCCT R: TCCCTTCGTTCCCGTTCTG	61/ 82	101
Chaperone	HO849934	F: TTTCGCCGACACCAACATC R: ACCTCTACCTTGACCTCCTCTTTCT	60/ 82	100
Catalytic/ glucosylceramidase	HO850028	F: GGGCGGTGTTGAATGAAGAT R: TTCACTTCGTTCCAGGGATCA	56/ 77	124
Stearoyl-acyl-carrier desaturase (SAD1)	HO849909	F: TGGAAAACTGGGCTGAAAA R: GTCTCATCTCTAACCCCGTC	56/ 81	247
Metallothionein protein type 3	HO850065	F: AGTGAATATGATGTTGAGA R: ACCAAACAGCACAATCTCACA	54/ 81	223
ACP-stearoyl desaturases (SAD2)	HO849908	F: GCACTACTTTCATCCCCATT R: TCTCCCTCACCGCATCCCC	58/ 85	250

2.3.12.3. Relative quantification through qPCR: experimental settings

Seven genes (5 % of the differentially expressed unigenes) were evaluated by qPCR (StepOne™ Real Time PCR, Applied Biosystems), following MIQE Guidelines (Bustin *et al.*, 2009, 2010). qPCR reactions were set up with the components supplied in the MaximaTM SYBR Green qPCR Master Mix (2x) kit (Fermentas, Ontario, Canada). A final concentration of 2.5 mM MgCl2 and 0.2 µM of each primer were used on 25 µL volume reactions, together with cDNA as template. Five biological replicates with two technical replicate reactions were used for each condition and time point. Three reference genes (40S ribosomal protein S10-like- HO849881; oligopeptidase, putative- HO850106- and 4-α-glucanotransferase- HO849990) were selected based on the RP statistics results. Thermal cycling for all genes was initiated with a denaturation step at 95°C for 10mins, followed by 45 cycles of denaturation at 95 °C for 15 s and annealing temperatures for 30 s. Each set of reactions included template and no template controls. Relative quantification was performed using the relative standard curve method (Applied Biosystems, 2008). Standard curves were generated, for both targets of interest and reference genes, from a ten-fold dilution series (10^{0} to 10^{-6}) starting with 1 ng concentration of the corresponding cDNA clone. Standard curves were plotted with Cq (quantification cycle) values generated by cDNA clone serial dilutions against the logarithm concentration of input template cDNA (Appendix III). The specificity of the qPCR amplicons was confirmed by melting curve analysis (Appendix IV) and gel electrophoresis (data not shown). For each target gene, efficiency of qPCR reaction was calculated from the slope of the standard curve (Appendix IV). Data were recorded with StepOne™ Software ver. 2.1. (Applied Biosystems) and final concentrations were calculated in Microsoft Excel® datasheets. First, for reference genes, median values were calculated for the five biological replicates in each time point (2 DAP and 4 DAP). Normalization was performed by using the median values of the 3 references genes against the 5 biological replicates of each of the evaluated genes. After, fold changes were calculated by dividing the median normalized quantity of pollinated by unpollinated samples at each time point. Mean absolute deviation (MAD) was calculated.

2.4. Results and discussion

2.4.1. Characterization of cDNA libraries

Total RNA yields ranged from 2µg- 150µg, per biological replicate. The yield was higher in unpollinated labella than in pollinated ones. Among time points, samples from 2 days after pollination gave a yield higher than samples from 4 days. Differences in total RNA yields may indicate decay in the RNA transcripts related to the pollination-enhanced mechanisms, i.e. petal senescence (O'Neill, 1997). For mRNA purification, aliquots of total RNA from the 10 replicates (5 replicates from 2 DAP and 5 replicates from 4 DAP) of unpollinated flowers were combined. The same procedure was followed with replicates of pollinated flowers. Poly (A)+ RNA was purified from 150 µg and 100 µg of total RNA in labella of unpollinated and pollinated flowers, respectively (Fig. 2.7).

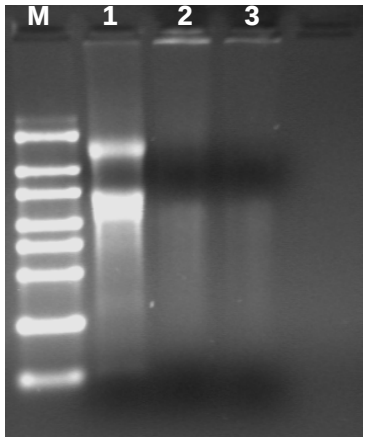

Figure 2.7- Gel electrophoresis of mRNA from unpollinated and pollinated *Ophrys fusca* labella. **M**- RiboRuler™ RNA ladder high range (Fermentas), lane **1**- total RNA from *Ophrys fusca*, lane **2**- mRNA pool from unpollinated *Ophrys fusca* labella, lane **3**- mRNA pool from pollinated *Ophrys fusca*.

cDNA libraries titer was calculated by serial dilutions (10^{-2}, 10^{-3}, 10^{-4}) of sample aliquots resulting from the transformation step. About 100 µL of each dilution was plated in LB containing the appropriate antibiotic. Through the titer of each plate, the average titer of the entire cDNA library was obtained. Finally, total number of **cfu** was calculated by using the ultimate volume of the library (12 mL) (Table 2.2).

Table 2.2. Titer determination of the two cDNA libraries constructed.

	Serial dilution titer (cfu/mL)	Average titer (cfu/mL)	Total cfu
Unpollinated library	**10^{-2}-** 6.6×10^5 **10^{-3}-** 7.3×10^5 **10^{-4}-** 9.0×10^5	7.6×10^5	9.2×10^6
Pollinated library	**10^{-2}-** 3.3×10^6 **10^{-3}-** 4.6×10^6 **10^{-4}-** 4.2×10^6	4.0×10^6	4.8×10^7

cDNA libraries constructed have an expected total cfu as established in CloneMiner ™ cDNA library Construction Kit (Invitrogen, Paisley, UK). Differences in final titers between constructed libraries may be related to the input of mRNA used, since quantification is difficult and sometimes inaccurate (mainly due to the low concentration). Qualitative characterization of the two libraries was carried out by clone restriction analysis and redundancy determined by sequencing. Clone restriction analysis allowed measurement of insert clone size (Table 2.3).

Table 2.3. Clone's digestion results for both unpollinated and pollinated libraries, with insert size information (kb) and recombinant's percentage.

	Analysed clones	Recombinants (%)	Average Insert Size (kb)	Insert Size Range (kb)
Unpollinated library	24	93	1.020	0.4-2.5
Pollinated library	23	100	1.5	0.6-5.1

Random sequencing of the cDNA libraries was done in order to evaluate redundancy within and between cDNA entry clones generated by the two constructed libraries. Forty-seven cDNA clones were randomly sequenced: 24 from unpollinated library (Table 2.4) and 23 from pollinated one (Table 2.5).

Random sequencing of the cDNA entry clones of both libraries allowed the identification of 34 unique sequences presenting homology with databases, 17 in unpollinated library and 17 in pollinated library. Unigenes identified can be included in different functional categories, mainly: metabolism [Cinnamate 4-hydroxylase (*Allium sativum*), 4E-19; Long-chain-fatty-acid CoA ligase, putative (*Ricinus communis*), 1E-5; Gl1 protein (*Zea mays*), 6E-50]; energy [Oxygen-evolving enhancer protein 1, chloroplastic (*Ricinus communis*), 6E-27; ATP synthase subunit D (*Ricinus communis*), 3E-33]; transcription [MADS box protein DOMADS3 (*Dendrobium* grex Madame Thong-In),

2E-57]; cell fate [Aspartic proteinase 12 (Fagopyrum esculentum), 1E-25; CK2 alpha subunit (Nicotiana tabacum), 3E-16; serine carboxypeptidase (*Matricaria chamomilla*), 4E-57; γ -expansin natriuretic peptide (*Alnus glutinosa*), 3E-41]; cell rescue, defense and virulence [Peptidyl-prolyl cis-trans isomerase (*Vanda* hybrid cultivar), 6E-72; Abscisic acid stress ripening protein homolog (*Prunus mume*), 3E-13]; protein fate [polyubiquitin (*Pinus sylvestris*), 2E-85]; and protein synthesis [Translation elongation factor 2 (*Andalucia godoyi*), 1E-42; 40S ribosomal protein S10 (*Oryza sativa* subsp. *japonica*), 3E-47]. Nine of the sequenced transcripts did not present any homology with databases.

Table 2.4. Unigenes resulting from randomly sequenced cDNA clones from unpollinated cDNA library from *Ophrys fusca* labella. GenBank Acc.No., Predicted function (species) retrieved by BLASTx from Uniref50, GO term (1st level- Biological Process), E-value and Clone/Contig information are presented.

GenBank Acc.No.	Predicted function (species)	GO term	E-value	Clone/Contig
Unpollinated library cDNA clones				
HO849876	Putative uncharacterized protein (*Vitis vinifera*)		4E-18	*Of*up#1
HO849880	Non-specific lipid-transfer protein (*Gymnadenia conopsea*)	GO:0006869	3E-27	*Of*ctgup#2(**2**)
HO849879	no homology			*Of*up#6
HO849880	Regulator of ribonuclease activity A (*Zea mays*)	GO:0051252	7E-21	*Of*up#11
HO849881	40S ribosomal protein S10 (*Oryza sativa* subsp. *japonica*)		3E-47	*Of*up#19
HO849882	ATP synthase subunit D, putative (*Ricinus communis*)	GO:0015991	9E-37	*Of*up#20
HO849897	no homology			*Of*up#49
HO849898	no homology			*Of*up#52
HO849899	Aluminum-induced protein (*Elaeis guineensis* var. *tenera*)		2E-23	*Of*up#53
HO849982	Peptidyl-prolyl cis-trans isomerase (*Vanda* hybrid cultivar)	GO:0006457	6E-72	*Of*up2353
HO849883	Protochlorophyllide reductase, chloroplastic (*Cucumis sativus*)	GO:0015979	9E-54	*Of*up#21
HO849886	Protein tipD, putative (*Ricinus communis*)		2E-21	*Of*up#29
HO849885	Putative uncharacterized protein (*Volvox carteri* f. *nagariensis*)		1E-15	*Of*up#27
HO849887	no homology			*Of*up#32
HO849888	Predicted protein (*Populus trichocarpa*)	GO:0006468	6E-14	*Of*up#34
HO849889	Cinnamate 4-hydroxylase (*Allium sativum*)	GO:0055114	4E-19	*Of*up#35
HO849890	Oxygen-evolving enhancer protein 3 (*Hyacinthus orientalis*)	GO:0015979	1E-17	*Of*up#36
HO849891	no homology			*Of*up#37
HO849896	no homology			*Of*up#47
HO849892	Translation elongation factor 2 (*Andalucia godoyi*)	GO:0006414	1E-42	*Of*up#39
HO849893	Oxygen-evolving enhancer protein 1, chloroplastic (*Ricinus communis*)	GO:0015979	6E-27	*Of*up#41
HO849894	Putative uncharacterized protein (*Vitis vinifera*)		6E-25	*Of*up#44
HO849895	Oxygen-evolving enhancer protein 1, chloroplastic (*Solanum tuberosum*)	GO:0015979	3E-41	*Of*up#46

(**n**)- **n** represents the number sequences contained in the corresponding contig.

Table 2.5. Unigenes resulting from randomly sequenced cDNA clones from pollinated cDNA library from *Ophrys fusca* labella. GenBank Acc.No., Predicted function (species) retrieved by BLASTx from Uniref50, GO term (1st level- Biological Process), E-value and Clone/Contig information are presented.

GenBank Acc.No.	Predicted function (species)	GO term	E-value	Clone/Contig
Pollinated library cDNA clones				
HO849994	Sterol desaturase, putative (*Ricinus communis*)	GO:0006633	1E-35	*Of*ctg459(**2**)
HO849995	CYP71D48v1 (*Nicotiana tabacum*)	GO:0055114	1E-5	*Of*p2624
HO849996	Cytochrome P450 protein (*Vanda* hybrid cultivar)	GO:0055114	1E-43	*Of*p#13
HO850015	no homology			*Of*p#5
HO850007	Abscisic acid stress ripening protein homolog (*Prunus mume*)	GO:0006950	3E-13	*Of*p#3
HO849997	Putative GDSL-like lipase/acylhydrolase (*Oryza sativa* subsp. *japonica*)	GO:0006629	2E-37	*Of*p1250
HO849999	Polyubiquitin (*Pinus sylvestris*)		2E-85	*Of*ctgp#9(**2**)
HO850000	Aspartic proteinase 12 (*Fagopyrum esculentum*)	GO:0006508	1E-25	*Of*p#12
HO850001	Long-chain-fatty-acid CoA ligase, putative (*Ricinus communis*)	GO:0008152	1E-5	*Of*p#14
HO850143	Ribulose-phosphate 3-epimerase (*Glycine max*)	GO:0008152	6E-55	*Of*p#23
HO850002	no homology			*Of*p#18
HO850003	MADS box protein DOMADS3 (*Dendrobium* grex Madame Thong-In)	GO:0006355	2E-57	*Of*p#19
HO850014	no homology			*Of*p836
HO850005	Snakin-like cysteine rich protein (*Phaseolus vulgaris*)		3E-24	*Of*p#24
HO850006	Endoxyloglucan transferase (*Arabidopsis lyrata* subsp. *lyrata*)	GO:0006073	3E-51	*Of*p#25
HO850010	Gl1 protein (*Zea mays*)	GO:0006633	6E-50	*Of*p#17
HO850008	CK2 alpha subunit (*Nicotiana tabacum*)	GO:0006468	3E-16	*Of*p#4
HO850009	Beta-ketoacyl-ACP synthase II (*Elaeis oleifera*)	GO:0008152	1E-13	*Of*p#8
HO850012	Gamma-expansin natriuretic peptide (*Alnus glutinosa*)		7E-41	*Of*ctg453(**2**)
HO850013	Serine carboxypeptidase (*Matricaria chamomilla*)	GO:0006508	4E-57	*Of*p743

(**n**)- **n** represents the number sequences contained in the corresponding contig.

Redundancy in unpollinated cDNA library was 4 % whereas in pollinated library about 13 % was encountered. No chimeric clones were detected. After clone sequencing, no redundancy was found between unpollinated and pollinated cDNA libraries. The results here presented, suggest that both constructed libraries are well represented with high titer, wide insert size range and with low redundancy.

2.4.2. cDNA microarrays

2.4.2.1. *Ophrys fusca* chip construction and hybridizations

A total of 3384 cDNA clones (1692 clones from each cDNA library) were selected based on the presence of a unique band higher than 500 bp. About 50 PCR reactions of 96 samples each (Fig. 2.8) were performed for both libraries to achieve the number of cDNA clones necessary for chip construction. Amplification efficiency was 71 % in both libraries.

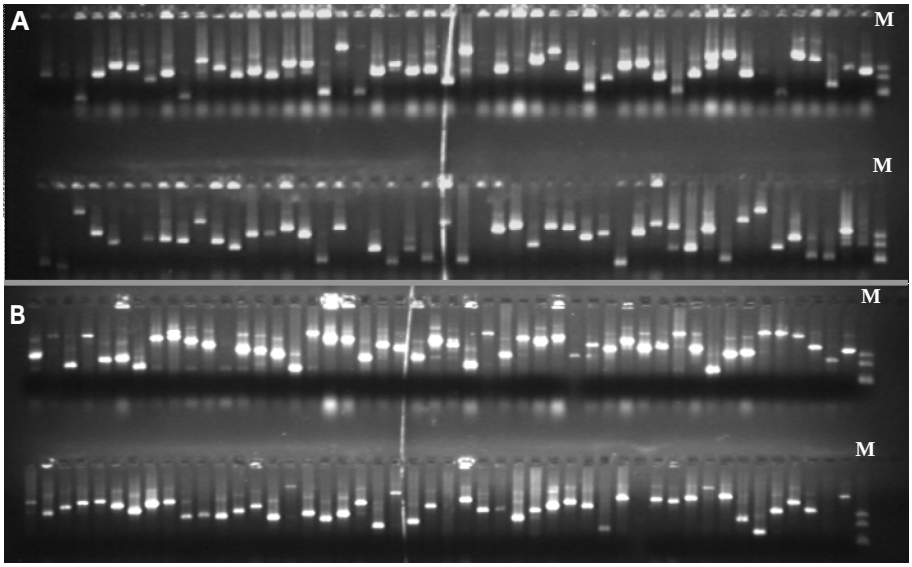

Figure 2.8- Gel electrophoresis of PCR products resulted from a 96- well reaction representing clones generated in unpollinated (**A**) and pollinated (**B**) cDNA libraries. **M**- DNA molecular marker (MtrnL: 1050 bp, 750 bp, 350 bp).

Before printing, two different surfaces for coating glass slides were tested: homemade poly-L-lysine slides and commercial UltraGAPS™ Coated slides (Corning, USA). When using the same printing parameters, poly-L-lysine covered glass slides (Fig. 2.9**A**) presented coalescence of the spots and higher background when compared to the commercial slides (Fig. 2.9**B**) tested.

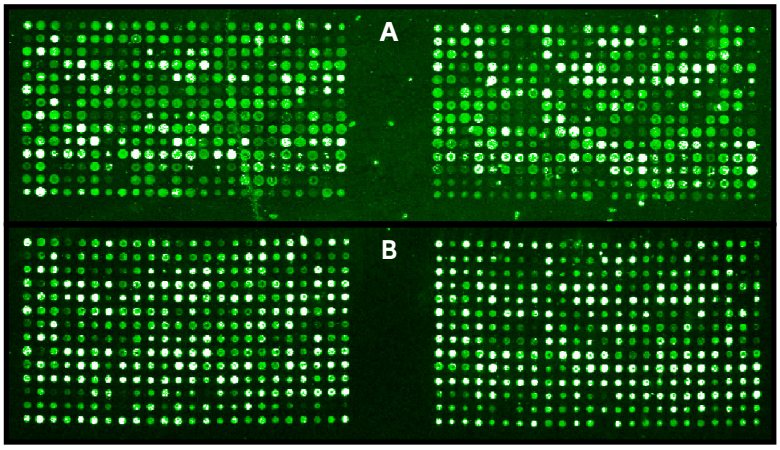

Figure 2.9- Gel Star® staining from a printing test performed on Poly-L-lysine (**A**) slides and on commercially UltraGAPS™ Coated Slides (**B**).

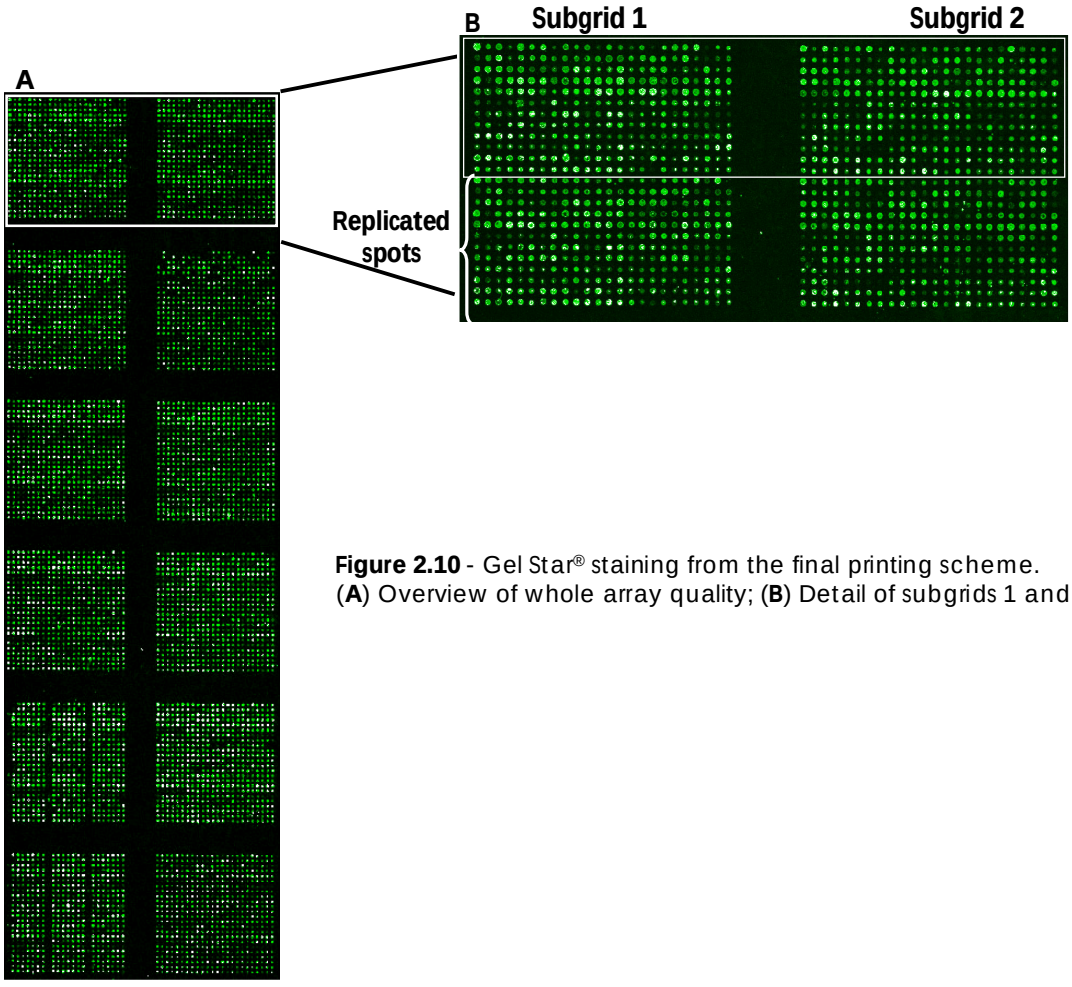

Figure 2.10 - Gel Star® staining from the final printing scheme. (**A**) Overview of whole array quality; (**B**) Detail of subgrids 1 and 2.

The final printing scheme established was 12 subgrids, each one with spots distributed in 24 columns and 24 lines, repeated twice (replicated spot) (Fig. 2.10**B**). Printing conditions were tested, namely temperature (20-23ºC) and relative humidity

(45-55%), using commercial slides. Gel Star® staining was performed in order to test printing quality (Fig. 2.10**A**). After Gel Star® staining of the final printing scheme, spots were uniformly distributed in the slide. No missing spots were detected.

To carry out the hybridizations, and due to limited amount of total RNA available, amplified RNA (Fig. 2.11) of all biological replicates from unpollinated and pollinated samples, in both 2 days and 4 days' time points, was assembled. aRNA technology was performed because it gives the quantity required for hybridizations and for additional quantitative real-time PCR (qPCR) analysis, with few technical errors introduced.

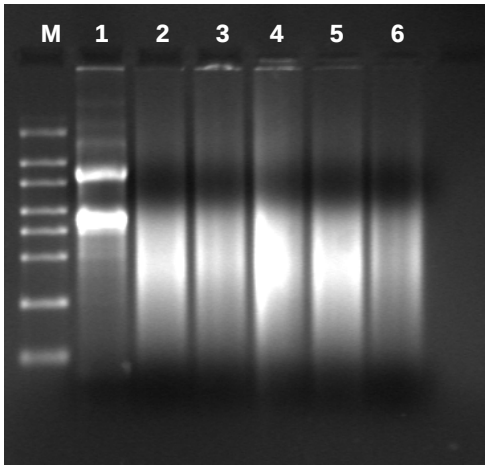

Figure 2.11- Gel electrophoresis of aRNA from biological replicates of unpollinated labella in 2DAP timepoint. **M**- RiboRuler™ RNA ladder high range (Fermentas), lane **1**- total RNA from *Ophrys fusca*, lanes **2-6**- aRNA from five biological replicates in unpollinated *Ophrys fusca* labella.

Li and co-workers (2004) compared the three major reverse transcription based RNA labeling methods used in microarrays: total RNA (T-RNA), mRNA, antisense RNA (aRNA). They found a ranking in terms of the number of differentially expressed genes for the three methods, as follows: aRNA >mRNA >T-RNA, consistent with previous reports (Puská *et al.*, 2002), and proved that the biases introduced in the processes of aRNA amplification and mRNA purification are sequence-dependent rather than abundance-dependent. Regarding target labelling, in direct labelled experiments, dye incorporation efficiency during mRNA reverse transcription may depend on the transcript's nucleotide sequences, and thus incorporation efficiency may be different for the two dyes used in the experiment. On the other hand, indirect labelling (Manduchi *et al.*, 2002), which was used in the experiments here presented, lessens this effect, despite differences in quantum efficiencies and stabilities of the dyes which can produce a phenomenon similar to differential incorporation efficiency (Dobbin *et al.*, 2005). As a result, indirect labelling in aRNA has proven to be a reliable method when

working with limited starting material. By increasing dyes incorporation efficiencies, differences in hybridizations may be mainly attributed to biological variation. Unpollinated biological replicates aRNA was labelled with Cy3 dye and used as control whereas pollinated biological replicates aRNA was labelled with Cy5 dye (test) (Fig. 2.12).

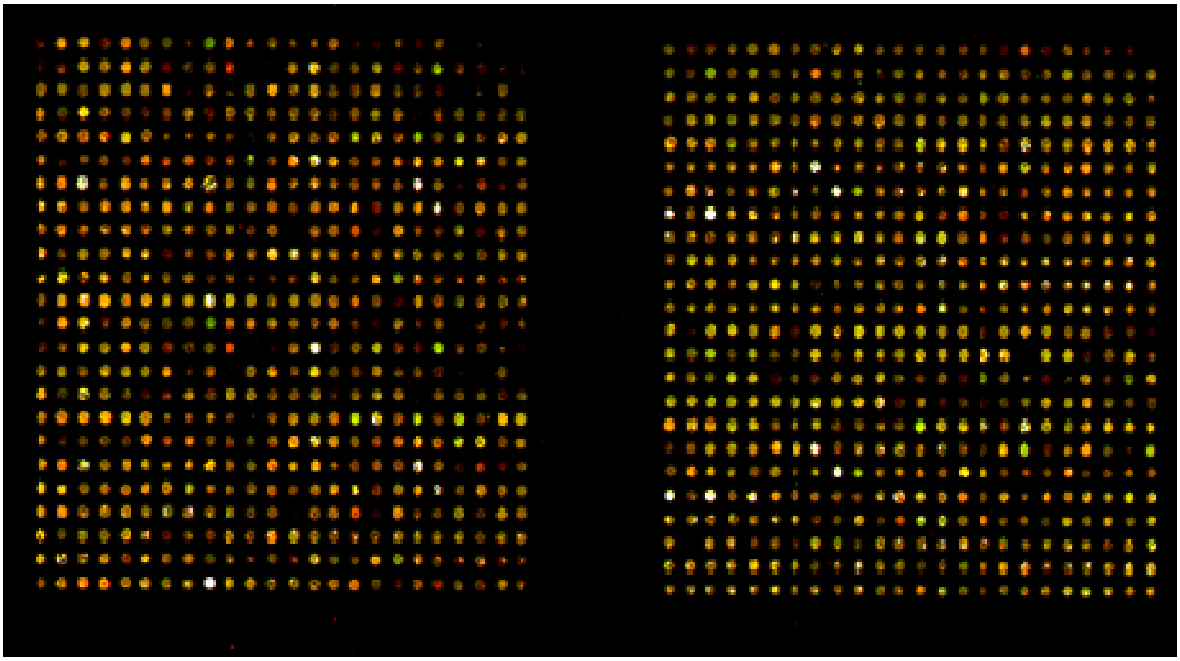

Figure 2.12- *Ophrys fusca* cDNA microarray (partial image) acquired with a VersArray ChipReader® vs 3.1. Build 1.68 (BioRad, USA) scanner. The image results from the overlay detection of Cy5 and Cy3 dyes after hybridization with the spotted cDNA chip. This microarray represents four subgrids and cDNA from unpollinated replicates was labelled with Cy3 and cDNA from Pollinated replicates labelled with Cy5. Red spots represent up regulated genes; green spots represent down regulated genes; genes with no differential expression are represented in yellow; saturated genes are represented as white spots.

2.4.2.2. Differentially expressed genes during pollination

Differential gene expression profiling in *O. fusca* at 2DAP and 4DAP was accessed through cDNA microarray comparison of labella from pollinated versus unpollinated flowers. Microarray data have been deposited in the NCBI's Gene Expression Omnibus (GEO) database (Edgar *et al.*, 2002) under the accession number GSE28273 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28273). Statistical analysis revealed 277 differentially expressed transcripts at both 2 DAP and 4 DAP (8.2 % of the total cDNA clones printed). Considering the two time points studied, the proportion of differentially expressed genes increased from 106 in 2DAP to 171 at 4DAP.

The majority of differentially expressed genes were up regulated (166 transcripts, 59.9 %) and this was more pronounced at 4DAP (Table V) with 112 transcripts up regulated (40.4 %). Fifty-four transcripts (19.5 %) were up regulated at 2DAP. Total down regulated transcripts accounted for 111 genes (40.1 %), 52 (18.8 %) at 2DAP and 59 (21.3 %) at 4DAP. Assemblage of the 277 genes differentially expressed revealed 140 unigenes (50.5 %) with 114 singletons and 26 contigs (Table 2.6). Twenty-four unigenes were common to 2DAP and 4DAP, with 11 up regulated and 6 down regulated. Highest fold changes were observed for clones with no identity retrieved: a transcript (acc.no. HO850109) with 2.61 -fold up regulation at 2DAP and a transcript (acc.no. HO849981) with 3.81 -fold down regulation at 4DAP.

Transcripts were grouped in functional categories according to their putative function. Functional categories include: (1) no homology (23 %); (2) metabolism (19 %); (3) cell rescue, defence and virulence (11 %); (4) unclassified (8.5 %); (5) cell fate (8.5 %); (6) energy (7 %); (7) protein fate (7 %); (8) non-plant, viral and plasmids proteins (5 %); (9) transcription factors and regulation of transcription (4 %); (10) biogenesis of cellular components (3 %); (11) cellular transport, transport facilities and transport routes (2 %); (12) cellular communication/signal transduction mechanism (1 %); (13) protein synthesis (1 %) (Fig. 2.13). The highest number of differentially expressed genes was recorded for the no homology category (23 %), followed by metabolism (19 %) (Fig. 2.13). Even though no virus infection symptoms were observed on *O. fusca* plants in the field, transcripts coding for non-plant, viral and plasmid proteins were detected as being up and down regulated, at both 2DAP and 4DAP, respectively. Transcripts coding for viral proteins have been detected previously on *Phalaenopsis* sp. flower buds (Chen *et al.*, 2005; Tsai *et al.*, 2006).

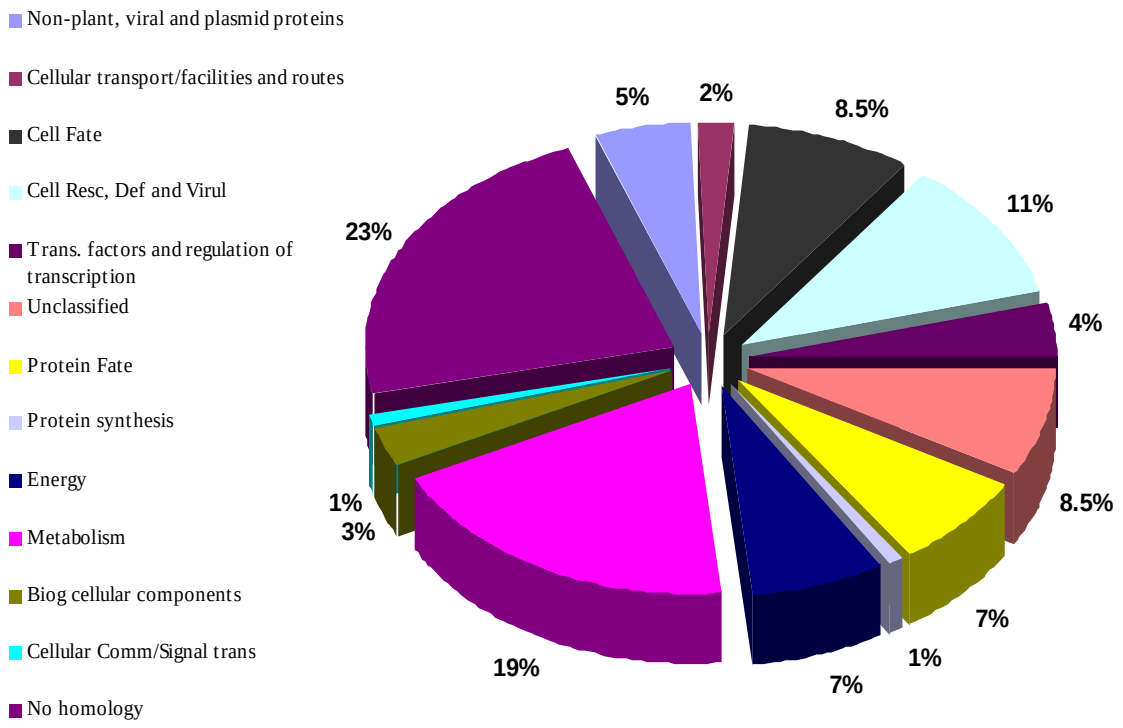

Figure 2.13- Differentially expressed genes in *Ophrys fusca* labellum at 2DAP and 4DAP, grouped into functional categories. Percentage of unique transcripts classified in each of the functional category is indicated.

Overall, at 2DAP, transcripts involved in cell fate and cell rescue, defence and virulence, were generally up regulated; whereas transcripts coding for metabolism and energy (photosynthesis and respiration) were down regulated. At 4DAP, alterations in gene expression were characterized by an up regulation of SAGs (cell fate; cell rescue, defence and virulence; and protein fate) and a down regulation of secondary metabolism related transcripts.

Table 2.6. Differentially expressed genes identified from *Ophrys fusca* labellum transcriptome, after pollination, at both 2 days after pollination (DAP) and 4DAP. Negative/green and positive/red values indicate down and up regulation, respectively. GenBank Acc.No., Predicted function (species) retrieved by BLASTx from Uniref50, GO term (1st level- Biological Process), E-value, Fold Change (normalized ratio of labella pollinated flowers vs. labella unpollinated flowers) and Clone/Contig information are presented.

GenBank Acc.No.	Predicted function (species)	GO term	E-value	Fold Change		Clone/ Contig
				2DAP	4DAP	
Metabolism						
Phenylpropanoid metabolism						
HO849900	Chalcone synthase (*Dendrobium nobile*)	GO:0009058	5E-58	**-1.3**		*Of*up2743
HO849901	Bibenzyl synthase (*Phalaenopsis* sp.)	GO:0009813	5E-41	**-1.53**		*Of*up510
HO849902	Flavonoid 3'- monooxygenase (*Zea mays*)	GO:0055114	9E-49	**-1.31**	**-1.69**	*Of*up975
HO850016	4-coumarate-CoA ligase (*Populus balsamifera* subsp. *trichocarpa*)	GO:0008152	2E-19	**-1.55**		*Of*p251
HO849951	stilbene synthase (*Vitis vinifera*)	GO:0008152	6E-06		**1.53**	*Of*up2706
HO850017	UDP-glucuronosyltransferase, putative (*Ricinus communis*)*****		3E-27		**-1.35**	*Of*p2607
HO849903	Polyphenol oxidase (*Ananas comosus*)	GO:0055114	1E-38		**1.73**	*Of*ctg1195 (**3**)
HO849905	Polyphenol oxidase (*Euterpe edulis*)	GO:0008152	1E-33		**1.45**	*Of*up1027
HO850019	Polyphenol oxidase (*Doritis pulcherrima Phalaenopsis*)	GO:0055114	2E-11	**1.85**		*Of*p2027
Alkaloid metabolism						
HO849906	tyrosine/DOPA decarboxylase (*Argemone mexicana*)	GO:0006520	1E-32	**-1.4**	**-1.42**	*Of*up1942
HO850020	salutaridinol 7-O-acetyltransferase, putative (*Ricinus communis*)	GO:0047180	2E-15		**-1.52**	*Of*p176
Metabolism of primary metabolic sugar derivatives						
HO850021	Myo-inositol 1-phosphate synthase (*Ricinus communis*)	GO:0006021	2E-32	**-1.31**		*Of*p2108
Sugar metabolism						
HO850022	Beta-amylase, putative (*Ricinus communis*)	GO:0005975	2E-33	**-1.34**		*Of*p963
HO850024	Beta-glucosidase 24 (*Oryza sativa* subsp. *japonica*)	GO:0005975	2E-28	**-1.46**		*Of*ctg2402 (**2**)
Lipid metabolism						
HO849907	TCER1 (*Triticum aestivum*)	GO:0006633	5E-37		**-1.81**	*Of*up1936
HO850025	Omega-6 fatty acid desaturase (*Cucurbita pepo*)	GO:0006629	4E-19	**-1.41**		*Of*p1524
HO849908	Stearoyl-ACP desaturase homologue 2 (*Ophrys* x *arachnitiformis* subsp. *archipelagi*)		2E-59		**-1.9**	*Of*ctg2559 (**2**)
HO849909	Stearoyl-acyl-carrier protein desaturase (*Elaeis oleifera*)	GO:0006633	3E-56		**-1.49**	*Of*up2825
HO849949	3-ketoacyl-CoA thiolase (*Arachis diogoi*)		2E-11		**1.47**	*Of*up3281

(**n**)- Represents the number sequences contained in the corresponding contig. *****- Homology by BLASTx non redundant proteins from NCBI database.

Table 2.6. Continued.

GenBank Acc.No.	Predicted function (species)	GO term	E-value	Fold Change		Clone/ Contig
				2DAP	4DAP	
Metabolism						
Lipid metabolism						
HO850027	Neutral ceramidase (*Hordeum vulgare*)		1E-38	**-1.31**		*Of*p336
HO850028	Non-lysosomal glucosylceramidase (*Oryza sativa*)	GO:0006665	1E-37	**-1.37**	**-1.4**	*Of*p179
HO850029	7-dehydrocholesterol reductase (*Zea mays*)		2E-15	**-1.31**		*Of*p1630
Nitrogen, sulfur and selenium metabolism						
HO850030	NifS-like protein (*Oryza ridleyi*)	GO:0008152	2E-18		**-1.54**	*Of*p2220
Amino acid metabolism						
HO850031	Ornithine aminotransferase (*Glycine max*)	GO:0008152	1E-38	**-1.39**		*Of*p3056
HO850032	Methylcrotonoyl-CoA carboxylase subunit alpha (*Zea mays*)	GO:0008152	3E-33	**-1.27**		*Of*p1184
Nucleotide/nucleoside/nucleobase metabolism						
HO850033	Putative allantoate amidohydrolase (*Phaseolus vulgaris*)	GO:0008152	3E-37		**1.36**	*Of*p1119
Cell Fate						
Cell aging and program cell death (PCD)						
HO850034	putative Hypersenescence1 (HYS1) (*Oryza sativa*)		1E-11	**-1.3**		*Of*p864
HO850035	Cysteine proteinase (*Phalaenopsis* sp. SM9108)	GO:0006508	1E-58	**1.32**	**1.52**	*Of*ctg1283(**4**)
HO849912	Cysteine proteinases (*Elaeis guineensis* var. *tenera*)	GO:0006508	1E-36		**1.56**	*Of*ctg3242 (**2**)
HO850038	RNase PD2 (*Prunus dulcis*)		2E-68	**1.59**	**1.54**	*Of*ctg2701 (**2**)
HO850042	Cysteine protease (*Medicago sativa*)	GO:0006508	1E-05		**1.43**	*Of*poll#11
HO850041	Cysteine proteinase (*Elaeis guineensis*)	GO:0006508	1E-59		**1.3**	*Of*ctg3347 (**3**)
HO849913	Cysteine protease (*Zea mays*)	GO:0006508	2E-42		**1.48**	*Of*up366
HO850043	Aspartic proteinase precursor, putative (*Ricinus communis*)	GO:0006629	2E-10		**-1.43**	*Of*p2286
HO849914	Triticain alpha (*Triticum aestivum*)	GO:0006508	3E-42	**1.37**	**1.4**	*Of*ctg915 (**4**)
HO850046	putative Tropinone reductase-14 (*Boechera divaricarpa*)	GO:0008152	1E-45		**1.42**	*Of*ctg2110 (**4**)
Cell enlargement						
HO850052	expansin B4, ATeXPB4, putative (*Ricinus communis*)		5E-40		**1.45**	*Of*ctg800 (**4**)
HO850050	Putative blight-associated protein p12 (*Oryza sativa* subsp. *japonica*)		9E-15		**1.44**	*Of*p1866
Cell Rescue, Defense and Virulence						
HO849916	Chitinase, putative (*Ricinus communis*)	GO:0005975	1E-18		**-1.55**	*Of*up2803

(**n**)- Represents the number sequences contained in the corresponding contig.

Table 2.6. Continued.

GenBank Acc.No.	Predicted function (species)	GO term	E-value	Fold Change		Clone/ Contig
				2DAP	4DAP	
Cell Rescue, Defense and Virulence						
HO850056	glutathione s-transferase gstf2 (*Oryza sativa* subps. *indica*)		2E-41	**1.57**	**1.66**	*Of*ctg1174 (**6**)
HO850058	phi class glutathione transferase GSTF3 (*Populus trichocarpa*)		3E-23		**1.3**	*Of*p1943
HO850059	MtN19-like protein (*Pisum sativum*)		2E-30		**1.35**	*Of*poll#20
HO850069	metallothionein-like protein type 2 (*Typha angustifolia*)		5E-18		**1.46**	*Of*ctg2943 (**9**)
HO849988	antimicrobial peptide snakin (*Capsicum annuum*)		3E-22		**1.4**	*Of*up1176
HO850078	antimicrobial peptide snakin (*Capsicum annuum*)		5E-11		**1.42**	*Of*ctg1262 (**2**)
HO849925	cytosolic cyclophilin (*Lupinus luteus*)*****	GO:0006457	3E-32		**1.59**	*Of*up529
HO849927	class III chitinase (*Bambusa oldhamii*)	GO:0005975	1E-51	**-1.43**	**1.57**	*Of*ctg1177 (**3**)
HO849917	Pathogenesis-related protein 10c (*Elaeis guineensis* var. *tenera*)	GO:0006952	6E-38	**1.59**	**1.74**	*Of*up2722
HO849938	Nonspecific lipid transfer protein (*Hordeum vulgare*)	GO:0006869	3E-17	**1.54**		*Of*up2151
HO849928	Abscisic stress ripening protein homolog (*Prunus armeniaca*)	GO:0006950	8E-43		**-1.39**	*Of*up2778
HO849952	ASR1 (*Solanum cheesmanii*)	GO:0006950	2E-9	**1.75**		*Of*up385
HO850060	Late embryogenesis abundant protein Lea5 (*Citrus sinensis*)	GO:0006950	1E-10		**1.46**	*Of*p1957
HO850063	Metallothionein-like protein type 3 (*Musa acuminata*)		1E-18	**-1.38**	**1.56**	*Of*ctg2343 (**7**)
Transcription factors and regulation of transcription						
HO849929	flavin-binding kelch domain F box protein FKF1 (*Allium cepa*)	GO:0007165	3E-23		**-1.47**	*Of*up2840
HO850072	Nam-like protein 14 (*Petunia hybrida*)	GO:0045449	1E-05	**1.34**		*Of*p2017
HO849930	Squamosa promoter-binding protein, putative (*Ricinus communis*)		1E-28	**-1.34**	**-1.54**	*Of*up100
HO850088	putative RNA-binding protein (*Oryza sativa*)*****		1E-21	**-1.31**		*Of*p1569
HO850089	Nucleic acid binding NABP (*Medicago truncatula*)		1E-11	**-1.3**		*Of*p966
Protein Synthesis						
HO850073	Ribosomal protein L17 (*Ricinus communis*)	GO:0006412	5E-20		**1.5**	*Of*poll#7
HO849931	translation initiation factor (*Oryza sativa* subsp. *indica*)	GO:0006412	2E-21		**1.56**	*Of*up2676
Biogenesis of Cellular Components						
HO850074	Actin 2 (*Glycyrrhiza uralensis*)		1E-36	**-1.47**		*Of*p28
HO849932	Peroxisomal membrane protein (PMP36) (*Arabidopsis thaliana*)	GO:0006810	1E-55		**-1.36**	*Of*up2813
HO849933	putative Profilin (*Phalaenopsis* hybrid cultivar)	GO:0007010	1E-46		**1.45**	*Of*up2694
HO850075	Pectin methyltransferase (PME)/invertase inhibitor-like protein (*Musa acuminata*)		5E-17		**1.51**	*Of*p706

(**n**)- Represents the number sequences contained in the corresponding contig. *****- Homology by BLASTx non redundant proteins from NCBI database.

Table 2.6. Continued.

GenBank Acc.No.	Predicted function (species)	GO term	E-value	Fold Change		Clone/ Contig
				2DAP	4DAP	
Protein Fate						
HO849934	Chaperone (*Agave tequilana*)		3E-45	**1.79**		*Of*up429
HO850076	Protein disulfide isomerase 2 precursor (*Elaeis guineensis* var. *tenera*)	GO:0045454	9E-56	**-1.51**		*Of*p2972
HO850077	Chloroplast small HSP (*Epilobium amurense*)	GO:0006950	3E-17		**-1.3**	*Of*p3009
HO849935	DnaJ protein homolog 1 (*Allium porrum*)	GO:0006457	1E-77		**-1.34**	*Of*up862
HO849936	E3 ubiquitin-protein ligase ATL15 (*Arabidopsis thaliana*)	GO:0016567	7E-27		**1.6**	*Of*up2830
HO849937	Spotted leaf protein (*Ricinus communis*)	GO:0016567	4E-10		**-1.49**	*Of*up1617
HO850080	Nucleotide pyrophosphatase/ phosphodiesterase (*Zea mays*)		7E-60	**-1.34**		*Of*p1539
HO850081	Purple acid phosphatase 1 (*Zea mays*)		7E-62	**1.81**	**1.82**	*Of*p705
HO850082	casein kinase, putative (*Ricinus communis*)	GO:0006468	2E-27	**-1.31**		*Of*p990
HO850083	Nucleotide pyrophosphatase/ phosphodiesterase (*Zea mays*)		2E-10		**1.34**	*Of*p1982
Cellular Transport, Transport Facilities and Transport Routes						
HO850084	Sterol carrier protein 2-like (*Oryza sativa japonica* group)*****		2E-39	**-1.3**		*Of*p887
HO850086	ATPUP3 (*Zea mays*)		7E-15	**-1.4**	**-1.37**	*Of*p574
HO849939	Adenine nucleotide translocator (*Cucumis melo* subsp. *melo*)		7E-76		**1.46**	*Of*up2719
Cellular Communication/Signal Transduction Mechanism						
HO850087	G protein beta subunit-like (*Medicago sativa* subsp. x *varia*)		4E-65	**-1.35**		*Of*p1546
HO850105	calmodulin (*Phaseolus vulgaris*)		3E-79		**-2.72**	*Of*p2982
Energy						
HO850090	Putative vacuolar ATP synthase subunit C (*Oryza sativa* subsp. *japonica*)	GO:0015991	3E-39	**-1.33**		*Of*p1128
HO850091	ribulose-1,5-bisphosphate carboxylase/oxygenase activase 1 (*Gossypium hirsutum*)		1E-62		**-1.45**	*Of*ctg3047 (**2**)
HO850092	Serine hydroxymethyltransferase (*Medicago truncatula*)	GO:0006563	4E-80	**-1.38**		*Of*p1559
HO849941	putative chlorophyll A-B binding protein of LHCI type II precursor (*Picea abies*)	GO:0015979	1E-40		**-1.49**	*Of*ctg1954 (**2**)
HO850103	putative chlorophyll A-B binding protein of LHCI type II precursor (*Picea abies*)	GO:0015979	2E-11		**-1.47**	*Of*p2503
HO849942	Chloroplast ferredoxin-$NADP^+$ oxidoreductase (*Capsicum annuum*)	GO:0055114	1E-55	**-1.37**	**-1.53**	*Of*up1656
HO849943	Photosystem I reaction center subunit V, chloroplastic (PSI-G)(*Arabidopsis thaliana*)	GO:0015979	2E-46		**-1.46**	*Of*up2749

(**n**)- Represents the number sequences contained in the corresponding contig. *****- Homology by BLASTx non redundant proteins from NCBI database.

Table 2.6. Continued.

GenBank Acc.No.	Predicted function (species)	GO term	E-value	Fold Change		Clone/ Contig
				2DAP	4DAP	
Energy						
HO849945	phosphoglycerate dehydrogenase, putative (*Ricinus communis*)	GO:0008152	1E-39		1.44	*Of*ctg1698 (**3**)
HO850095	chloroplast photosystem II 10 kDa protein-like protein (*Wolffia arrhiza*)	GO:0015979	2E-54		1.5	*Of*p2102
HO850096	Protoheme IX farnesyltransferase (*Zea mays*)	GO:0048034	1E-18	-1.3		*Of*p1955
Unclassified						
HO850144	tropinone reductase/dehydrogenase, putative (*Arabidopsis thaliana*)*		1E-05		1.75	*Of*p447
HO850097	Transferase, putative (*Ricinus communis*)		3E-11		1.39	*Of*p1292
HO850098	S-adenosylmethionine-dependent methyltransferase, putative (*Ricinus communis*)	GO:0032259	5E-52	-1.41		*Of*p1516
HO849946	S-adenosylmethinonine synthetase (*Camellia sinensis*)	GO:0006730	5E-79		1.65	*Of*up1593
HO850099	Fiber protein Fb34 (*Zea mays*)		3E-52	-1.27		*Of*p1547
HO850100	Conserved hypothetical protein (*Ricinus communis*)		2E-21	-1.33		*Of*p1562
HO849947	cytochrome P450-1 (*Musa acuminata*)	GO:0055114	7E-13	-1.6		*Of*up2976
HO849948	Protein with unknown function (*Ricinus communis*)		1E-10		-1.4	*Of*up1252
HO850101	unnamed protein product (*Vitis vinifera*)		2E-10	-1.33		*Of*p1530
HO850108	Putative uncharacterized protein (*Zea mays*)		9E-10	-1.55		*Of*p266
HO849978	Putative uncharacterized protein (*Vitis vinifera*)		8E-6	1.62		*Of*up3257
HO849919	Whole genome shotgun sequence (*Vitis vinifera*)		2E-35	1.81	1.65	*Of*p2044
No Homology						
HO849950					1.54	*Of*up2187
HO850104					1.49	*Of*p428
HO850140					-2.45	*Of*ctg2252 (**2**)
HO849967				1.65	-3.02	*Of*ctg1542 (**2**)
HO850124					2.02	*Of*ctg99 (**2**)
HO850112				1.93	-2.97	*Of*ctg1742 (**12**)
HO849884				1.21	-2.29	*Of*ctgup#24 (**7**)
HO849963					1.43	*Of*ctg3270 (**4**)
HO849981				1.7	-3.81	*Of*up86
HO849968					1.5	*Of*up999
HO850085				-1.32		*Of*p3031
HO850126				-1.45		*Of*p1148

(**n**)- Represents the number sequences contained in the corresponding contig. *- Homology by BLASTx non redundant proteins from NCBI database.

Table 2.6. Continued.

GenBank Acc.No.	Predicted function (species)	GO term	E-value	Fold Change		Clone/Contig
				2DAP	4DAP	
No Homology						
HO849971					-1.33	*Of*up963
HO849969					1.58	*Of*up2677
HO849972					1.46	*Of*up2551
HO850129					-1.33	*Of*p2109
HO849973					1.47	*Of*up3230
HO849970					1.5	*Of*up170
HO850131					1.43	*Of*poll#1
HO849975					1.41	*Of*up1808
HO849976				-1.74		*Of*up1909
HO850132				-1.39		*Of*p2122
HO849977				-1.33		*Of*up2750
HO850133					1.97	*Of*p168
HO850134					1.65	*Of*p3171
HO849979					1.49	*Of*up2624
HO850135					1.45	*Of*p2757
HO850136					1.45	*Of*p1099
HO850127					1.5	*Of*p1998
HO850137				1.44	1.59	*Of*p479
HO849980				1.44	1.49	*Of*up2030
HO850138					-1.41	*Of*p2417
Non-plant, viral and plasmid Proteins						
HO850141	putative polyprotein (Peach mosaic virus)	GO:0019079	1E-47		-2,25	*Of*p2719
HO850142	RNA-dependent RNA pol (African oil palm ringspot virus)	GO:0019079	6E-11	1.74	-2.59	*Of*p1261
HO850122	Putative uncharacterized protein (*Puccinia graminis* f.sp.*tritici* CRL75-36-700-3)		1E-19		2.45	*Of*ctg3249 (**3**)
HO850119	Putative uncharacterized protein (*Colletotrichum graminicola* (strain M1.001 / M2 / FGSC 10212)		3E-11	1.46	1.38	*Of*ctg783 (**3**)
HO850128	Pherophorin-C1 protein (*Chlamydomonas reinhardtii*)		2E-12	-1.41		*Of*p1090
HO850130	Pherophorin-C1 protein (*Chlamydomonas reinhardtii*)		2E-12		-1.36	*Of*p906
HO849974	Putative uncharacterized protein (*Rhodopirellula baltica*)		3E-6	1.39	1.34	*Of*up3301

(**n**)- Represents the number sequences contained in the corresponding contig.

2.4.2.2.1. Metabolism

Genes involved in metabolism revealed to be affected by pollination and account for 19 % of unique transcripts detected in the expression study performed. Differentially expressed genes involved in metabolism are generally down regulated by pollination, at 2DAP and 4DAP**.** Soon after pollination (2DAP), genes accounting for secondary metabolism pathways are repressed suggesting that the flower no longer needs its by-products. As a result, at 4DAP, pollination event seems to initiate a stress-related response, which is detectable by overexpression of transcripts coding for phytolexins and phenolic compounds biosynthetic genes. Several secondary metabolism biosynthetic pathways are discussed below: phenylpropanoid, alkaloid, starch catabolism, lipid metabolism, nitrogen, sulfur and selenium metabolism, amino acid metabolism and nucleotide/nucleoside/nucleobase metabolism. Genes encoding enzymes involved in both phenylpropanoid and lipid metabolisms are the most prevalent. Regarding the phenylpropanoid pathway, genes are generally repressed at 2DAP and their expression decline continues at 4DAP. Flavonoid biosynthesis, which is essentially related to labellum colour traits, is the pathway most influenced by pollination. Lipid metabolism appears to be mediated by pollination, since all genes differentially expressed are down regulated.

A general overview of secondary metabolism pathways and putative function of differentially expressed genes detected in the gene expression study performed in *Ophrys fusca* pollination are discussed below.

2.4.2.2.1.1. Phenylpropanoid m*etabolism*

Phenylpropanoids display vital developmental roles in plant structure integrity, UV photoprotection, reproduction and internal regulation of plant cell physiology and signalling (Lucheta *et al.*, 2007). They also function as key chemical modulators of plant communication with insects and microbes, playing both attractive and repellent roles, in defensive phytoalexin responses to infection and herbivory, attraction of insect pollinators via flower color, and induction of root nodulation by symbiotic nitrogen-fixing rhizobial colonies (Ferrer *et al.*, 2008). In the phenylpropanoid pathway, three enzymatic transformations redirect the carbon flow from primary metabolism, transforming phenylalanine into Coenzyme A (CoA)-activated hydroxycinnamoyl (phenylpropanoid) thioester capable of entering the two major downstream pathways, monolignol and flavonoid biosynthesis (reviewed in Ferrer *et al.*, 2008). After deamination of L-phenylalanine by phenylalanine ammonia-lyase (PAL), the produced cinnamic acid is

further catalised by cinnamic acid 4-hydroxylase (C4H), with the introduction of a hydroxyl group in the phenyl ring of cinnamic acid at the *para* position, producing *p*-coumaric acid. The carboxyl group of *p*-coumaric acid is then activated by formation of a thioester bond with CoA, a process catalyzed by *p*-coumaroyl: CoA ligase (4CL) (Douglas, 1996; Kaneko *et al.*, 2003; Lucheta *et al.*, 2007). Finally, 4CL is responsible for the production of CoA thiol esters from cinnamates and coumarate, which are used in biosynthesis of numerous phenylpropanoid-derived compounds, such as stilbenes, lignins, lignans, suberins, flavonoids, isoflavonoids, and various small phenolic compounds (Kaneko *et al.*, 2003). These compounds have diverse functions in plants, including mechanical support and rigidity to cell walls, attractants of insect pollinators which help pollination and fertilization, protection against biotic and abiotic stresses, and also participate in signalling processes (Douglas, 1996; Mayer *et al*., 2001; Lucheta *et al.*, 2007). Various phenolic compounds such as stilbenes and naringenin chalcone are formed through reactions catalysed by stilbene synthase (STS) and chalcone synthase (CHS), respectively (Hanhineva *et al.*, 2009). These two enzymes, STS and CHS, belong to the type III polyketide synthase superfamily, which is a highly versatile group of enzymes that also include 2-pyrone synthase (Eckermann *et al.*, 1998), bibenzyl synthase (Preizig-Müller *et al.*, 1995), and acridone synthase (Junghanns *et al.*, 1995).

Many phenylpropanoids, classified as phytolexins, function as antimicrobial compounds synthesized in response to pathogen or herbivore attack (Chong *et al.*, 2009). Stilbenes are phenylpropanoid derivatives produced by plants and referred as phytoalexins since they contribute to the defense response against predators and pathogens (Treutter, 2005). The pivotal enzyme for their synthesis is stilbene synthase (STS) which catalyzes, in a single reaction, the biosynthesis of the stilbene backbone from three malonyl-CoA and one CoA-ester of a cinnamic acid derivative (most frequently cinnamoyl-CoA or *p*-coumaroyl-CoA) (Goodwin *et al.*, 2000; Chong *et al.*, 2009). High contents of stilbenes are synthesized after microbial attack as part of both constitutive and inducible defense responses (Chong *et al.*, 2009). In addition to the well-known function of stilbenes as phytoalexins, these compounds may also be involved as chemical signals in response to oxidative stress generated by UV irradiation (He *et al.*, 2008).

The enzyme chalcone synthase (CHS) is crucial for downstream enzymatic machinery in the flavonoid compounds biosynthesis pathway (Buer *et al.*, 2010). Flavonoid compounds, produced as a branch of the phenylpropanoid pathway, are classified into different groups based primarily on the oxidation degree of the three-carbon bridge (Taiz and Zieger, 1998). Flavonoids play roles in many plant physiology mechanisms, including: influence in the transport of auxins (Peer and Murphy 2007), defense (Treutter, 2005), allelopathy (Bais *et al.*, 2006) and modulation of reactive

oxygen species (ROS) levels (Bais *et al.*, 2006). Flavonoids also provide flower coloring important to attract pollinators (Mol *et al.*, 1998) and in many species they are required for pollen viability (Taylor and Jorgensen, 1992). In flowers, flavonoids can additionally provide protection against UV radiation (Dixon and Paiva, 1995), and act as chemical defense compounds against pathogens (Bednarek and Osbourn, 2009). Anthocyanins (from Greek: ἀνθός (anthos) = flower + κυανός (kyanos) = blue) are water-soluble vacuolar pigments that can appear red, purple, or blue according to pH. They belong to the class of flavonoids and can occur in all types of higher plants tissues, including leaves, stems, roots, flowers, and fruits (Taiz and Zieger, 1998). These pigments are produced by the phenylpropanoid biosynthesis pathway, the key enzymes being chalcone synthase (CHS) at the top of the pathway, dihydroflavonol 4-reductase (DFR) and the flavonoids hydroxylases (F-3'H and F-3'5'H) that turn orange to lavender/purple and/or to blue by a simple chemical reaction (Bednarek and Osbourn, 2009). The resulting unstable anthocyanidins are further coupled to sugar molecules by enzymes like UDP-3-O-glucosyltransferase to produce the final relatively stable anthocyanins (Bednarek and Osbourn, 2009). The anthocyanidins are the basic structures of the anthocyanins. When the anthocyanidins are found in their glycosilated form (bonded to a sugar moiety) are known as anthocyanins (Castañeda-Ovando *et al.*, 2009). There is a huge variety of anthocyanins mainly due to the number of hydroxylated groups, the nature and the number of bonded sugars to their structure, the aliphatic or aromatic carboxylates bonded to the sugar in the molecule and the position of these bonds (Kong *et al.*, 2003a). Up to know, about 500 different anthocyanins and 23 anthocyanidins have been described (Castañeda- Ovando *et al.*, 2009 and references therein). The glycosilated derivatives of the three non-methylated anthocyanidins (Cy, Dp and Pg) are the most common in nature, being found in 80 % of colored leaves, 69 % in fruits and 50 % in flowers (Castañeda- Ovando *et al.*, 2009). The most frequent anthocyanidins are pelargonidin Pg (orange red color), cyaniding Cy (purplish red), delphinidin Dp (bluish purple), peonidin Pn (rosy red) and petunidin Pt (purple) (Clifford, 2000). Anthocyanins play important roles in flowers and fruits pigments from numerous plants across the plant kingdom, by promoting attraction insects for pollination thereby ensuring plant survival (Babar and Khatun, 2006; Colquhoun *et al.*, 2010), seed dispersion and acting as protectants against UV-B radiation (Chiou and Yeh, 2008). Floral pigmentation provides the basis for mimicking the colours of female insect's body in the *Ophrys* pollination system (Schlüter and Schiestl, 2008). Generally, flower colour is determined by the presence of carotenoids and anthocyanins and by their interaction with the molecular environment and by the way light is reflected on differentially shaped epidermal cells at the flower surface (Dyer *et al.*, 2007). Previous studies in the purple orchid *Dendrobium,* as well as in other orchids, showed that anthocyanins,

mainly cyanidins, pelargonidins and delphinidins are the most predominant pigments (Kuehnle, 1997). In the gene expression study in *Ophrys fusca* after pollination, flavonoid biosynthetic genes (chalcone synthase, a flavonoid 3' monooxygenase and UDP-glucuronosyltransferase) were down regulated, both at 2DAP and 4DAP.

2.4.2.2.1.1.1. Coumarate-CoA ligase (4CL)

From gene expression analysis, *Of*p251 transcript coding for a 4-coumarate: coenzyme A (CoA) ligase (4CL, EC 6.2.1.12) protein homolog was down regulated at 2DAP. This enzyme catalyzes the conversion of 4-coumarate (4-hydroxycinnamate) and other substituted cinnamates, such as caffeate (3,4-dihydroxycinnamate) and ferulate (3-methoxy-4-hydroxycinnamate), into the corresponding CoA thiol esters (Boerjan *et al.*, 2003). Thus, it catalyzes the formation of CoA esters of hydrocinnamic acid, these activated intermediates being used in the biosynthesis of diverse compounds via specific branch pathways, such as those leading to flavonoids, stilbenes and lignin biosynthesis (Douglas, 1996; Boudet, 2007). This multiple function might explain why 4CL is encoded by a gene family in both angiosperm and gymnosperm species (Wagner *et al.*, 2009 and references therein). In *Arabidopsis thaliana*, 4CL is encoded by a gene family of four members (4CL1-4) which frequently exhibit distinct substrate affinities that could be coincident with specific metabolic functions (Hamberger and Hahlbrock, 2004). For instance, 4CL2 has higher specificity for caffeate than for coumarate, and ferrulate is not a substrate of 4CL2 (Ehlting *et al.*, 2001).

The down regulation of 4CL at 2DAP suggests that orchid lips altered the regulation of genes associated with the phenylpropanoid metabolism by repressing the production of major phenylpropanoids biosynthetic genes, which are no longer required for secondary compounds production (e.g. lignins and flavonoids). The immediate precursors of lignin, monolignols, are biosynthesized via the cinnamate/monolignol pathway, and changes in 4CL expression modifies the metabolic flow of this pathway and ultimately affects the lignin amount, which is the final product of the pathway (Suzuki *et al.*, 2010). In this study, down regulation of 4CL can, as well suggest a decrease in lignin content and an accumulation of *p*-hydroxycinnamic acids (*p*-coumaric, ferulic, and sinapic acid), as reported for tobacco (Boerjan *et al.*, 2003 and references therein), *Arabidopsis* (Boerjan *et al.*, 2003), and poplar (Caihong *et al.*, 2004; Suzuki *et al.*, 2010). The activity of 4CL is essential for the production of the lignin precursor's 4-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Tamagnone *et al.*, 1998). Therefore, 4CL down regulation probably decreased the conversion of *p*-coumaric acid to lignin (Schneider *et al.*, 2003). As a result, down regulation of 4CL may suggest that most hydroxycinnamic acids were glycosylated,

and the resulting hydroxycinnamic acid glucosides as previously reported may be a result of a common detoxification mechanism employed in plants (Meyermans *et al.*, 2000; Suzuki *et al*., 2010). Yet, this assumption has to be confirmed since 4CL belongs to a gene family with 4 isoforms (4CL1-4), with different putative function depending on its substrate specificity (Stuible and Kombrink, 2001; Hamberger and Hahlbrock, 2004).

2.4.2.2.1.1.2. Chalcone synthase (CHS) and bibenzyl synthase (BBS)

In *Ophrys fusca* labellum a transcript coding for a chalcone synthase (CHS, EC 2.3.1.74) was found down regulated at 2DAP. Chalcone synthase, a key enzyme in the flavonoid biosynthesis pathway, catalyses the condensation of 3 acetate residues, from malonyl-coenzyme A (CoA) with 4-coumaroyl-CoA, to form naringenin chalcone (Holton and Cornish, 1995; Ferrer *et al.*, 2008). Because of its key function in the anthocyanin biosynthetic pathway, cDNAs and genes encoding CHS have been used to manipulate flower color (Han *et al.*, 2006b). CHS is encoded by a multigene family in the majority of higher plants, except parsley, *Arabidopsis*, and snapdragon which have only one copy of *chs* gene in their haploid genomes (Winkel-Shirley, 2001). In *Petunia hybrida*, two of the four *chs* genes are expressed in floral tissues and other two genes are induced in young seedlings by UV-radiation (Koes *et al.*, 1987). In previous studies, *Pchs1* gene was moderately transcribed in petals and lips of *Phalaenopsis* at developmental stages where anthocyanins are accumulated at a fast rate (Han *et al.*, 2006b). In apple, *CHS* transcripts were highly expressed also in flowers (Dong *et al.*, 1998). Previous studies on expression of *Phalaenopsis* CHS in tobacco demonstrated that *Pchs* can play a role in anthocyanin biosynthesis as well as in male fertility (Han *et al.*, 2005), thus it can be used to manipulate flower colours of *Phalaenopsis* and, most likely, of other orchid genus. Down regulation of a CHS transcript suggest that at 2 days after pollination labellum has decreased its expression probably related to anthocyanins, a downstream pathway. This may indicate that after successful pollination, flower has fulfilled its reproductive purpose and maintainance of lip attractability is no longer required.

A transcript coding for a bibenzyl synthase (BBS) protein was found down regulated at 2DAP. BBS belong to the type III polyketide synthase superfamily and in *Phalaenopsis* cultivars revealed >98% identity with several CHS sequences (Preisig-Müller *et al.*, 1995). In *Phalaenopsis* sp., this enzyme performs a series of reactions to ultimately render dihydrophenanthrenes (Preisig-Müller *et al.*, 1995). Stressed or wounded orchid tissues, especially those infected by endomycorrhizal fungi, accumulate bibenzyl stilbenes and their tricyclic derivatives, 9,10-dihydrophenanthrenes, to presumably function as antifungal agents (Gehlert and Kindl, 1991; Austin and Noel, 2003 and

references therein). Down regulation of a BBS at 2DAP may be related to an antifungal response thereby stimulating the production of bibenzylstilbenes, which are soon inactivated after pollination. Yet, other putative functions may not be discarded, since high homology to CHS gene has been described for *Phalaenopsis* (Preisig-Müller *et al.*, 1995). Also, according to Sparvoli *et al.* (1994) the enzymes acting early in the phenylpropanoid and flavonoids pathways are encoded by a larger gene family due to the diverse metabolic functions which require a diverse control of gene expression. Gene duplication and molecular divergence may have contributed to the establishment of functionally distinct genes, each one with its own ability to respond to different environmental and/or functional stimuli (Sparvoli *et al.*, 1994). As a result, BBS could be a CHS-like protein or a bibenzyl-producing enzyme, thus until its characterization on *Ophrys fusca* labellum, no specific function may be related to pollination event.

2.4.2.2.1.1.3. Flavonoid 3' monooxygenase/hydroxilase (F3'H) and UDP-Glucuronosyltransferase

Microarray analysis revealed, for both 2DAP and 4DAP, downregulation of two different transcripts coding for flavonoid 3'-hydroxylase (F3'H EC 1.14.13.21), and at 4DAP a transcript coding for an UDP-glucoronosyltransferase protein homolog. These transcripts are involved in the biosynthetic pathway of anthocyanins, important as flower pigments, and its transcription is indicated to be down regulated by pollination.

Flavonoids are subdivided into several classes, the three major ones: flavonols, anthocyanins, and proanthocyanidins (PAs, condensed tannins) (reviewed in Lepiniec *et al.*, 2006). The biosynthesis of anthocyanins begins with the condensation of malonyl-CoA with 4-coumaroyl-CoA, leading to the formation of naringenin chalcone, this reaction being catalysed by chalcone synthase (CHS) (Kleindt *et al.*, 2010). Chalcone is converted into naringenin by chalcone flavanone isomerase (CHI). Naringenin is then hydroxylated, at the 3' position of the central ring, by flavanone 3-hydroxylase (F3H) to produce dihydrokaempferol (DHK). DHK can be further hydroxylated at the 3' position or at both 3' and 5' positions of the B-ring to produce dihydroquercetin and dihydromyricetin, respectively (Han *et al.*, 2010). Dihydrokaempferol, dihydroquercetin and dihydromyricetin lead to the production of the brick-red/orange pelargonidin-, red/pink cyanidin-, and blue/violet delphinidin-based pigments, respectively (Grotewold, 2006). The hydroxylation pattern of the B-ring plays an important role in coloration, and is controlled by two members of cytochrome P450 family, flavonoid 3-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H), by introducing hydroxyl groups at the 3' position or at 3' and 5' positions of the B-ring of the flavonoid molecule,

respectively (Han *et al.*, 2010). In plants, flavonoid hydroxylases highly influence flower coloration (Han *et al.*, 2010). Down regulation of a transcript coding for a F3'-hydroxylase reveals that 3' hydroxylation to dihydroquercetin may be the main flavonoids hydroxylation process occuring in *Ophrys fusca*. This hydroxylation is also widely documented in other higher plants (Han *et al.*, 2010 and references therein).

Epicatechin and anthocyanins share a common biosynthetic pathway from phenylalanine to anthocyanidin. Further, anthocyanins are synthesized via glycosylation by UDP-dependent glucosyltransferases (UGTs), whereas epicatechin by anthocyanidin reductase (Chiou and Yeh, 2008). Anthocyanidins undergo several modifications, such as glycosylation or methylation, by UDP-glucoside: flavonoids 3-O-glucosyltransferase (3GT) and anthocyanin methyltransferase (AMT), to yield the final relatively stable anthocyanins (Chiou and Yeh, 2008). Glycosylation acts on a wide range of biological processes of plants, by the action of glycosyltransferases (GTs) which can transfer single or multiple activated sugars from nucleotide sugar donors to a wide range of small molecular acceptors of plants (Wang and Hou, 2009). A transcript coding an UDP-glucuronosyltransferase matching as an anthocyanidin 3-O-glucosyltransferase (3GT, EC 2.4.1.115), was down-regulated at 4DAP. This enzyme uses preferentially cyanidin, delphinidin and peonidin as substrates and does not catalyze glycosylation at the 5' position of cyanidin. Anthocyanidin 3-O-glucosyltransferase catalyzes the chemical reaction: UDP-D-glucose + an anthocyanidin → UDP + an anthocyanidin-3-O-β-D-glucoside. Thus, the substrates of this enzyme are UDP-D-glucose and anthocyanidins and the resulting products being UDP and anthocyanidin-3-O- β -D-glucoside. Previous studies demonstrated that in almost European orchids, petals present cyanidin glucosides, namely: cyaniding 3-monoglucoside (chrysanthemin), cyanidin 3-diglucoside (mecocyanin), and cyanidin 3,5- diglucoside (cyanin) and unusual anthocyanins of more complex chemical structure (Uphoff, 1979). They are pigments characteristic of the subfamily Orchidoideae and contain the fundamental structure of cyanin. Orchid characteristic pigments are named ophrysanin (3-oxalylglucoside), orchicyanin I (oxalyl-3,5-diglucoside-kaempferol 7-glucoside) and orchicyanin II (oxalyl-3,5-diglucoside (orchicyanin) and its concentrations is genera- or species- specific (Uphoff, 1979). Reports regarding anthocyanin contents on *Ophrys* sp. labellum pointed out for a high pigment similarity between different species mainly dominated by ophrysanin (50-70 %) and with additional chrysanthemin (< 30%) (Strack *et al.*, 1989). Chemically, ophrysanin (cyanidin 3-oxalylglucoside) and chrysanthemin (cyanidin 3-monoglucoside) are similar pigments. It is reasonable to assume that UDP-glucoronosyltransferase may be responsible for the stability of cyanidin derivatives responsible for flower pigmentation in *Ophrys fusca*. To our knowledge, this is the first report regarding the identification of enzymes responsible for lip coloration in *Ophrys*

and even in the subfamily Orchidoideae. Anthocyanins determine the visible and ultraviolet light (UV) patterns of orchids (Kong *et al.*, 2003b), and play important roles in the attraction of pollinators, other than scent and morphology (Stintzing and Carle, 2004). Following pollination, anthocyanins may be destroyed in *Ophrys* as it occurs in other orchids, such as *Vanda* sp. In contrary, in *Cymbidium* sp. they are produced (Kong *et al*., 2003b). Downregulation of anthocyanin related genes (F3'H and UDP-glucoronosyltransferase) at both timepoints suggest that transcription of pigment biosynthesis pathways are regulated by pollination. Since pigmentation, an important visual cue in *Ophrys* pollination system, is required for attracting pollinators, transcription of the regulating enzymes is no longer active after successful pollination.

2.4.2.2.1.1.4. Stilbene synthase (STS)

Up regulation of a transcript coding for a stilbene synthase was detected at 4DAP. Stilbene synthase (STS, EC 2.3.1.95) is a member of the chalcone synthase (CHS) superfamily of type III polyketide synthases (PKSs) (Chong *et al.*, 2009). Chalcone synthase is the most ubiquitous PKS in plants, catalyzing the first committed step of flavonoid biosynthesis. In contrast, in a limited number of plants, e.g. grapevine (Vitaceae), peanut (Cyperaceous), and pine (*Pinus*), stilbene synthases have been identified and revealed to be essential for the synthesis of resveratrol used in the stilbenoid biosynthesis (Schöppner and Kindl, 1984; Melchior and Kindl, 1990; Kodan *et al.*, 2002). STS genes share 75–90 % of its amino acid sequence identity with CHS (Chong *et al.*, 2009). The resveratrol synthesis in grape is catalyzed by stilbene synthase (STS) enzyme which uses *p*-coumaryl- CoA and malonyl-CoA as substrates (Versari *et al*., 2001). Resveratrol (*trans*- 3,5,4'-trihydroxystilbene), its *cis* isomeric configurations and their glucosides (piceides) and oligomers (viniferins) are stilbene compounds classified as phytoalexins due to their role in plant defense mechanisms against fungal pathogens (Hain *et al.*, 1990). Up regulation of a transcript coding for a stilbene synthase at 4DAP, may reveal phytoalexins production enhanced by pollination. Given that phytoalexins are antimicrobial compounds known to be involved in plant defense response, this may be indicative of a defense reaction enhanced by pollination event. To date, enzymes performing STS-like cyclizations (e.g. bibenzyl synthases) have been isolated from *Phalaenopsis* sp. (Preisig-Müller *et al.*, 1995). Stilbene synthases and the related bibenzyl synthases are plant polyketide synthases, which biological functions rely on the formation of antimicrobial phytoalexins (Preisig-Müller *et al.*, 1997). To our knowledge, this transcript may be the first stilbene synthase sequence found in an orchid species, yet needs functional identification and/or confirmation. Being resveratrol the most well studied stilbene-type compound, crucial in a wide range of

biological and pharmacological properties (reviewed in Camins *et al.*, 2009), it will be of extreme interest to further confirm resveratrol synthesis on *Ophrys* labellum.

2.4.2.2.1.1.5. Polyphenol oxidase (PPO)

Three transcripts coding for polyphenol oxidase (PPO, EC 1.14.18.1) homologs were found to be up regulated at 2DAP and 4DAP.

In higher plants, polyphenol oxidase, a nuclear-encoded copper metalloprotein, is the major cause of tissue enzymatic browning and is involved in plant cell walls lignification as well as in polyphenols oxidation producing reactive quinones (Mayer, 2006). The quinone products can then polymerize and react with amino acid groups of cellular proteins, resulting on black or brown pigment deposits (Mayer and Harel 1979; Goldman *et al.*, 1998). Quinones formed during PPO-oxidation reactions may undergo redox recycling, which generate free radicals, and can damage DNA, proteins, amino acids or lipids (Mazzafera and Robinson, 2000; Yoruk and Marshall, 2003). Plant PPO is located in the chloroplast thylakoid membranes and is activated in the cytosol when plant tissues undergo physical damage such as bruising, cutting, ripening, or senescence (Mayer, 2006; Thipyapong *et al.*, 2007). Studies have indicated that phenol-oxidizing enzymes may participate in defense reactions, inducing resistance to abiotic and biotic stresses (Stewart *et al.*, 2001). PPO catalyzes two different oxidative reactions in combination with molecular oxygen: the hydroxylation of monophenols into *o*-diphenols (monophenol oxidation) and the oxidation of *o*-diphenols into *o*-quinones (diphenol oxidation) (Yoruk and Marshall, 2003). In C*amellia sinensis*, the highest activity of this enzyme was reported in cross-pollinated styles rather than in selfed ones (Neog *et al.*, 2004). Studies on *Arachnis* flowers after pollination and emasculation revealed a rise in PPO activity (Hew *et al.*, 1989). A close relationship between ethylene and PPO was suggested in tobacco flowers, where it was shown that ethylene enhanced PPO activity after pollination (Sheen, 1973; Hew *et al.*, 1989). A tobacco flower-specific gene coding for a polyphenol oxidase was considered as having defense functions by controlling phenolic compounds production that will act as signalling molecules (Goldman *et al.*, 1998). Up regulation of three transcripts coding for PPOs at 2DAP and 4DAP may suggest an increased production of quinones, formed by PPO-oxidation reactions, as a result of the physical damage (i.e. RNA degradation, proteinases) promoted by senescence process in labellum enhanced by pollination. This assumption can be supported by studies on rice that related an elevated level of polyphenol oxidase as a consequence of leaf senescence process (Kar and Mishra, 1976). Yet, PPO cannot be considered as an indicator of senescence since its activity

follows irregular patterns and fluctuations of enzyme activities are species specific (Patra and Mishra, 1979).

2.4.2.2.1.2. Alkaloid metabolism

Alkaloids are a diverse group of low-molecular-weight, nitrogen-containing compounds. As secondary metabolites, alkaloids are thought to play a defensive role in protecting plants against herbivores and pathogens (Facchini and St-Pierre, 2005). Thus, due to their potent biological activity, more than 12 000 known alkaloids have been exploited as pharmaceuticals, stimulants, narcotics and poisons.

Down regulation of alkaloid biosynthetic genes (a transcript coding for L-tyrosine/ L-Dopa decarboxylase (TYDC; EC 4.1.1.25) at 2DAP and 4DAP and other coding for salutaridinol 7-*O*-acetyltransferase at 4DAP) was found in *Ophrys fusca* labellum (Table 2.6).

2.4.2.2.1.2.1. Tyrosine/ L-Dopa decarboxylase (TYDC)

In plants, aromatic L-amino acid decarboxylases (AADCs), are involved in the biosynthesis of several types of secondary metabolites, that are defined as compounds that are not essential for normal growth and development, but are often involved in key interactions between plants and their biotic and abiotic environments (Facchini *et al.*, 2000). L- tyrosine/ L-Dopa decarboxylase (TYDC; EC 4.1.1.25) and L- tryptophan decarboxylase are AADCs that have attracted considerable attention due to their roles in the biosynthesis of several important groups of pharmaceutical alkaloids (Facchini *et al.*, 2000). TYDC is involved in the biosynthesis of complex alkaloids, mainly benzylisoquinoline alkaloids, which include more than 2500 known compounds that are pharmacologically active including morphine and codeine (analgesic and antitussic drugs) (Facchini *et al.*, 2000; Schäfer and Wink, 2009). TYDC also participates in cell wall-bound hydroxycinnamic acid amide biosynthesis which suggests a role in defense response since in the cell wall deposition of hydroxycinnamic acid amides, and other phenolics, may create a barrier against pathogens by reducing cell wall digestibility and/or by directly inhibiting fungal hyphae growth (Liyama *et al.*, 1994; Facchini *et al.*, 2000). In flowers, hydroxycinnamic acids may play functional roles as antimicrobial defense compounds against pathogens and as cell wall reinforcement during anthesis (Kang and Back, 2006). Down regulation of TYDC transcript at 2DAP and 4DAP may suggest an inhibition of the alkaloid biosynthetic pathway after pollination. It is known that herbivory can decrease pollination success by reducing resources available for floral displays or rewards (e.g. petal attractability and scent emission), or by damaging

attractive tissues (usually pollinators have preference for less damaged tissues) (Adler *et al.*, 2001). In *Ophrys* pollination system, TYDC can eventually be related to antimicrobial defense response and may be responsible for creating a barrier for herbivory. Ultimately, this would increase pollination events in orchids with a deceptive pollination system, such as *Ophrys fusca*, characterized by low pollination rates (Ayasse *et al.*, 2000; Cozzolino *et al.*, 2005).

2.4.2.2.1.2.2. Salutaridinol 7-*O*-acetyltransferase

At 2DAP, no differential expression of a salutaridinol 7-*O*-acetyltransferase (EC 2.3.1.150) was detected. Down regulation at 4DAP of a salutaridinol 7-*O*-acetyltransferase (EC 2.3.1.150) protein was observed. This enzyme catalyzes the conversion of the phenanthrene alkaloid salutaridinol to salutaridinol-7-*O*-acetate, the immediate precursor of thebaine along the morphine biosynthetic pathway (Lenz and Zenk, 1995; Grothe *et al*., 2001). Since morphine is a benzylisoquinoline alkaloid, its biosynthesis is enhanced uptream by TYDC and ultimately by the salutaridinol 7-*O*-acetyltransferase, the specific enzyme in morphine biosynthesis (Grothe *et al*., 2001). The discovery of a transcript coding for salutaridinol 7-*O*-acetyltransferase in orchids is pioneer. Previous studies performed on *Epipactis helleborine* flowers, a nectar rewarding orchid species, established a correlation between narcotic compounds production such as 3-{2-{3-{3-(benzyloxy)propyl}-3-indol, 7,8-didehydro-4,5-epoxy-3,6-D-morphinan and oxycodone (semisynthetic morphinan) with potential pollinators attraction (Jakubska *et al.*, 2005). Despite the fact that *Ophrys fusca* pollination is achieved by mimicking sex pheromones of the female pollinator species, it cannot be discarded the hypothesis of alluring insects through narcotic compound synthesis, since production of narcotic compounds would increase the chance of pollinators to visit a larger number of flowers. Down regulation of a transcript coding for salutaridinol-7-*O*-acetyltransferase at 4DAP suggest a decrease on morphine biosynthesis, thus potentially reducing pollinator alluring and attraction soon after pollination.

Besides, a down regulation of salutaridinol 7-O-acetyltransferase transcript at 4DAP may emphasize the gene expression decrease of alkaloid biosynthetic pathway after pollination. The creation of a barrier to herbivory/florivory before pollination is mainly based on alkaloids biosynthesis (Adler *et al.*, 2001). Since florivory (McCall and Irwin, 2006) can affect pollination by damaging attractive tissues such as petals (McCall and Irwin, 2006; Kessler and Halitschke, 2009), alkaloid synthesis would constitute a chemical defense to reduce florivore damage, and therefore, is disengage after successful pollination.

2.4.2.2.1.3. Sugar metabolism

Starch is the major storage polysaccharide in plants. Apart from sucrose, starch is also a photosynthesis primary product transiently deposited in the chloroplast. It is composed of glucose polymers, namely amylose and amylopectin. Besides its structural and nutritional function, polysaccharides are involved in plant growth and development (Wang *et al*., 2008). In photosynthesis-competent cells of higher plants, starch degradation of transitory starch results on the formation of glucose and maltose (Fettke *et al.,* 2009). The latter is formed by plastidial β-amylases that act either on glucan chains located at the surface of native starch granules or hydrolyse soluble glucans released by the action of isoamylases (Smith *et al.*, 2005; Edner *et al.*, 2007). Starch degradation can be divided in two steps: initiation of degradation and digestion (reviewed in Fettke *et al.,* 2009). In digestion step, a key role is attributed to β-amylase (Orzechowski, 2008).

2.4.2.2.1.3.1. β-amylase

The primary function of β-amylase (BMY) is starch breakdown. RNAi and studies on mutants have shown that β-amylases are enzymes important for starch degradation (Scheidig *et al.*, 2002; Kaplan and Guy, 2005). BMY expression and activity may be regulated by light, sugars, phytohormones, proteolytic cleavage and abiotic stresses (e.g. osmotic, salt, cold and heat stress) (Kaplan and Guy, 2004). β-amylase is an exo-amylase, responsible for the hydrolysis of the starch granule with the final product being maltose. Maltose is exported to the cytosol and further metabolized to glucose and/or sucrose and maltodextrins by the activity of cytosolic glucosyltransferases during transitory starch degradation (Kaplan *et al.*, 2006). In *Arabidopsis thaliana*, nine genes are known to encode β-amylases mainly expressed in leaves (Orzechowski, 2008). Starch reserves do play a role in reproductive processes. Carbohydrates accumulated in the style support pollen tube growth and starch within the ovule is involved in the development of both ovular structures and embryo (Rodrigo *et al*., 2000 and references therein). Down regulation of a chloroplast-localized β-amylase by antisense mRNA resulted on a starch-excess phenotype in potato leaves compared to wild type plants (Scheidig *et al*., 2002). Studies on gene expression and enzyme activities performed on *Oncidium* sp. pseudobulbs highlight the putative mobilization of starch development by the action of β-amylase to fulfil energy requirement for floral development (Wang *et al.*, 2008). Reports in *Arabidopsis thaliana* (Okada and Shimura, 1994) and in *Vitis vinifera* (Lebon *et al.*, 2005; Lebon *et al.*, 2008) suggest a close relationship among flower development, sexual reproduction and carbohydrate degradation. Therefore, it is

reasonable to suggest a possible role of BMY in *Ophrys fusca* flower development, since down regulation of a transcript coding for this enzyme, at 2 days after pollination, may be related to a deactivation of starch degradation that remains as an energy storage reserve useful for ovule maturation and/or germinating seeds.

2.4.2.2.1.3.2. β**-glucosidase**

β-glucosidases belong to the glycoside hydrolases family, which catalyse the hydrolysis of glycosidic linkages in aryl and alkyl β-glucosides and cellobiose, displaying a broad specificity with respect of both aglycone and the glycone moieties as substrates (Opassiri *et al.*, 2003; Morant *et al.,* 2008). Plant β-glucosidases (EC 3.2.1.21) play important roles in defense, phytohormone regulation, oligosaccharide catabolism, lignification, activation of metabolic intermediates and release of volatiles from their glycosides (Sasanuma and Hirakawa, 2010). These enzymes help defend against herbivores and invasive fungi by hydrolyzing relatively inert glycosides to produce toxic compounds, such as hydrogen cyanide (HCN), saponins, coumarins, quinones, hydroxynamic acid and rotenoids (Poulton, 1990; Opassiri *et al.*, 2003 and references therein). *Arabidopsis thaliana* genome revealed that β-glucosidases are a multigenic family of 48 members (Xu *et al*., 2004), and sequence variations are likely to reflect differences in substrate specificity (Opassiri *et al*., 2003). Also, β-glucosidases are receiving increased attention due to their use in biotechnological and industrial applications, mainly by their importance in aroma formation, e.g. in wine (Fia *et al.*, 2005) and strawberry (Orruño *et al.*, 2001). Studies performed in *Narcissus* flowers, correlated the high activity of β-glucosidase with the increase in scent production (Reuveni *et al*., 1999). In some flowers the aromatic compounds are stored as non-scented glycoside precursors in the vacuoles (Dudareva and Pichersky, 2000). β-glucosidases can remove sugar moiety thereby allowing the release of the volatile fraction (Reuveni *et al*., 1999). Scent plays a ubiquitous role in plant–insect interactions. Plant volatiles display numerous functions since they can attract pollinators, provide learning cues for foraging insects, serve as herbivore repellents or attractants, or attract natural enemies of herbivore (Andrews *et al.*, 2007). Flower scents are prone to exploitation by herbivores. Studies performed on moth-pollinated flowers of *Nicotiana attenuata* suggest the existence of a distinct rhythmicity in release of volatile organic compounds, the emission being high at times of high pollinator activity and low when floral herbivores are most active (Theis, 2006). So far, several herbivore derived elicitors inducing plant defense have been identified, namely fatty acid-amino acid conjugates and a β-glucosidase (Dicke *et al.*, 2009). Also, the removal of the sugar moiety from these compounds has shown to be assisted by the activity of the β-glucosidases in floral

tissue, which in turn correlates with scent production (Reuveni *et al.*, 1999). In *O. fusca*, down regulation of a β-glucosidase at 2DAP may suggest a putative role in scent emission, either by activating plant defense mechanism after herbivory events (Dicke *et al.*, 2009) and/or through its roles addressed in breakage of sugar moieties in non-volatile forms for incrementing its volatility. Studies on *Dendrobium crumenatum* orchid demonstrated a significant decrease of β-glucosidase activity during flower senescence (Yap *et al.*, 2008), which is in accordance with results here reported for *O.fusca*. Other putative roles attributed to β-glucosidase cannot be ruled out. β-glucosidases described in barley (Leah *et al.*, 1995) and rice (Akiyama *et al.*, 1998) appears to be involved in cell wall-derived oligosaccharides recycling produced during germination. Yet, to determine the function of the β-glucosidase of *Ophrys fusca* after pollination, detailed analysis of substrate-specificity and cloning/expression analysis is advised.

2.4.2.2.1.3.3. *Myo*-inositol 1-phosphate synthase

Myo-inositol-1-phosphate synthase (MIPS; EC 5.5.1.4), involved in the *de novo* inositol biosynthesis pathway, catalyzes the conversion of D-glucose 6-phosphate to 1-L-*myo*-inositol-1-phosphate, the rate-limiting step of *myo*-inositol (MI) biosynthesis (Abreu and Aragão, 2007). MI is precursor of compounds that function in phosphorus storage, signal transduction, stress protection, hormonal homeostasis and cell wall biosynthesis (Abid *et al.*, 2009). Compounds derived from *myo*-inositol contribute to mechanisms such as signal transduction (phosphatidylinositols), stress response (pinitol and ononitol), and seed storage (raffinose, phytic acid) (Chiera and Grabau, 2007). MIPS is encoded by a multigenic family that vary among plant species (Meng *et al*., 2009; Eckardt, 2010). Donahue and co-workers (2010) characterized three MIPS genes in *Arabidopsis thaliana*: MIPS1 is expressed in most cell types and developmental stages, being required for seed development and cell death suppression. MIPS2 and MIPS3 are mainly restricted to vascular tissues and, to our knowledge; no putative function has been assigned. MIPS3 sequences from soybean were found predominantly in flowers, but were also found in leaves, buds and germinated cotyledons (Hegeman *et al.*, 2001). Down regulation of a transcript coding for a MIPS gene, suggest a general decrease of *myo*-inositol biosynthesis at 2DAP, which could be related to a possible role on plant development, as shown by studies where down regulation of MIPS activity revealed to be unfavourable for plant development (Meng *et al.*, 2009 and references therein). A recent report shown that MIPS1 has a significant impact on *myo*-inositol levels which is critical for maintaining levels of ascorbic acid, phosphatidylinositol, and ceramides that regulate growth, development and cell death (Donahue *et al.*, 2010). In *Arabidopsis,*

MIPS1 is required for cell death suppression (Donahue *et al.*, 2010). Since ceramidases were down regulated in *Ophrys fusca* labellum at 2DAP (see Lipid metabolism, Sphingolipids section), and ceramides (sphingolipid precursors associated with cell death) accumulation may be responsible for enhancing program cell death cascades, this may suggest a cross-talk between *myo*-inositol levels and cell death program. Yet, MIPS role needs to be further investigated for addressing specific function in development and pollination-enhanced events.

2.4.2.2.1.4. Lipid metabolism

Several transcripts coding for proteins involved in lipids metabolism were encountered in the *Ophrys fusca* pollination microarray under study. Particularly, all transcripts involved in lipid metabolism were down regulated at both 2DAP and 4DAP. Transcripts coding for wax biosynthesis (TCER1), fatty acids (FAD2 and SAD), sphingolipids (ceramidase and glucosylceramidase) and sterol metabolism (7-dehydrocholestrol reductase) are discussed regarding pollination in *Ophrys fusca*.

Lipids are molecules soluble in nonpolar organic solvents naturally occurring in plants or animals. Lipids can be divided in fatty acids (saturated and unsaturated), glycerides, non glycerydes lipids (including waxes, steroids and sphingolipids) and complex lipids (lipoproteins). They can display a wide range of functions, namely: as components of plant cell membranes, as second messengers in signal transduction pathways that elicit plant growth, development and stress responses by creating a physical barrier to environmental assaults, and in photosynthesis (Shah, 2005).

2.4.2.2.1.4.1. Wax biosynthesis

In aerial plant organs, epidermal waxes form a hydrophobic coating which is deposited either outside of the cuticle (epicuticular wax) or within the cuticular matrix (intracuticular wax) (Broun *et al.*, 2004). In addition to its role in protecting plants from water loss, epidermal wax function also in defense response against biotic stresses, such as UV radiation (Nawrath, 2006) and in plant-insect interactions (Post-Beittenmiller, 1996). Epicuticular waxes are complex mixtures of very long chain (VLC, >C18) fatty acids, hydrocarbons (alkanes and alkenes), alcohols, aldehydes, ketones esters, triterpenes, sterols, and flavonoids. Very long fatty acids (VLCFAs), the precursors of most wax components, are produced by elongation of fatty acids (C16 or C18) through a series of four reactions (condensation, reduction, dehydration, and second reduction) catalyzed by the fatty acid elongation (FAE) system (reviewed in Post-Beittenmiller, 1996). Long chain products (C16, C18) are processed by enzymes as

stearoyl ACP- desaturases (SAD) and plastidial acyl transferases, among others. Other components of the wax are produced by three elongation steps: the **decarbonylation pathway**, which leads predominantly to the production of alkanes from long-chain fatty acids and aldehydes; the **acyl reduction pathway** leading to primary alcohols, and **β-ketoacyl elongation** resulting on production of β-diketones and their derivatives (Post-Beittenmiller, 1996; Ohlrogge and Jaworski, 1997). Secondary alcohols and ketones result from enzymatic modification of alkanes.

2.4.2.2.1.4.1.1. Eceriferum 1 (CER1)

Until date, 11 genes have been identified as being involved in wax biosynthesis or in wax regulation, including seven genes that are predicted to encode enzymes of the wax secretory pathway: CER1 (Aarts *et al.*, 1995), CUT1/CER6 (Fiebig *et al.*, 2000), KCS1 (Todd *et al.*, 1999), WAX2 (Chen *et al.*, 2003), FIDDLEHEAD (Yephremov *et al.*, 1999), GLOSSY1 (Sturaro *et al.*, 2005), and GLOSSY8 (Xu *et al.*, 1997). CER1 encodes a fatty aldehyde dercabonylase, a protein involved in the selective secretion of alkanes in epidermal cells (Post- Beittenmiller, 1996; Fiebig *et al.*, 2000; Mariani and Wolters-Arts, 2000). Cheesbrough and Kolattukudy (1984) described for the first time the decarbonylation of an aldehyde intermediate to form alkane. However, conclusive biochemical and molecular genetic evidences are lacking to unravel alkane formation pathways; the less understood part of wax biosynthesis. Sorting alkane pathways would be of extreme importance since cuticular alkanes have potential interest in the fuel sector that until now is based on fossil hydrocarbons (Jetter and Kunst, 2008).

Dowregulation of a gene coding for TCER1 at 4DAP suggests a post pollination reduction in alkane's compounds functioning as olfactory mimic cue, particularly important in plant-insect interaction in *Ophrys* pollination systems (Schiestl and Ayasse, 2001). This result suggests that the emission of compounds triggering *Ophrys* pollinator insect copulatory behaviour is transcriptionally regulated and its decrease was only revealed at 4DAP. One hypothesis for no differential expression detected at 2DAP may be related with: pollinated lips still produce compounds triggering pollination, most likely for attracting pollinators to other flowers of the same inflorescence. Studies on *Ophrys sphegodes* revealed that production of odour compounds that trigger pollination (alkanes and alkenes) was not ceased after pollination (Schiestl and Ayasse, 2001), since alkanes are part of the desiccation-preventing wax layer on the flowers. Therefore, downregulation of TCER1 transcript suggests that production of cuticular waxes compounds, namely alkanes, is transcriptionally decreasing at 4DAP. Cozzolino and Schiestl (2008) revealed that these compounds (alkanes and alkenes) are common components of cuticular waxes that can be regarded as pre-adaptations for

the emission of pseudopheromones (mimicking female pheromones from pollinator species), thus revealing an energy-saving system by exploiting of wax general functions. Bradshaw and co-workers (2010) suggested that cuticular waxes may provide structural support for the elongate trichomes that ornaments all *Ophrys* labella that, in fact, mimic the trichomes of the insect female of pollinator species.

2.4.2.2.1.4.2. Fatty acids Metabolism

Fatty acids are long-chain hydrocarbons which have an acidic end (-COOH). The chain can be either saturated or unsaturated, and fatty acids serve as the hydrophobic portion of the cellular membrane. The desaturation of long-chain fatty acids is a ubiquitous transformation which plays a critical role in the biosynthesis of lipids, and is achieved by double bonds introduction at specific positions in a fatty acid chain by means of different desaturases.

2.4.2.2.1.4.2.1. Desaturases: Omega-6 Fatty Acid Desaturase (FAD2) and Stearoyl ACP-Desaturase (SAD)

The pathway catalyzed by omega 6-fatty acid desaturase, also known as Fatty acid desaturase 2 (FAD2), is the primary route of polyunsaturated fatty acids (PUFAs) production in plants , by performing the first extra-plastidial desaturation by converting oleic acid (18:1, a monounsaturated fatty acid) to linoleic acid (LA) (Byfield and Upchurch, 2007). Linoleic acid (18:2), also known as omega-6 fatty acid, is one of the two essential fatty acids together with α-linoleic (18:3 or omega-3 fatty acid), which cannot be synthesized by mammals and therefore must be obtained from dietary sources (Dyer *et al.*, 2008; Wu *et al.*, 2009). The resulting fatty acids products may constitute structural components of cell membranes (e.g. phospholipids of the endoplasmatic reticulum (ER) and galactolipids of plastids), and a proportion can be used for triacylglycerol (TAG) production (Dyer *et al.*, 2008), which is the main constituent of vegetable oil and animal fats (Wertz, 2000). Stearoyl ACP-desaturase (SAD), commonly known as Δ9 desaturase or as fatty acid biosynthesis 2 (FAB2), is a key enzyme that catalyzes the conversion of stearic (18:0) into oleic acid (18:1) by introducing the first double bond in stearoyl ACP between carbon 9 and 10 (Liu *et al.*, 2009). Linoleic and oleic acids are important in determining the quality of plant oils, since oils with high proportion of this two acids are of nutritional interest for human and animals (Khadake *et al.*, 2009). Δ-9 stearoyl-ACP desaturases are found in all plant cells and are essential for the biosynthesis of unsaturated membrane lipids (Byfield and Upchurch, 2007). SAD is exclusive to plant kingdom since all other desaturases are

known to be membrane proteins (Ohlrogge and Browse, 1995; Shanklin and Cahoon, 1998). SAD plays a key role in C18 fatty acid biosynthesis and its expression and/or enzyme activity have shown to modulate the relative oil levels of both stearic and oleic acids (Byfield and Upchurch, 2007). Recent studies performed in breeding maize lines (Liu *et al.*, 2009) and in *Arabidopsis thaliana* (Kachroo *et al.*, 2007) demonstrated that high SAD mRNA expression play an important role in increasing oleic acid levels. Down regulation at 2DAP of a transcript coding for an omega-6 fatty acid desaturases suggests a decrease of linoleic acid production and the downregulation of two copies coding for SADs at 4DAP suggests a decrease in oleic acid production. Since activity of fatty acid desaturases regulates the levels of unsaturated fatty acids on membrane lipid fluidity (Upchurch, 2008), it can be considered that a decrease on unsaturated fatty acids occurred as a result of senescence-related changes in cell membranes. During flower senescence, membrane properties have been shown to undergo several changes such as increase in the saturation:unsaturation index of fatty acids and membrane phospholipids losses (Leverentz *et al.*, 2002). Our results suggest a decrease in PUFAs biosynthesis transcripts may be related to membrane lipid losses, as reported earlier (Wu *et al.*, 2009). These fatty acids can be further converted into aldehydes by hydroperoxide lyase, jasmonic acid and methyl jasmonate by allene oxide synthase or into epoxides by peroxygenase, all of which can be recycled by the plant (Brash, 1999; Koch *et al.*, 1999).

Recent evidences point out for the possibility of different desaturases, introducing double bonds at specific positions in fatty acids, may give rise to a vast array of unsaturated fatty acids and, ultimately, to alkenes (Schlüter and Schiestl, 2008). In *Ophrys*- pollinator crosstalk, a blend of alkanes and alkenes (e.g. saturated and unsaturated hydrocarbons, respectively) with different carbon chain lengths are responsible for chemical mimicry of the pollinator insect species (Schiestl, 2005). Alkane and alkene biosynthesis are part of the wax biosynthetic pathway, until now poorly understood. As alkane formation pathway seems to undergo decarbonylation in FAE system, alkenes production pathway has been linked to an elongation system which includes a desaturase (von Wettstein-Knowles, 2007). Schlüter and Schiestl (2008) suggested that alkene biosynthetic pathway should follow alkane's synthesis, except on alkene formation that would need additional desaturation steps. For instance, in *Ophrys sphegodes*, pollinated by *Andrena nigroaenea*, pseudocopulation is elicited mostly by 9-alkenes (Schiestl *et al.*, 2000), and recently a SAD2 has been attributed for introducing double bonds at 9- and 12-positions (Schlüter *et al.*, 2011). Thus, a SAD from *Ophrys fusca* coding for an homologue for SAD 2 (Table 2.6.) may be related to a putative function on 9- and 12 alkenes double bonds introduction (Schlüter *et al.*, 2011). This assumption is corroborated by recent studies performed in barley, where the synthesis

of the 9-alkene compounds depends on a desaturase acting at the 9-10 position, as it happens with the introduction of the first double bond into the C18 products of the fatty acid synthase in plastids (von Wettstein-Knowles, 2007). It is also supported by studies on *Cistus* sp. petals, where the 9-10 double bonds in the C23-C33 alkenes depends on a desaturases (Gülz, 1980). Therefore, it is conceivable that, as in *Drosophila melanogaster* (Dallerac *et al.*, 2000), alkenes with double bonds depend on enzymes acting preferentially on different acyl chains (C14, C16, C18) as substrates to yield a vast array of alkenes. Furthermore, the possibility of omega 6-desaturase to have a putative role on alkene biosynthesis cannot be discarded, given that previous studies revealed that alkanes and alkenes, according to their chain length and positions of double bonds, may be derived from palmitic, stearic, oleic, linoleic or linolenic acid (Ney and Boland, 1987). The down regulation of two transcripts coding for different desaturases suggests that both alkane and alkene biosynthesis decreased in labella 4 days after pollination. This cessation could be related to a decrease in compounds production, no longer needed after pollination. Previous reports on *Ophrys sphegodes* pollination verified a slight decrease in odour bouquet on labella extracts (mainly composed of alkanes and alkenes) after pollination yet with no statistical significance (Schiestl and Ayasse, 2001). The involvement of both desaturases in *Ophrys* pollination ecological relationship, through the insertion of double bonds at different positions at fatty acid substrates of distinct carbon chain lengths, seems to be a promising challenge that yet needs to be confirmed. Recently, expression of two SAD isoforms, SAD1 and SAD2, in two *Ophrys* species revealed to be flower-specific and thus SAD2 has shown a significant association with alkene production (Schlüter *et al.*, 2011).

2.4.2.2.1.4.2.2. 3-ketoacyl-CoA thiolase (KAT)

The breakdown of fatty acids, performed by the β-oxidation cycle, is crucial for plant germination and sustainability (Pye *et al.,* 2010). β-oxidation involves four enzymatic reactions. The final step, in which a two-carbon unit is cleaved from the fatty acid, is performed by a 3-ketoacyl-CoA thiolase (KAT) (Castillo and Leon, 2008). In our microarray, up regulation of a KAT transcript at 4DAP suggest that fatty acids are being degraded via β-oxidation cycle. This process can mobilize carbon stored for further use and can eliminate deteriorative effects of free fatty acids released from lipid turnover (Gerhardt, 1992). Fatty acid breakdown in peroxisomes have been observed in both leaf and petal senescence (Pistelli *et al.*, 1991; Graham and Eastmond, 2002; Hoeberichts *et al.*, 2007; van Doorn and Woltering, 2008).

2.4.2.2.1.4.3. Sphingolipids metabolism

The basic building block of sphingolipids is an amino alcohol long chain base (LCB). In plant cells, ceramide (which is the basic element for more complex shingolipids) is formed by acylation of the LCB amine group with a fatty acid (FA), generally composed of 14–26 carbon atoms (Pata *et al.*, 2010). Plant sphingolipids can be divided into four classes: glycosylinositolphosphoceramides (GIPCs), glycosylceramides (GlcCer), ceramides (Cer) and free long-chain bases (LCBs) (Zäuner *et al*., 2009). Uncovering possible functions for each of the sphingolipids classes is complex and challenging, since sphingolipid metabolites are being continuously converted into each other via *de novo* synthesis, through condensation of serine with an acyl-CoA, and salvage pathway, where ceramides and free LCBs are released from more complex sphingolipids (Zäuner *et al*., 2009). In the past few years, sphingolipids from plants have been subject of interest for their putative functions as plasma membrane and lipid rafts components, as being involved in signaling of plant response to drought and in regulation of programmed cell death (PCD) (Shah, 2005; Markham *et al*., 2006).

2.4.2.2.1.4.3.1. Glucosylceramidase and neutral ceramidase

In plants, ceramide (Cer) can be formed via two pathways: the FA-CoA dependent and free FA-dependent pathways, being the FA-CoA dependent pathway the major route through which ceramide is synthesized (reviewed in Pata *et al*., 2010). Cer can undergo further modifications resulting on the formation of complex sphingolipids such as GIPCs and glycosylceramides (GlcCer). Ceramide is a major intracellular messenger that mediates cellular responses in apoptosis, senescence, cell cycle arrest and differentiation (Hannun *et al.*, 2001; Choi *et al*., 2003). Ceramidases, known as key regulators of sphingolipid signaling metabolites, hydrolyzes ceramide into a long chain shinganine (in animals, sphingosine) and a free FA (Kono *et al.*, 2006). Ceramidases are considered acid, alkaline and neutral, based on the pH at which their activity is optimal. In *Arabidopsis thaliana*, three ceramidase-like genes encoding neutral and alkaline ceramidases were described (Liu and Yu, 2009). Since neutral ceramidases regulate the availability of the Cer and LCB polls, functions as key modulators of cellular processes and signaling have been addressed (Pata *et al.*, 2010). In animals, GlcCer, the simplest member of glycosphingolipids, has been involved in many cellular processes such as cell proliferation, oncogenic transformation, differentiation and tumour metastasis (Bleicher and Cabot, 2002 and references therein; Sandhoff and Kolter, 2003). In plants, GlcCer are extremely abundant in plasma

and vacuolar membranes (Pata *et al.*, 2010), and may function in chilling/ freezing tolerance, pathogenesis, membrane permeability and stability (Sperling and Heinz, 2003). Glycosylceramidase is a lysozomal enzyme, which cleaves glucosylceramide to glucose and ceramide. Down regulation of two sphingolipid -related genes was detected in our study, namely of neutral ceramidase (2 DAP) and glucosylceramidase (EC: 3.2.1.45, 2 DAP and 4 DAP) transcripts. Ceramidases can regulate the levels of ceramide (substrate) and/or its by-products sphingosine and sphingosine 1-phosphate (S1P) (Hannun and Obeid, 2002, 2008). Glucosylceramidases hydrolyze glucosylceramides, complex sphingolipids, to render *de novo* ceramide (Pata *et al.*, 2010). While ceramide is a well-established inducer of apoptosis/PCD in animals (Hannun and Obeid, 2008), the related S1P has been shown to suppress PCD in animals (Cuvillier *et al.*, 1996). It has been described that maintenance of sphingolipid homeostasis is important for regulating apoptosis in plant cells (Shi *et al.*, 2007), namely regarding the pool balance of ceramide and its derived compound S1P. In plants, the first evidence for the involvement of ceramide in PCD was reported by Liang and co-workers (2003), and later studies validate the same feature (Gechev *et al.*, 2004; Shi *et al.*, 2007). In our microarray, down regulation of different ceramidases suggest a decrease of ceramide hydrolysis to render S1P (an anti-apoptotic sphingolipid) and also a decline on glucosylceramide hydrolysis to ceramide, which may point out to an imbalance on ceramide and S1P pools that could be related to a sphingolipid-regulated PCD enhanced after pollination.

2.4.2.2.1.4.4. Sterol metabolism

Phytosterols (also called plant sterols) are a group of steroid alcohols, phytochemicals naturally occurring in plants (Hartmann, 1998; Weingärtner *et al.*, 2009). They are synthesized via melavonate pathway of the isoprenoid metabolism (Schaller, 2004) and, in plant cells, phytosterols contribute to the regulation of the fluidity and permeability of cell membranes and affect growth processes, such as embryogenic growth (Shaller, 2003). Phytosterols also sustain the domain structure of cell membranes where they are considered as membrane reinforcers, since sterols are critical for the formation of lipid rafts (Dufourc, 2008). Certain sterols, such as campesterol in *Arabidopsis thaliana*, are precursors of oxidized steroids acting as growth hormones collectively named brassinosteroids (Schaller, 2003). The crucial importance of brassinosteroids upon growth and development has been established through the study of a set of dwarf mutants affected in brassinosteroids synthesis or perception (Choe *et al.*, 1999, 2000; Nam and Li, 2002).

2.4.2.2.1.4.4.1. 7-dehydrocholestrol reductase

End products of the sterol biosynthetic pathway differ depending on species: cholesterol is encountered in animals; ergosterol is the most common sterol in fungi; while sitosterol, campesterol, cholesterol and stigmasterol are typical plant sterols (Lecain *et al.*, 1996; Weingärtner *et al.*, 2009). In mammals, the sequential steps of cholesterol biosynthesis, 7-dehydrocholestrol reductase (Δ7SR, EC 1.3.1.21) catalyzes the reduction of the Δ7 double bond in sterol intermediates (Zhang *et al.*, 2007). Plants that have an inefficiency of this enzyme can display dwarfism resulting in severely altered growth and development (Choe *et al.*, 2000). Brassinosteroids (BRs) are a group of steroid hormones that uses mainly campesterol as precursor for its biosynthesis and are known for roles in regulation of plant growth and development, such as stem elongation, tracheary element differentiation and regulation of gene expression (Fujioka and Yokota, 2003; Nomura *et al.*, 2004) and also by acting as hormone signals (He *et al*., 2003). Cellular defects in BRs biosynthesis or response, often result in a characteristic dwarf syndrome due to a defect on cell expansion (Nomura *et al.*, 2004 and references therein). The deficiency in Δ7SR is the main cause of dwarfism phenotype in *Arabidopsis*, known as *dwf5* (Choe *et al.*, 2000). Down regulation of a transcript coding for a Δ7SR in *Ophrys fusca* labella at 2DAP suggests that growth and development are compromised after pollination, not neglecting that the event may have occurred before. Since brassinosteroids are important plant hormones that are involved in cell expansion and elongation (working together with auxin) (Mockaitis and Estelle, 2004), in promoting vascular differentiation, and are necessary for pollen elongation (Hewitt *et al.*, 1985; Ye *et al.*, 2010), the observed down regulation of this transcript suggests that all these developmental processes were compromised after pollination.

2.4.2.2.1.5. Nitrogen, sulfur and selenium metabolism

Sulfur (S) is an essential macronutrient for plants, and is present at 0.1-1% of plant dry weight, depending on the plant family and soil type (Pilon-Smits and Pilon, 2007). Sulfur is an essential element for plant primary metabolism as a structural component of proteins and lipids, antioxidants, regulatory molecules, metal-binding molecules and cofactors (Pilon *et al*., 2006). NifS-like proteins, also known as cysteine desulfurases (EC 2.8.1.7), catalyze the conversion of the amino acid cysteine into alanine and elemental sulfur (S) (Mihara and Esaki, 2002; van Hoewyk *et al*., 2007). In plants, NifS-like proteins may play roles in Fe-S formation, biosynthesis of thiamine, biotin, molybdenum cofactor (MoCo), also in seleno(Se)-protein and Se tRNA synthesis due to its selenocysteine lyase

activity (Xu and Møller, 2006; van Hoewyk *et al.*, 2008). Down regulation of a transcript coding for a Nifs-like protein at 4DAP may suggest that Fe-S protein formation, which is dependent on NifS-like proteins activity, was disrupted by the pollination event. Consequently, as previously described, functions including protein import, chlorophyll metabolism, nitrogen and S reduction, respiration and photosynthetic electron transport chain are compromised (Xu and Møller, 2006). Also, it has been addressed a possible role of a NifS-like protein in Fe–S cluster development in pollen (van Hoewyk *et al.*, 2008), however more studies are needed to disclose a putative function in orchid reproductive biology.

2.4.2.2.1.6. Amino acid metabolism

2.4.2.2.1.6.1. Ornithine-δ-aminotransferase

Organic solutes such as proline, glycine betaine, sugars, and polyols, accumulating at high concentrations in the cytoplasm in response to abiotic stress without interfering with primary metabolism, are classified as 'compatible' solutes, (Verbruggen and Hermans, 2008). High levels of compatible solutes are found not only under stress conditions, but also in plant organs such as pollen and seeds undergoing dehydration as part of their development (Lehmann *et al.*, 2011). In higher plants, proline (Pro) biosynthesis involves conversion of glutamate into pyrroline-5-carboxylate (P5C) by P5C synthase (P5CS) and subsequent reduction of P5C to proline by P5C reductase (P5CR) (Trovato *et al.* 2008; Verbruggen and Hermans, 2008). In an alternative pathway of proline synthesis, the activity of ornithine-δ-aminotransferase (δOAT; EC 2.6.1.13) converts ornithine and α-ketoglutarate to P5C and glutamate by transamination, yet this biosynthetic pathway does not contribute to stress-induced proline accumulation (Funck *et al.*, 2008; reviewed in Stránská *et al.*, 2008). In *Arabidopsis* mutants lacking δOAT activity, plants were unable to mobilize nitrogen from arginine or ornithine but they showed proline accumulation, which supports the role of δOAT mainly in arginine degradation (Funck *et al.*, 2008). *Arabidopsis* uses arginine (Arg) as a storage and transport form of nitrogen and proline (Pro) as a compatible solute in the defense against water deprivation abiotic stresses (Funck *et al.*, 2008). Arg catabolism produces ornithine (Orn), which is a controversial precursor alternative to glutamate (Glu) for Pro biosynthesis in mitochondria (Goldraij and Polacco, 2000). Down regulation of a transcript coding for an ornithine-δ-aminotransferasewas observed in *Ophrys fusca* at 2DAP. Previous studies suggest that proline plays an important role in plants development, especially in flowers and pollen, serving as an accessible source of energy (Kavi Kishor *et al.*, 2005). In flowers with nectars as rewards,

its content is usually proline-rich (Carter *et al.*, 2006). Since *Ophrys* is a nectarless orchid genus this possibility has to be discarded. In the absence of stress, *Arabidopsis thaliana* revealed high content and active transport of Pro during flowers development (Hua *et al.*, 1997). High expression levels of ProT1and LeproT encoding a proline transporter have been identified in *A. thaliana* (Rentsch *et al.*, 1996) and tomato (Schwacke *et al.*, 1999) flowers. Recent studies revealed that proline biosynthesis and transport contribute to proline accumulation in flower buds and flowers (Xue *et al.*, 2009), and that higher Pro turnover in flowers than in other organs of the plant may be responsible for providing energy and/or carbon and nitrogen sources for flower development (Hua *et al.*, 1997). The role of proline in plant cells response to stress is well documented, and Pro has been proposed to display a function in flowering and development both as a metabolite and as a signal molecule (Mattioli *et al.*, 2009). Proline has multiple functions, such as scavenger of toxic substances, protector of different enzyme systems and membranes stabilizer (Fricke and Pahlich, 1990). Down regulation in *O.fusca* at 2DAP of a transcript coding for a putative ornithine-δ-aminotransferase suggests that proline is no longer synthesized via the alternative Pro biosynthesis pathway after pollination. Since higher accumulation of proline allows plants to maintain an osmotic balance preventing tissue dehydration (Ali *et al.*, 2006), after pollination labella are no longer experiencing a developmental process and thus, hydration of flower tissues are not required for organ maintenance. Accumulation of proline in floral organs of different species has been described by different authors, suggesting that this amino acid may play a role in flowering process (Hua *et al.*, 1997; Mattioli *et al.*, 2008, 2009; Xue *et al.*, 2009).

The significance of the alternative pathway and δOAT activity in proline biosynthesis has been questioned, since proline levels were not affected in *Arabidopsis thaliana* knock out mutants (Funck *et al.*, 2008; Szabados and Savouré, 2010). Instead, δOAT may facilitate nitrogen recycling from arginine catabolism (Funck *et al.*, 2008; Stránská *et al.*, 2008). Thus, the main function of δOAT in *Arabidopsis* may be related to ornithine catabolism rather than to proline synthesis (Funck *et al.*, 2008). The first step of Arg breakdown is the cleavage into Orn and urea by arginase and Orn catabolism seems to depend on δOAT activity (Shargool *et al.*, 1988; Funck *et al.*, 2008). In *Arabidopsis,* the conversion of Orn to Glu, through δOAT activity, is an essential route for nitrogen recycling from arginine but not for stress-induced proline accumulation (Funck *et al.*, 2008). L-arginine is the amino acid with the highest content of N (four atoms per molecule) and represents the major source of N for the biosynthesis of nitrogenous compounds. Down regulation of a transcript coding for δOAT, an enzyme putatively involved in Orn catabolism, may suggest a role of accumulating Orn in *O. fusca* at 2 days after pollination. Available Orn can re-enter the Arg biosynthetic pathway

(Slocum, 2005) and Arg can be used as N sink, as it has been proposed for poplar senescence in which Arg accumulation in stems may function as N storage compound (Couturier *et al.*, 2010).

2.4.2.2.1.6.2. Methylcrotonyl-CoA carboxylase α-subunit

Plants catabolize leucine (Leu) via two separated pathways: a peroxisomal pathway not requiring methylcrotonyl-CoA carboxylase (MCC) and a mitochondrial one, a MCC-requiring pathway that catabolizes Leu to acetoacetate and acetyl- CoA (Che *et al.*, 2002, 2003). MCC (EC 6.4.1.4) is a biotin enzyme, composed of two subunits: biotin-containing α- subunit (MCC-A) and non-biotin-containing β-subunit (MCC-B) (Alban *et al.*, 2000; Nikolau *et al.*, 2003). This enzyme has a pivotal role in both leucine catabolism and carbon recycling from isoprenoids via the mevalonate pathway (Anderson *et al.*, 1998; Che *et al.*, 2002). Leucine (Leu) catabolism revealed to be down regulated at 2DAP, through the expression of mitochondrial methylcrotonyl-CoA carboxylase enzyme (MCC). Leucine catabolism is known to provide an alternative source of acetyl-CoA to sustain respiration and metabolic processes in the absence of photosynthesis (Mentzen *et al.*, 2008). Elevated MCC mRNA levels in flowers have been related to increases on Leu catabolism to guarantee ATP demands essential for growth (Mckean *et al.*, 2000). As a result, down regulation at 2DAP of MCC transcript suggest that Leu catabolism was inhibited after pollination, since catabolically derived ATP was no longer required. Also, if Leu is not being catabolized after pollination, we can speculate that this amino acid could be further used for newly protein synthesis required for proper enhancement of senescence (see protein synthesis section below).

2.4.2.2.1.8. Nucleotide/nucleoside/nucleobase metabolism

Ureides result from nitrogen fixation via purine degradation, the majority being associated with ammonia assimilation (Werner *et al.*, 2010; Witte, 2011). Ureides are produced in senescing tissues, such as seedling cotyledons, which reserves support the developing seedlings (Todd and Polacco, 2006). The three major ureides are allantoin, allantoic acid and citrulline. In *Arabidopsis*, allantoin is converted to ureidoglycolate and ammonia by allantoinase or allantoate amydohydrolase (AAH, EC 3.5.2.5.) (Todd and Polacco, 2006). In specific ureide-accumulating organs (e.g. roots), allantoin and/or allantoate are translocated to other plant organs, such as shoots and leaves, where they are completely degraded (Todd *et al.,* 2006*)*. In plants, allantoate degradation is required for recycling the nitrogen purine-ring (Werner *et al.*, 2008). Up regulation of a transcript coding for AAH at 4DAP suggests the mobilization of nitrogen

resulting from purine catabolism. This feature is especially important during flower senescence, in which nitrogen source tissues (i.e. senescing flowers) provide nitrogen for metabolic sinks (Witte, 2011). Increased levels of purine catabolism enzymes have been reported in senescent leaves (Brychkova *et al.*, 2008).

2.4.2.2.2. Cell fate

2.4.2.2.2.1. Cell aging and program cell death (PCD)

Programmed cell-death (PCD) is defined by the death of a cell or cells mediated by an intracellular program. Senescence can be considered a type of PCD, since it involves structural, biochemical and molecular changes similar to those of PCD (Tripathi and Tuteja, 2007). Petals constitute an excellent model for studying senescence since they have a finite lifespan and their death relies on a highly coordinated developmental control. Once a flower is pollinated or is no longer receptive for pollination, its maintenance is extremely costly in terms of respiratory and water loss requisites (Jones *et al*., 2005). Petal senescence is controlled by growth factors and hormones, like ethylene, auxin and abscissic acid (Mea *et al.,* 2007; Bai *et al.*, 2010). In ethylene- sensitive flowers, like orchids, the first response to pollination encompasses an increase of ethylene production by the stigma often followed by increased ethylene production in ovaries and petals (Klee and Clark, 2010). The pollination-induced ethylene in different floral organs is responsible for coordinating pollination-associated events such as ovary growth and perianth senescence (Llop-Tous *et al.*, 2000). In carnation and *Phalaenopsis* orchid spp., genes encoding ACC oxidase and ACC synthase are responsible for the post-pollination ethylene burst that induces petal senescence (Porat *et al*., 1994; Tang *et al*., 1994; Nadeau and O'Neill, 1995; Woltering *et al*., 1995). Ethylene-inducible transcripts from petals including mRNAs with homology to glutathione *S* transferase (Itzhaki *et al*., 1994), phosphoenolpyruvate mutase (Wang *et al.*, 1993) and cysteine proteinase were also identified (Jones *et al*., 2005). Previous results on orchid flowers revealed low activity of ethylene biosynthetic enzymes in labellum, with a peak at 12h after pollination (Bui and O'Neill, 1998).

Induction of cysteine proteinases during leaf (De Michele *et al*., 2009) and flower (Xu and Hanson, 2000) senescence have been considered as an outcome of the PCD event. According to their catalytic mechanisms, proteases are classified into serine (Ser), cysteine (Cys), aspartic, and metallo proteases (van der Hoorn *et al.*, 2004). Cys proteases (EC 3.4.22) respond to a variety of stimuli and may be involved in protein maturation, degradation and rebuilt. They also function as a housekeeping to remove

misfolded proteins (Grudkowska and Zagdańska, 2004). Proteolysis by cysteine proteases is a highly regulated process. Cysteine proteases include different families such as: calpains, caspases and papain-type proteinases. The papain-type cysteine proteases (e.g. triticain α, β and γ) are the most thoroughly investigated group among cysteine proteases, mainly in *Brassica oleracea* (Eason *et al.*, 2005) and barley (Kiyosaki *et al.*, 2009 and references therein). Aspartic proteases have been involved on protein processing and/or degradation in different plant organs, in plant senescence, stress responses, programmed cell death and sexual reproduction (Simões and Faro, 2004). An aspartic protease functioning as anti-cell-death component was identified *in Arabiodopsis*, revealing a putative involvement on enhancing PCD event (Ge *et al.*, 2005). Down regulation of a transcript coding for an aspartic proteinases in *O. fusca* labellum at 4DAP may be related to a function as an anti-cell death component that was inhibited after pollination, and as result, PCD and, consequently, senescence has been enhanced. A role on *O. fusca* stress response cannot be discarded, since the plants under study were originated from natural populations possibly challenged by pathogens and other biotic and/or abiotic agents. Up regulation of a different set of cysteine proteases in *O.fusca* at 2DAP, and mostly at 4DAP, reveals that pollination triggers proteolytic activity. Since the flower is essential for sexual reproduction success, its lifespan is closely related to pollination which ultimately triggers floral death by proteolysis activation.

In *O. fusca* microarray, up regulation of a transcript coding for a tropinone reductase-14 at 4DAP was detected. Tropinone reductase-like (TRL) enzymes may have an array of putative functions (reviewed in Dräger, 2006). TRL enzymes, which are short-chain dehydrogenases, act on alkaloid biosynthesis in Solanaceae. In *Arabidopsis thaliana* as well as in other *Brassicaceae*, these enzymes may have yet unknown functions, since tropane alkaloids have been detected neither in *Arabidopsis thaliana* nor in other species studied (Sato *et al.*, 2001; Oksman-Caldentey *et al.*, 2007). since TRL-14 has been shown to be a SAG13 homolog, one of the best markers of senescence before beginning of wilting in *Arabidopsis* (Miller *et al.*, 1999) and on grapevine (Espinoza *et al.*, 2007). As a result, TRL-14 may be associated with senescence event, as supported earlier on leaf senescence studies (Miller *et al.*, 1999; Espinoza *et al.*, 2007).

Down regulation of a transcript coding for an *Hypersenescence* (HYS) 1 protein was detected in *O. fusca* labellum at 2DAP. Studies on *Arabidopsis* (Aki *et al.*, 2007) suggest that HYS1 plays a role in sugar signalling and defense response, thereby controlling growth and development. Also, HYS1 was recognized as playing a role in inhibiting leaf senescence and pathogen-defense responses (Yoshida *et al.*, 2002b). Down regulation of this gene in *O. fusca* labellum at 2DAP may suggest an important

role in flower senescence signalling, by acting as a putative senescence initiation signal.

Up regulation of two transcripts coding for ribonucleases (RNases) was detected 2 and 4 days after pollination. RNases are RNA-degrading enzymes associated with different physiological processes such as senescence and phosphate remobilization (Ma and Oliveira, 2000 and references therein) and responses to environmental stresses (Chen and Deutcher, 2005). Plant RNases family is divided in S-like RNases and S-RNases, being the second specifically involved in self-incompatibility control (Bariola *et al.*, 1999). S-like RNase genes constitute the major family of RNA-degrading enzymes in plants (Green, 1994). In contrast to the S-RNase genes, which expression is generally restricted to the style, S-like RNase genes are often related to several physiological processes including phosphate starvation, senescence, wounding, cell death pathway, defense against pathogens and the light signalling pathway induced in other organs (Taylor *et al.*, 1993, Liang *et al.*, 2002, Lers *et al.*, 2006). The activation of two RNase PD2 transcripts at both 2DAP and 4DAP suggests that RNA degradation is occurring early after pollination, as a consequence of the pollination enhanced-senescence. The RNase PD2 belongs to a class of S-like RNases family (Ma and Oliveira, 2000), and RNase activity has been shown to increase during petal senescence (Panavas *et al.*, 2000; Xu and Hanson, 2000). Roles have been addressed to RNase PD2 on phosphate (Pi) mobilization (Hu *et al.*, 2011) and removal of RNA from the dying cells (Lers *et al.*, 1998).

2.4.2.2.2.2. Cell enlargement

Expansins are important regulators of wall extension during plant cell growth (reviewed in Sampedro and Cosgrove, 2005) being involved in cell wall loosening and extension with possible roles in fruit ripening (Civello *et al.*, 1999) and in softening of the maternal tissues for faster pollen tube penetration during pollination (Cosgrove, 1998). Expansins have been associated to wall loosening in maternal tissues in order to assist rapid penetration of pollen tubes, rather than stimulating pollen tube growth itself (Sanchez *et al.*, 2004; Yoshida *et al.*, 2005). Expansins are pH-dependent wall-loosening proteins frequently associated with the acid growth hypothesis, which refers to the ability of plant cells and/or cell walls to elongate or expand quickly at acidic pH (reviewed in Rayle and Cleland, 1992). Auxin pumps protons out of the cell decreasing apoplastic pH, and thus activating expansins activity (Rayle and Cleland, 1992). Several roles have been suggested for auxin in the pollination process (Sundberg and Østergaard, 2009). Ethylene and auxin are known to play key roles in regulating the biochemical and anatomical changes in orchid's post pollination phenomena and flower senescence mediation (Zang and O'Neill, 1993; Attri *et al.*, 2008). Thus it is

possible that these two hormones are active during postpollination events. Up regulation of transcripts coding for expansins in *O. fusca* at 4DAP may be a consequence of auxin action inducing a post pollination phenomenon, which is critical for ovary maturation and growth in orchids (Ketsa *et al.*, 2006). This hypothesis is supported by studies performed in *Dendrobium* and other orchids, according which ovule development is triggered by pollination and ovary growth starts within two days after pollination (Ketsa *et al.*, 2006), a timeline rather unique comparing to the majority of flowering plants in which the ovules are mature and the egg cells are ready to be fertilized at anthesis (Yu and Goh, 2001). Since transcripts coding for auxin- responsive genes (Jain and Khurana, 2009) were not detected in *Ophrys fusca* pollination microarray, auxin action can be suggested only based on assumptions of their role in mediating post pollination phenomena in orchids proposed by other authors.

2.4.2.2.3. Cell rescue, defense and virulence

Microarray studies and analyses of individual genes have revealed a set of genes up regulated during petal senescence generally assumed to be stress related (van Doorn *et al.*, 2003; Wagstaff *et al.*, 2010). Those include metallothioneins, abscisic acid (ABA)-responsive genes (Breeze *et al.,* 2004) as well as glutathione *S*-transferases genes (Meyer *et al.*, 1991; Price *et al.*, 2008). A significant number of up regulated senescence genes are also pathogenesis related (Hanfrey *et al.,* 1996; Thomas *et al.*, 2003). Previous authors reported that senescence of *Iris* petals is accompanied by the induction of a considerable number of putative defense-related genes (van Doorn *et al.*, 2003). Regarding *Ophrys fusca* pollination, up regulation of defense-related genes may be related with flower senescence, as proposed in leaf senescence (Bhalerao *et al.*, 2003; Hoeberichts *et al.*, 2007).

Pathogenesis- related (PR) proteins are usually induced by pathogens and abiotic stresses (Xie *et al.*, 2010). Unlike most other PR proteins with an extracellular destination, PR10 proteins, typically intracellular and small (16-18 kDa), are slightly acidic and resistant to proteases (Liu and Ekramoddoullah, 2006; Xie *et al.*, 2010). Up regulation of a transcript coding for PR10 protein was detected at 2DAP and 4DAP. Also, previous studies found PR10 mRNA expression in senescing soybean (Crowell *et al.* 1992) and *Betula pendula* (Valjakka *et al.*, 1999) leaves. It has been described that PR genes are also co-expressed with cyclophilins (Godoy *et al.*, 2000; Kong *et al.*, 2001). In fact, up regulation of a transcript coding for a cytosolic cyclophilin was detected in *O. fusca* at 4DAP. Cyclophilins (Cyps) are ubiquitous proteins originally identified as intracellular targets for the immunosuppressant cyclosporin A (CsA) (Kiełbowicz-Matuk *et al.*, 2007). Plant CyPs are supposed to play essential roles in protein

maturation/trafficking and in the processing of nucleic acids (Kiełbowicz-Matuk *et al.*, 2007). In flowers, high levels of cytosolic cyclophilins transcripts have been reported (Chou and Gasser, 1997). Up regulation of a cytosolic cyclophilin in *O. fusca* labellum at 4 DAP suggest a potential role in PCD machinery, since cyclophilins are known to display endonuclease activity, responsible for supercoiled DNA degradation and, ultimately, PCD (Montague *et al.*, 1997; Kong *et al.*, 2001).

Two different Abscicic Stress Ripening (ASR) genes were regulated by pollination: ASR down regulated at 4DAP and ASR1 up regulated at 2DAP. ASRs are transcription factors whose likely targets are hexose transporters and abscicic acid (ABA) responsive genes (Frankel *et al.*, 2006). Since ASR transcripts accumulate following stress conditions, their expression suggests a downstream action of a common signal transduction pathway involved in responses triggered by different abiotic stresses (Carrari *et al.*, 2004). The *ASR* gene family includes *ASR*1, *ASR*2, *ASR*3, and *ASR*4. *ASR*1, *ASR*3, and *ASR*4 are induced by dehydration, ABA, and cold stresses, whereas *ASR*2 is ABA-independent and is specifically induced by dehydration (Dóczi *et al.,* 2005). As a result, up regulation of an ASR1 at 2DAP may be related to petal dehydration. Recent studies have shown an early senescence induction of a petunia *ASR4* gene expression at 2DAP (Bai *et al.*, 2010), which is in accordance with our results. Putative function attributed to *PhASR4* supports a potential role in senescence signaling, whether by petal dehydration or increased ABA levels. Regarding, down regulation at 4DAP of a transcript coding for an *ASR* could be related to one of the signaling pathways of ABA during adaptation of plants to drought (Frankel *et al.*, 2003, 2006). Since *O. fusca* labella were harvest from plants occurring in the wild, it is not surprising to found transcripts related to stresses imposed by the natural habitat environment (i.e. drought stress). However, measurements of ABA levels during *Ophrys fusca* petal senescence were not performed.

Plant chitinases (EC 3.2.1.14) belong to a relatively large gene family divided in classes according to specific functions (Passarinho and de Vries, 2002; Kasprzewska, 2003). The fungicide mode of action of chitinases consists on degradation of fungal cell walls by cleaving the β-(1,4)-glycoside bonds of chitin, the major component of fungal cell walls (Takakura *et al.*, 2000). The specificity of some chitinase genes suggests that they could also play a role in developmental processes such as pollination (Wagner *et al.*, 2007), senescence, seed germination (Santos *et al.*, 2004) root and root nodule development (Kim and An, 2002) and somatic embryogenesis (Passarinho and de Vries, 2002). Chitinases content increased during parsley leaf senescence (Lers *et al.*, 1998). Also during *Brassica napus* leaf senescence (Hanfrey *et al.*, 1996), increased expression of a PR1 protein and a chitinase was detected but it is not clear whether these proteins have a specific function in senescence or whether they are present to protect the

senescing leaf from opportunistic pathogen attack (Buchanan-Wollaston and Ainsworth, 1997). Down regulation of a chitinase in *O. fusca* labellum at 4DAP may suggest a putative role of this protein in flower development, as previously demonstrated in tobacco (Neale *et al.*, 1990) and rice (Takakura *et al.*, 2000) healthy flowers in which accumulation of chitinase was not related to pathogenesis response. According to Neale *et al.* (1990), chitinase mRNA levels increase in most floral parts along flowers maturation. Down regulation at 2DAP and up regulation at 4DAP of a transcript coding for a class III chitinase in *O. fusca* may suggests that its expression was promoted as part of a generalized defense-related response, not directely related to pollination enhanced-mechanisms.

Up regulation at 2DAP of a transcript coding for a non specific lipid transfer protein (LTP) was found. Roles in plant–pathogen interactions and/or pollen tube growth regulation have been proposed for LTPs (Lan *et al*., 2004). Lipid transfer proteins are responsible for shuttling phospholipids and other fatty acid groups between cell membranes (Choi *et al.*, 2008). Other roles proposed for plant LTPs include participation in cutin biosynthesis, antimicrobial activity, symbiosis, embryogenesis and adaptation to different stresses (reviewed in Kader, 1996). Up regulation in *O.fusca* of a transcript coding for a LTP at 2DAP, in combination with up regulated transcripts coding for PR-10c (see table 2.6), may be related to a defense response for protecting senescing petal from pathogen attack. This hypothesis is supported by previous studies on pollination/fertilization, according which up regulated genes such as LTP were related to defense responses (Lan *et al.*, 2004, 2005).

Such as for leaf senescence, during petal senescence free radicals/metal ions are often released causing oxidative stress. As a result, a membrane/protein breakdown occurs (Buchanan-Wollaston, 1994; Hanfrey *et al.*, 1996). During metal release, plants engage detoxification actions thereby reducing the risk of metal toxicity to the cells of surrounding tissues (Navabpour *et al.*, 2003; Breeze *et al.*, 2004). These degenerative processes often result on increased production of reactive oxygen species (ROS) and the plant responds by enhancing senescence-non-enzymatic antioxidants production such as ascorbate and glutathione, as well as enzymatic antioxidants such as catalase, superoxide dismutase and ascorbate peroxidase (Navabpour *et al.*, 2003). ROS may function as a general signal that induces the expression of a set of genes involved in detoxification during senescence. Environmental pollution with heavy metals also requires mechanisms of metal homeostasis and detoxification by the plant (Heise *et al*., 2007). Metals like Zn, Fe, and Cu, which are essential in trace amounts, and nonessential metals such as Pb, Al, and Cd, which are not required for plant cell functions, are considered toxic in low quantities. Metallothioneins are the most important metal binding factors in plant metal

homeostasis and detoxification. Metallothioneins (MTs) are metal binding proteins, low molecular weight and cysteine-rich polypeptides encoded by a gene family (Cobbett and Goldsbrough, 2002). Specific functions in plant development, in specific tissues (e.g. developing seeds) and in stress response have been assigned to MTs (Zhou *et al.*, 2005). Expression of MT genes has been shown to be induced in senescing leaves (Guo *et al.*, 2003) and petals (Breeze *et al.*, 2004), in ripening fruits (Moyle *et al.*, 2005) and in tapetum cells during microsporogenesis (Charbonnel-Campaa *et al.*, 2000). Gene expression in *Ophrys fusca* pollination revealed nine and seven sequences, matching to metallothioneins type 2 and type 3, respectively. Down regulation of transcripts coding for metallothioneins type 3 at 2DAP was detected, whereas at 4DAP up regulation of both metallothioneins type 2 and 3 was observed. High number of metallothioneins was also encountered in microarray studies performed in *Astroemeria* petal senescence (Breeze *et al.*, 2004). Down regulation of transcripts coding for metallothioneins type 3 at 2DAP suggests that soon after pollination metal homeostasis is not required for plant development. On the other hand, up regulation of a diverse array of ESTs with homology for metallothioneins (type 2 and 3) at 4DAP, may indicate a function in sequestering metal ions to prevent increase in ROS levels during senescence (Reddy *et al.*, 2002; van Doorn and Woltering, 2008; Wagstaff *et al.*, 2010).

Genes associated with floral senescence, mainly those involved in ethylene biosynthesis and also expression of glutathione *S*-transferase (GST) genes, have been isolated (Meyer *et al.*, 1991; Tripathi and Tuteja, 2007). GSTs display key roles in defense or in cellular protection producing proteins as a response to pathogen attack, wounding, senescence and lipid peroxidation resulting from these processes (Rubinstein, 2000). Studies in carnation petals demonstrated that ethylene-regulated GST1 and GST2 genes were expressed during senescence (Marrs, 1996). Since senescence promotes lipoxygenase mediated membrane disruption, a putative function of glutathione *S*-transferase in detoxification of lipid has been addressed (Meyer *et al.*, 1991; Marrs, 1996). As a result, glutathione S-transferase expression has been attributed as a result from the detoxification of lipid resulting from the senescence-induced oxidative processes (O'Neill, 1997; Rubinstein, 2000). Up regulation of two transcripts coding for glutathione *S*- transferase at 2DAP and 4DAP is in accordance with previous studies in petal senescence in carnation (Meyer *et al.*, 1991) and wallflower (Price *et al.*, 2008). GST gene expression may have a putative role in detoxification of lipid (co-expression of a transcript related to the breakdown of fatty acids, see Lipid metabolism) (Itzhaki *et al.*, 1994; Rubinstein, 2000; Chakrabarty *et al.*, 2009). Thus, up regulation of GST at 2DAP and 4DAP may be related to prevention of cell integrity in the sequence of lipid peroxidation occurring during senescence. PCD is

a controlled process in which cell integrity is central for nutrient remobilization, until ultimate cell death (Mea *et al.*, 2007).

Up regulation at 4DAP of a stress-related gene, with homology for a LEA (Late Embryogenesis Abundant) 5 protein was found. LEA proteins were first discovered in the cotton plant *Gossypium hirsutum* and originally described as being expressed during later stages of embryo development (post abscission) in plant seeds (Galau *et al.*, 1986). Also, LEA proteins are known to be a large number family with different functions addressed, yet they have been generally considered as stress-related proteins (Tunnacliffe and Wise, 2007). Since LEA proteins are involved in desiccation resistance, a variety of mechanisms for achieving this end have been proposed including protecting cellular structures from the effects of water loss by retention of water, sequestration of ions, direct protection of other proteins or membranes, or renaturation of unfolded proteins (Wise, 2003 and references therein). Specifically, *Lea*5 gene was postulated as being involved in both stress and hormone responses, and studies have shown to be transcriptionally regulated during fruit ripening (Jeon *et al.*, 2006; Tao et *al.*, 2006). Up regulation at 4DAP of a LEA5 protein may be related to a stress response triggered by pollination induced-senescence.

A transcript coding for a MtN19 protein was detected as being up regulated at 4DAP. MtN19 was originally identified as a gene up regulated in 4-day-old nodules of *Medicago truncatula*, and referred to as a nodulin gene (Gamas *et al.*, 1996). It has been demonstrated that MtN19 gene expression is influenced by nodulation (Gamas *et al.*, 1996), senescence (Breeze *et al.*, 2004), light and drought stresses (Kimura *et al.*, 2003), and nitric oxide (NO) (Huang *et al.*, 2002). However, no specific function has been assigned to this protein. It is noteworthy that the above processes comprise a common feature, the production of reactive oxygen species. Expression of pathogen-related genes may be activated as a result of ROS accumulation that enhances a defense response (Huang *et al.*, 2002). Treatment of pea pots with the insect elicitor Bruchin B also induces MtN19 expression, which may elicit a defense response (Doss, 2005). High light intensities increase endogenous levels of ROS due to leakage of electrons from the overloaded photosynthetic machinery (Kimura *et al.*, 2003). Finally, senescence elicits lipids and proteins degradation thus contributing to oxidative stress (Navabpour *et al.*, 2003). In *O. fusca* labella, up regulation at 4DAP of a transcript coding for MtN19 may be associated to oxidative stress, as a result of lipid and protein degradation that generate ROS which, in turn, is an outcome of the senescence process.

In our microarray, high number of stress- and pathogen-related genes up regulated (i.e. gluthatione *S*-transferase, MtN19-like protein, cyclophilin, metallothioneins types 2 and 3, antimicrobial snakins proteins, late embryogenesis

abundant Lea5, chitinase, lipid transfer proteins (LTPs), and PR10c protein) were found. In senescence studies, it has been hypothesized that these genes may protect tissues (e.g. ovule, ovary, pollen tube development) from pathogen attack (Lan *et al.*, 2004, 2005) or from the damaging effects of ROS accumulation, to allow degradation of cellular constituents and remobilization of nutrients (Buchanan-Wollaston *et al.*, 2005). Transcripts related to both stress- and defense-responses identified in our microarray analysis are in accordance with previous studies on petal senescence (Thomas *et al.*, 2003; van Doorn *et al.*, 2003; Breeze *et al.*, 2004; Hoeberichts *et al.*, 2007; Price *et al.*, 2008; Wagstaff *et al.*, 2010).

2.4.2.2.4. Transcription factors and regulation of transcription

Transcription factors (TFs) are DNA-binding proteins that regulate gene expression at the mRNA transcription level. Many transcription factors families have been identified. *Arabidopsis* genome revealed 29 transcription factor's classes, 16 of which appear to be unique to plants (Riechmann *et al*., 2000; The *Arabidopsis* Genome Initiative, 2000).

Two transcripts coding for different RNA- binding proteins were down regulated at 2DAP: RNA- binding protein and a Nucleic acid binding NABP protein. RNA binding proteins can regulate basic cellular processes like synthesis, transport, translation and degradation of mRNAs (Fedoroff, 2002). The regulation of gene expression during development is achieved through transcriptional regulation and post transcriptional control of RNAs, which in turn is ruled by RNA-binding proteins (RBPs) (Lorković, 2009). These interactions are important in mediating mRNA maturation events such as splicing, capping, polyadenylation and export from the nucleus. RNA-binding proteins also contribute to post transcriptional regulatory events in the cytoplasm, such as mRNA localization, stability and decay, as well as translation (Tam *et al.*, 2010). One group of RNA-binding proteins that are important regulators of cytoplasmic post transcriptional control is the PUF protein family (Goldstrohm *et al.*, 2006). NABP is a motif characteristic of the Puf family of RBPs (Goldstrohm *et al.*, 2006; Tam *et al.*, 2010), with roles addressed in cell growth, division, differentiation and development (Wickens *et al.*, 2002). PUFs bind to 3′ untranslated regions of mRNAs repress their expression and, in many cases, promote shortening of poly(A) tails (deadenylation) (reviewed in Wickens *et al.*, 2002; Traven *et al.*, 2010). Puf proteins can regulate the stability or translation of their target mRNAs in response to environmental stimuli in a rapid and coordinated manner. In *O. fusca*, down regulation of two different RBPs, RNA- binding protein and a putative Puf (Pumilio) protein, at 2DAP suggests that regulation of the basic cellular processes in mRNAs have been compromised by pollination. This hypothesis is coherent with the up

regulation of S-like RNAses at 2DAP and 4DAP (see Cell Fate). Our data on *Ophrys fusca* pollination suggest that at 2DAP, transcriptional regulation and post transcriptional control of RNAs are soon deactivated via down regulation of RNA binding proteins, and up regulation of S-like RNases (major family of RNA-degrading enzymes) at both 2DAP and 4DAP, reveal that RNA degradation is activated to ensure removal of RNA from the dying cells and to allow Pi remobilization.

Light has two pivotal roles in regulating flowering time: (1) enhancing the circadian clock and (2) interacting with an output from the clock preventing or inducing flowering process (Thomas, 2006). Daylength is an important regulator of flowering time, allowing sexual reproduction to occur at an appropriate time, ensuing cross-pollination and, consequently, fertilization. Circadian clock involves the recognition of light and darkness cycles by means of multiple photoreceptor proteins, phytochromes (phy) and cryptochromes (cry). The phy family absorbs in the red region of the spectrum, whereas the cry family absorbs in the blue region (Devlin and Kay, 2000). Both photoreceptors mediate light input to the clock regulation mechanism. In *Arabidopsis*, at least four of the five phytochromes (PHYA, B, D, and E) and both cryptochromes (CRY1,2) contribute to clock regulation (Thomas, 2006 and references therein). Phytochrome regulates a range of developmental processes in response to red and far-red light, including seedling establishment, shade avoidance and transition to flowering (Somers *et al.*, 1998). In *Arabidopsis*, forward and reverse genetic studies identified ZEITLUPE (ZTL), FLAVIN-BINDING KELCH REPEAT F-BOX1 (FKF1) and LOV, KELCH PROTEIN2 (LKP2) as contributors to the period of circadian oscillation (Baudry *et al.*, 2010 and references therein). They share a unique combination of motifs: PAS/LOV domain, an F-box domain and six kelch repeats (Yanovsky and Kay, 2001). In *Arabidopsis*, ZTL family of F-box proteins is involved in the turnover of clock and flowering time proteins (Chae *et al.*, 2008). This ZTL family of F-box proteins that also includes FKF1, a protein involved in the regulation of flowering time (Nelson *et al.*, 2000; Imaizumi *et al.*, 2003, 2005), contains a LOV/PAS domain that mediates blue light dependent protein-protein interactions. Although not yet demonstrated, FKF1 might act as blue-light receptor (Mizoguchi and Coupland, 2000). Nelson and co-workers (2000) and, more recently, Baudry and co-workers (2010) determined that the FKF1 mRNA abundance is circadian clock regulated, namely diurnally. FLAVIN-BINDING KELCH REPEAT F-BOX1 mediates the cyclic degradation of CYCLING DOF FACTOR1 (CDF1), a Dof transcription factor involved in repression of the flowering-time gene CONSTANS (CO) (Imaizumi *et al.*, 2003, 2005). Genetic models showed that the clock acts to establish the rhythm of the CO gene expression, which is regulated by FKF1 and GIGANTEA (GI) in *Arabidopsis* (Sawa *et al.*, 2007). Down regulation in *O. fusca* at 4DAP of a transcript coding for FKF1 reveals for the first time, a regulation mechanism in orchid flowering time by means of FKF1-

mediated cyclic degradation of the CO repressor CDF1, as described for *Arabidopsis* (Sawa *et al.*, 2007). Although floral development and the acquisition of floral traits have been extensively characterized in model species, they have yet to be deciphered in orchids, which flowers are complex and differ markedly from those of the other plant families (Park *et al.*, 2010).

A transcription factor which contains a DNA binding domain referred as *SQUAMOSA* promoter binding protein (SBP) domain was down regulated in *Ophrys fusca* labella 2 and 4 days after pollination. SBP proteins are encoded by the SBP-box genes, a feature characteristic of the *Arabidopsis SQUAMOSA* promoter binding protein- like (SPL) gene family (reviewed in Cardon *et al.*, 1999; Wang *et al.*, 2009). SBP-box genes have been identified in a wide variety of plants, e.g. 16 genes in *Arabidopsis* (SPL1 to SPL16) (Wang *et al.*, 2009) and 19 in rice (Yang *et al.*, 2008). SBP-domain proteins were first isolated from *Antirrhinum majus* by their capacity to interact *in vitro* with a promoter sequence element of the floral meristem identity gene *SQUAMOSA*, the *Antirrhinum* ortholog of the *Arabidopsis* gene APETALA1(AP1) (Klein *et al.*, 1996; Wang *et al.*, 2009). The ABC model of flower development shows how the presence or absence of different classes of transcription factors in the different parts of the flower regulates the development of floral organs (Schwarz-Sommer *et al.*, 1990; Coen and Meyerowitz, 1991). One of the floral organ identity genes, APETALA1 (AP1) together with APETALA2 (AP2), APETALA3 (AP3), PISTILLATA (PI), AGAMOUS (AG), several AGAMOUS-LIKE (AGL) genes and SEPALLATA1, 2, 3, 4, are specific for the development of petals, sepals, stamens and pistils (Thiruvengadam and Yang, 2009). Functions of SBP-box genes are largely unknown, but they are predicted to act as transcriptional regulators based on the presence of plant-specific putative DNA binding domains (Stone *et al.*, 2005). For instance, SPL1 and SPL2 genes from *Antirrhinum* interact with a promoter sequence element of the floral meristem *SQUAMOSA* (SQUA) (Cardon *et al.*, 1999). *Arabidopsis* SPL family can be divided into subfamilies, based on genomic organization and sequence similarities. SPL1, SPL7, SPL12, SPL14 and SPL16 form one subfamily representing the largest members of the gene family (Stone *et al.*, 2005; Zhang, 2005). They are characterized by their constitutive expression throughout plant development, namely during flower development (Cardon *et al.*, 1999; Birkenbihl *et al.*, 2005). In *O. fusca*, down regulation of a transcript coding for a SBP-domain at 2DAP and 4DAP, suggest a putative function in flower development, rather than in pollination event and in senescence related process. However, other putative function may not be discarded, since several SPL proteins have been related to plant development in general (Ruokolainen *et al.*, 2010). Further studies are advised to entangle SPL putative function in orchid flower development, namely in *Ophrys fusca* labellum.

Also, up regulation of a NAM-like protein in *O. fusca* was found. The genes NO APICAL MERISTEM (NAM) in *Petunia hybrida* and CUP-SHAPED COTYLEDON (CUC) 1-3 in *Arabidopsis thaliana* are highly homologous, and members of the NAC family of plant-specific transcription factors (Souer *et al.*, 1996; Ishida *et al.*, 2000; Olsen *et al.*, 2005). In *Arabidopsis*, 105 NAC genes have been identified. Some of them have been shown to play diverse roles during embryonic, floral and vegetative development (Souer *et al.*, 1996; Aida *et al.*, 1997; Mao *et al.*, 2007), involvement in auxin and abscisic acid signal transduction (Xie *et al.*, 2000; Aida *et al.*, 2002) and also in plant responses to biotic and abiotic stresses (Olsen *et al.*, 2005; Zhong *et al.*, 2006). Members of the NAC transcription-factor family are required for establishing boundaries between lateral organ primordia resulting on organ separation (Kikuchi *et al.*, 2000; Krizek and Fletcher, 2005). Inactivation of petunia NO APICAL MERISTEM (NAM), *A. thaliana* CUC1 and CUC2 and of *A. majus* CUPULIFORMIS (CUP) results in fusion of cotyledons, leaves and/or floral organs (Souer *et al.*, 1996; Aida *et al.*, 1997). CUC2 has high homology with petunia (*Petunia hybrida*) NAM gene which is required for floral organ development, ovule development, cotyledon separation and embryonic SHOOT APICAL MERISTEM (SAM) formation (Souer *et al.*, 1996; Aida *et al.*, 1997, 1999; Hibara *et al.*, 2006). In *Arabidopsis thaliana*, CUC2 mRNA was detected in bud and flower and its expression pattern addresses involvement in organ separation in shoot and floral meristems (Ishida *et al.*, 2000) and also in the establishment of the SAM as well as in correct positioning of the cotyledons in maize (Zimmermann and Werr, 2005) and *Arabidopsis* (Nardmann and Werr, 2007). Previous studies in *Arabidopsis*, point out that CUC2, together with CUC1, are required for ovule development (Ishida *et al.*, 2000; Skinner *et al.*, 2004) and both genes have shown to be controlled posttranscriptionally by miR164 (Larue *et al.*, 2009). Further the requirement of NAM and CUC for ovule development has been described (Ishida *et al.*, 2000; Skinner *et al.*, 2004; Vialette-Guiraud *et al.*, 2011). Up regulation of a NAM-like protein in *O. fusca* at 2 DAP may, thus, be related with a putative role on ovule development triggered by pollination, as in most orchid species the ovule development is accomplished only after pollination (Zhang and O'Neill, 1993; Nadeau *et al.*, 1996; Yu and Goh, 2001). It is of major interest to fully understand the putative function of this transcription factor in the orchid ovule developmental program.

2.4.2.2.5. Protein synthesis

Protein synthesis (or translation) is accomplished in the cytoplasm by ribosomes that translate the information from mRNA to proteins. Eukaryote ribosomes are composed of two subunits, each one consisting of ribosomal RNA (rRNA): the small

subunit 40S has one molecule of 18S rRNA and 33 proteins, and the larger subunit 60S contains three types of rRNA (5.5S, 5.8S and 28S) and 49 proteins (Preiss and Hentze, 2003; Rabl *et al.*, 2011). The ribosomal proteins are named in accordance with the subunit of the ribosome which they belong to - the small (S1 to S33) and the large (L1 to L49). Usually they decorate the rRNA cores of the subunits (Preiss and Hentze, 2003). Up regulation at 4DAP of a transcript coding for a putative ribosomal protein L17, with a key role in protein synthesis (Gao *et al.*, 1993; Meng *et al.*, 2010), may indicate that translation has been activated with pollination. The dynamic process of mRNA translation is usually divided into three phases: initiation, elongation and termination (Browning, 2004). In eukaryote mRNAs, translation is initiated in cytoplasm and the translation process being accomplished with the RNA 3' poly(A) tail. Gene expression data on *O. fusca* labellum upon pollination here presented revealed a transcript coding for a translation initiation factor, which is necessary for accurate initiator codon recognition (Fletcher *et al.*, 1999). Up regulation of genes related to protein synthesis at 4DAP may suggest that pollination induced protein synthesis through activation of the translation process. Recent studies in petal senescence point out to a requirement of *de novo* protein synthesis in the timely initiation and progression of a senescence program (van Doorn and Woltering, 2008; Bai *et al.*, 2010). Therefore, it is not surprising the up regulation of genes involved in protein synthesis after pollination. Since PCD programming seems to be fully recognized within 4 days after pollination, it was expected to find genes related to enhancement of protein translation. Recent proteomic studies on petal senescence in petunia pointed out to a 64 % increase in abundance of differentially regulated proteins (Bai *et al.*, 2010).

2.4.2.2.6. Biogenesis of cellular components

In *Arabidopsis*, about ten actin (ACT) genes are widely distributed in the genome with different functions attributed. For example, actins ACT2, ACT8, ACT7 are considered to be vegetative, whereas ACT11, ACT1, ACT3, ACT4, and ACT12 are believed to be reproductive (reviewed in McKinney and Meagher, 1998). Reorganization of the actin cytoskeleton in eukaryotic cells is regulated by a variety of actin binding proteins that modulate the behaviour of both filamentous (F-actin) and globular actin (G-actin) (Gibbon *et al.*, 1998). Profilin is a G-actin binding protein (Witke, 2004). The function of these actin-binding proteins may be controlled by numerous signals, including calcium, phosphoinositides, pH or reversible phosphorylation (Winder and Ayscough, 2005). Actin-binding proteins display several functions, namely regulation of filament assembly and disassembly (e.g. profilin, thymosin 4, ADF/cofilin, gelsolin, capping protein), *de novo* nucleation of actin filaments (e.g. ARP2/3, WASP),

regulation of actin polymerization and interactions among actin filaments (Winder and Ayscough, 2005). In pollen, one of the most abundant actin-binding proteins, profilin, is known to regulate microfilament formation and a significant role in regulating the dynamics of the actin cytoskeleton has been addressed (Gibbon *et al.*, 1998; Hussey *et al.*, 2002). In maize pollen, at least three profilin isoforms are expressed: ZmPRO1, ZmPRO2, and ZmPRO3 (Staiger *et al.*, 1993). Up regulation of a transcript coding for a profilin at 4DAP suggests a putative function during pollen germination, namely in rearrangement of actin cytoskeleton, revealed by downregulation of ATC2 gene at 2DAP. This hypothesis is supported by studies in *Lilium longiflorum*, according which profilin increase was detected during pollen germination, its expression being associated with actin rearrangement (Miki-Hirosige *et al.*, 2004).

Pectin represents about 30% of polysaccharides of the cell wall in dicotyledonous plants (Caffall and Mohnen, 2009). Monocots and dicots are significantly different with respect to pectin composition and distribution. In monocots, pectin and pectate represent only a small fraction of the cell wall components (1- 6 %), the relatively small amount of pectin being tightly bound to the cell wall. In dicots, pectin is a major cell wall component and exists primarily as a homogenous layer within the cell wall (Carpita, 1996). Pectin methylesterase (PME) is the first enzyme acting on pectin, a component of plant cell wall. PME hydrolyze pectin with different structural and functional properties, having an important role on plant physiology (Giovanea *et al.*, 2004). PME activity is regulated by specific PME inhibitor (PMEI) protein either by differential expression or by posttranslational control (Zhang *et al.*, 2010). Up regulation in *O. fusca* of a transcript coding for a PMEI at 4DAP suggests that pectin hydrolysis was inhibited by pollination, probably due to cell wall changes enhanced by senescence event. Considering that about 50 PMEs are transcribed in flowers (Bosch and Hepler, 2005) and both PMEs and PMEIs are expressed in flower tissues and pollen grains (Di Matteo *et al.*, 2005), one explanation is that PMEI has a broad inhibitory activity against PMEs, thus affecting cell wall integrity. These changes reflect directly or indirectly the biochemical and physical events of wall loosening, expansion, and retightening (Carpita, 1996). Since cross-pollination induces actin cytoskeleton rearrangements while self-pollination disrupts actin structure (Iwano *et al*., 2007), it was expected to identify transcripts coding for actin and profilin resulting on cytoskeleton rearrangements and for changes of cell wall integrity (PMEI).

Plant peroxisomes are organelles essential to physiological processes such as lipid metabolism, photorespiration, hormone biosynthesis and metabolism (Reumann *et al.*, 2009). The peroxisomal membrane forms a permeability barrier through peroxisomal membrane proteins (PMPs), which are critical for different functions, namely the import

proteins into the organelles (Hettema and Tabak, 2000). Peroxisomes/glyoxysomes[6] participate in the fatty acid β-oxidation, since fatty acid chains greater than C22 are too long to be handled by the mitochondria (reviewed in Poirier *et al.*, 2006). In *O. fusca*, down regulation at 4DAP of a transcript coding for a PMP36, may suggest an inactivation of the peroxisomes membrane proteins synthesis, essential for providing a permeability barrier for protein transport, thus compromising efficient peroxisome biogenesis.

2.4.2.2.7. Protein fate

In daylily petal senescence, an involvement of the ubiquitin pathway through the degradation of proteins was postulated (Courtney *et al.*, 1994). This may entail a causal role for the ubiquitin-proteasome system (UPS) in petal senescence (Rubinstein, 2000). UPS is part of a wide diversity of functions, namely in regulating different steps of cell cycle, embryogenesis, senescence, defense, environmental responses and hormone signaling (Santner and Estelle, 2010). This allows plants to effectively and efficiently alter their proteasome to ensure developmental plasticity and environmental adaptation (Dreher and Callis, 2007; Yee and Goring, 2009). The ubiquitination pathways function as the main proteolytic system in eukaryotes and mediate posttranslational modifications of cellular proteins commonly targeting them for degradation by the proteasome (Cho *et al.*, 2008; Craig *et al*., 2009). Thus, target proteins are first modified through the articulated actions of E1[7], E2[8] and E3[9] enzymes and the polyubiquitylated proteins are then degraded by 26S proteasome (reviewed in Frugis and Chua, 2002; Vierstra, 2003). E3 ubiquitin ligases are of particular interest as they are responsible for substrate specificity, being categorized accordingly their mechanism of action and presence of specific domains as RING, HECT, F-box, and U-box (Yee and Goring, 2009). Plant U-Box (PUB) gene family can be divided in five subclasses suggesting that they display diverse roles (Azevedo *et al.*, 2001). Based on the induction of these PUB genes in response to cold, drought and salt treatments, it has been generalized that these E3 ligases may function as regulators in various abiotic stress responses (Yee and Goring, 2009). Up regulation of an E3 ligase ATL15 transcript belonging to the ATL (Arabidopsis tóxicos en levadura) gene family, a conserved group of RING zinc-finger proteins (Salinas-Mondragon *et al.*, 1999), and down regulation of a spotted leaf protein with U-BOX domain (PUB), a characteristic domain from E3 ligases

[6] Glyoxysomes are specialized peroxisomes found in plants, particularly in the fat storage tissues of germinating seeds.
[7] Ubiquitin- activating enzyme.
[8] Ubiquitin- conjugating enzyme.
[9] Ubiquitin- protein ligase.

(Smalle and Viestra, 2004), point out to a role of the ubiquitin–proteasome system (UPS) in senescence modulation through selective protein degradation. Indeed, ubiquitin conjugation occurs through enzymatic cascades by the action of E3 ubiquitin ligases (Yee and Goring, 2009). A role on selective protein degradation by the UPS has been reported before on petal senescence (Rubinstein, 2000; Hunter *et al.*, 2002; Breeze *et al.*, 2004; Buchanan-Wollaston *et al.*, 2005; Yamada *et al.*, 2007; Xu *et al.*, 2007a,b; Hoeberichts *et al.*, 2007; van Doorn and Woltering, 2008; Bai *et al.*, 2010). Detection in *O. fusca* pollination of differentially expressed transcripts coding for two distinct E3 ligases (U-BOX and RING-type) suggests that ubiquitin-proteasome system action is depended of E3-substrate specificity to modulate specific responses. Studies in leaf senescence demonstrated the importance of the selective protein removal route mediated by the ubiquitin mediated- proteolysis pathway via 26S proteasome (Yoshida *et al.*, 2002a).

In addition to UPS mediated process, proteins can also be modulated through the action of heat shock proteins acting in a myriad of biological process. Plant small heat shock proteins (sHsps) range in size from 15 to 42 kDa and constitute the most abundant and diverse group of proteins synthesized as a response to heat stress (Neta-Sharir *et al.*, 2005). These proteins can be induced by different environmental stresses such as drought (Maqbool *et al.*, 2005), cold, salinity and in development processes such as embryogenesis, germination and fruit development (Wang *et al.*, 2004). Heat-shock proteins (Hsp) are commonly denominated as molecular chaperones and display important roles in protein–protein interactions (Wang *et al.*, 2004). sHSPs bind to partially folded or denatured proteins and prevent their aggregation thereby facilitating folding by other chaperone. Two different transcripts coding for Hsps were encountered: chaperone up regulated in *O. fusca* at 2DAP and a small Hsp down regulated at 4DAP. In *Ophrys fusca* pollination, down regulation of a sHSP transcript can be related to stress response as a mean of adaptation and modulation of proteins in natural habitats (Mahmood *et al.*, 2010). In *O.fusca*, up regulation of a chaperone at 2DAP suggests a response to a stress either triggered by pollination event or by the environment itself, as plants were collected from natural populations that face continuous requirement towards adaptation on their hostile habitat. Among the diverse group of molecular chaperones, the highly conserved 70 kDa heat shock proteins (Hsp70s) and their partner J-proteins are the most specialized group of heat shock protein machinery expressed under abiotic stress conditions (Rajan and D'Silva, 2009). Most of the functional diversity of the Hsp70s is driven by a diverse class of cofactors, J proteins, required as accessory factors (Kampinga and Craig, 2010). Hsp70:J-protein complex is involved in a variety of essential cellular processes including: *de novo* protein folding, translocation of polypeptides across cellular membranes and

degradation of misfolded proteins (Walsh *et al.*, 2004). Refolding of damaged proteins after exposure of cells to stress is ensured by this machinery by controlling the activity of many regulatory proteins. Down regulation in *O. fusca* of a transcript coding for DNA J protein at 4DAP suggests that cell homeostasis is not being maintained after pollination. Whenever successful pollination is achieved, the flower as a reproductive organ has fulfilled its role in plant survival by ensuing offspring and the processes required for maintenance of such an energy-consuming organ are nonessential.

Down regulation in *O .fusca* of a transcript coding for a protein disulfide isomerase (PDI; EC 5.3.4.1) at 2DAP was detected. PDI has the ability to modulate redox responses and chaperone proteins (Ondzighi *et al.*, 2008). PDI is an essential protein for formation, reduction or isomerization of protein disulfide bonds in the endoplasmic reticulum (ER) lumen (Huang *et al.*, 2005). Besides, it catalyzes the formation and breakage of disulfide bonds between cysteine residues in proteins as they fold (Noiva *et al.*, 1993). Since protein folding is an error prone process, PDI provides a mechanism to correct errors in disulfide pairing when they occur (Gilbert, 1997). Disruption of protein folding causes ER stress and activates signaling cascades, and the unfolded protein response (UPR) restores folding capacity (Lu and Christopher, 2008). Misfolded proteins are removed by conjugation to ubiquitin for proteasomal degradation, through the UPS. Down regulation in *Ophrys fusca* of a transcript coding for a PDI at 2DAP suggests that restoration of protein folding capacity is disengaged soon after pollination.

Several transcripts coding for protein phosphatases and a kinase were differentially regulated in *Ophrys fusca* pollination. Down regulation was determined for a casein kinase transcript at 2 DAP and up regulation of several transcripts encoding protein phosphatases (nucleotide pyrophosphatase/ phosphodiesterase and a purple acid phosphatase (PAP)) at both 2 DAP and 4 DAP (Table 2.6). Protein phosphorylation, a posttranslational modification of proteins, is one of the most common ways of protein function regulation. Phosphorylation is achieved by a protein kinase (PK) that adds a phosphate group to a substrate, this process being reverse by protein phosphatases (PPs) that removes the phosphate group (Chevalier and Walker, 2005). Addition or removal of a phosphate group to or from an enzyme either activates or deactivates the enzyme (Singh *et al.*, 2010). In most cases, change of the protein form between phosphorylated and unphosphorylated, represents an active or inactive protein. Protein kinases can be classified according to their primary sequences and to the residue being subjected to phosphorylation activity: serine/threonine (ser/thr), histidine (his) or tyrosine (tyr). Down regulation in *O. fusca* of a transcript coding for a casein kinase (CK) at 2DAP was detected. Casein kinases (I or II) are involved in various biological processes: it phosphorylates different proteins, including transcription factors; can modulate DNA-binding ability, intracellular localization and protein stability (Ogiso

et al., 2010). Recent studies revealed that CK2 has a conserved function as a circadian clock component in *Arabidopsis thaliana* (Ogiso *et al.*, 2010). PPs can also be classified into serine/threonine and tyrosine phosphatases, based on the amino acid residue they preferentially dephosphorylate. In *O. fusca*, a transcript coding for a purple acid phosphatase (PAP) was up regulated at 4DAP (Table 2.6). PAPs are binuclear metalloenzymes that catalyse the hydrolysis of a wide range of phosphate esters and anhydrides (Schenk *et al.*, 2000), but functional roles have not been fully addressed. Phosphatases are responsible for the phosphate (Pi)-hydrolysis from organic compounds thereby favoring Pi mobilization and translocation from senescent tissues (Duff *et al.*, 1994; Fang *et al.*, 2009). According to Chapin and Jones (2009), being phosphorus (P) a growth-limiting nutrient, Pi remobilization from unneeded tissues, like corolla, will allow the plant to use nucleic acids as P storage molecules. Inhibition of phosphorylation by kinase (at 2 DAP) and activation of dephosphorylation (2 DAP and 4 DAP) by phosphatases after pollination can be likely related to Pi remobilization from the labellum to younger and/or actively growing tissues (e.g. ovule, ovary, other flowers from the same inflorescence), thus allowing Pi recovery by the plant (Chapin and Jones, 2009).

2.4.2.2.8. Cellular transport, transport facilities and transport routes

During senescence, up regulation of transcripts involved in catabolism for nutrient remobilization should be expected.

Down regulation, at 2 DAP and 4 DAP, of a transcript coding for purine permease 3 (ATPUP3) may be associated with cytokinin -mediated delay senescence. Cytokinins biosynthesis pathway is partially shared with purine metabolism (Hirose et al., 2008) and translocation of cytokinins is apparently mediated by nucleoside transporters and purine permeases (PUPs) (Gillissen *et al.*, 2000; Kudo *et al.*, 2010). Up regulation of cytokinins was shown to delay senescence in both leaf (Gan and Amasino, 1995) and petal (Chang *et al.*, 2003), while its down regulation was suggested to increase ethylene sensitivity in unpollinated ethylene-sensitive species during petal senescence (Lara *et al.*, 2004; Price *et al.*, 2008; van Doorn and Woltering, 2008). In *O. fusca* PUP3 abundance decreased soon after pollination, thus, the senescence delay mediated by cytokinins could have been dismantled.

Up regulation at 4DAP of a transcript coding for Adenine nucleotide translocator (ANT) was found (Table 2.6). This transcript is involved on ADP/ATP exchange during oxidative phosphorylation and is located at the inner membrane of the mitochondria (Picault *et al.*, 2004; reviewed in Dahout-Gonzalez *et al.*, 2006). In animals, apoptotic factors released from the mitochondria are a result of the

permeability transition pore (PTP) opening (Kroemer *et al.*, 2007). Activity of the PTP, usually formed following cellular stress (e.g. changes ATP levels, ROS production), results on losses of the mitochondrial inner membrane (MIM) potential ($\Delta\psi m$), activity of pro-apoptotic proteins, and disruption of the outer membrane, in a process designed as mitochondrial membrane permeability transition (MPT) (Kroemer *et al.*, 1998; Crompton, 1999; Le Bras *et al.*, 2005; Masuda, 2011). In apoptosis, evidence of MPT as the committed step is well documented; and similar task has been considered in plants (Jones, 2000; Tiwari *et al.*, 2002; Reape and McCabe, 2010). PTP is a multicomponent protein complex which include an adenine nucleotide translocator (ANT) MIM-located, cyclophilin D (CyP-D) and the voltage-dependent anion channel (VDAC) in the outer membrane (Le Bras *et al.*, 2005). ANT plays an important role in oxidative phosphorylation, via the import of ADP and the export of ATP (Laloi, 1999). Recently, two ANTs proteins have been identified in rice (Taylor *et al.*, 2010), yet no functions have been addressed. In animals, overexpression of ANT has been shown to be cytotoxic (Zamora *et al.*, 2004; Jang *et al.*, 2008; Baines and Molketin, 20009) and studies using ANT inhibitors have pointed out to a putative function of ANT as a link between MPT and apoptosis (Green and Reed, 1998; Kroemer *et al.*, 1998). Up regulation of this transcript at 4DAP may be related to an increase in ADP/ATP exchange during oxidative phosphorylation, which ultimately increase ROS production, a major consequence of senescence event. A recent study on animals indicated that ANT mediates cell death, not through the MPT pore, but rather via ROS production (Baines and Molkentin, 2009). Also, it cannot be discarded an involvement on PTP-forming protein complex, thereby suggesting a possible role of MTP on petal senescence.

2.4.2.2.9. Cellular communication/signal transduction mechanisms

Signal transduction is a mechanism that converts a mechanical/chemical stimulus into a specific cellular response. Signal transduction is elicited by a signal at a receptor, which, in turn, mediates a change in cell function. Cells communicate and adapt to changes to the physical environment using various signals. The ability of receptors to transfer signals to the cytosol is achieved by a series of conformation changes in the receptor itself and the activation of the heterotrimeric G-protein (Neves *et al.*, 2002). Down regulation in *O. fusca* of a transcript coding for G- protein β-subunit at 2DAP was detected. G proteins (guanidine nucleotide-binding proteins) are a family of proteins involved in chemical signals transmission from the outer cell, through G protein-coupled receptors which pass through the cell membrane for signal amplification (Jones and Assmann, 2004). G proteins belong to a large family of proteins, the GTPases. Roles in regulating ions channels, enzymes transport, as well as

transcription control, secretion, contractility and motility have been addressed to G proteins (Guo *et al.*, 2009). These cellular processes regulate functions such as control of seed germination, light responses, cell division and elongation, as well as response to phytohormones (Neves *et al.*, 2002). In plants, 4 heterotrimeric G-protein subunits have been identified in *Arabidopsis thaliana*: one α-, one β- and two γ-subunits (Jones, 2002). G-proteins are involved in numerous processes including phytochrome (Romero and Lan, 1993) and gibberellins signalling (Jones *et al.*, 1998), and regulation of stomatal movement (Li and Assmann, 1993; Chen *et al.*, 2004). Several studies point out to an involvement of G protein β-subunit on normal leaf, flower and fruit development (Ma *et al.*, 1999; Lease *et al.*, 2001; Peškan-Berghöfer *et al.*, 2005). Since an AGB1, encoding a β subunit of G protein in *Arabidopsis*, has been recently described as a regulatory gene of the reproductive trait plasticity (Nilson and Assmann, 2010), down regulation of a G protein β may illustrate a decrease on G-protein-mediated signalling regarding phenotype plasticity related to environmental adaptation.

Down regulation of a transcript coding for a putative Calmodulin (CAM) protein was found at 4DAP. In plants, calmodulin (CAM) is one of the major classes of calcium (Ca^{2+}) sensors characterized so far (reviewed in Yang and Poovaiah, 2003). CAMs are the most conserved proteins serving as a universal calcium sensor in eukaryotes, mediating calcium action by regulating the function of many targets in diverse cellular pathways (Zielinski, 1998; Bey *et al.*, 2004). No enzymatic activity has been attributed to CAMs. After Ca^{2+} sensing, they can regulate the activity of target protein in numerous processes (Bouché *et al.*, 2005). In *A. thaliana*, 7 *CAM* genes and 50 *CAM*- like genes, with multiple roles have been described (McCormack *et al.*, 2005). Increasing evidence supports a role for CAM proteins in plant development and in responses to biotic and abiotic stimuli (Ranty *et al.*, 2006). In *A. thaliana* floral buds, a highly expressed *CAM2* gene showed to be essential in male and female gametophytes function (Landoni *et al.*, 2010). In *O. fusca* down regulation of a transcript coding for a CAM2 at 4DAP may be related to cell signalling processes required for petal differentiation, as reported for *Antirrhinum majus* (Bey *et al.*, 2004). This CAM transcript may be associated to protein target regulation in petal development, yet other processes may not be discarded.

Pollination appears to inactivate/disengage signal transduction mechanisms, by chemical signal perception of G-proteins and CAM -mediated pathways.

2.4.2.2.10. Energy

Photosynthesis and carbon fixation are two of the most important cellular processes (Fig. 2.14). Oxygenic photosynthesis (i.e. the conversion of sunlight into chemical energy by plants) uses two multi-subunit photosystems (I and II) located in

plants chloroplasts thylakoid (Nelson and Ben-Shem, 2004). Photosystem I (PSI) has a P[10]700 reaction centre containing chlorophyll that takes the electron and associated hydrogen donated from PSII to reduce NADP+ to NADPH (Nelson and Yocum, 2006). Photosystem II (PSII) has a P680 reaction centre containing chlorophyll that uses light energy to carry out the oxidation (splitting) of water molecules, and to produce ATP via a proton pump (Nelson and Yocum, 2006; Diller *et al.*, 2007). Both ATP and NADPH are subsequently used in the light-independent reactions to convert carbon dioxide to glucose using the hydrogen atom extracted from water by PSII, releasing oxygen as a by-product (reviewed in Szabó *et al.*, 2005). Many *Arabidopsis* genes participating in the light-harvesting reactions of photosynthesis are under clock control. Among them are the light- harvesting complex I (LHCI) and II (LHCII) gene families which encode chlorophyll *a*/*b* binding polypeptides for photosystems I and II, respectively (Yakir *et al.*, 2007). Genes involved in the biosynthesis of chlorophyll and RuBisCO small subunit (RBCS) and RuBisCO activase (RCA) that participate in carbon fixation are also clock-controlled (Yakir *et al.*, 2007). The most prominent event in leaf senescence is the disassembly of the photosynthetic apparatus with consequent decrease of photosynthetic capacity (Weng *et al.*, 2005), which may be associated with reduced photochemical activities of photosystems (Lu *et al.*, 2002; Zhang *et al.*, 2006). Plants absorb light but this event also damages the photosynthetic machinery, primarily PSII, by causing PSII photoinactivation that is referred as photoinhibition (Takahashi *et al.*, 2007).

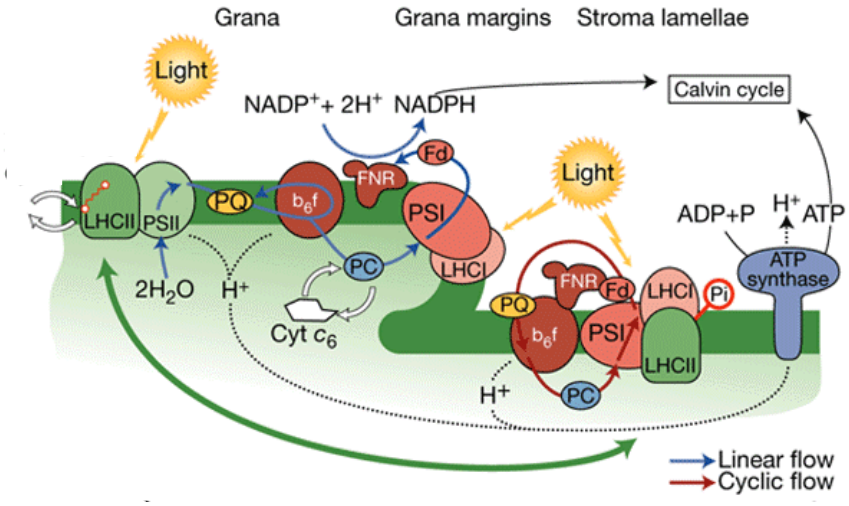

Figure 2.14- Photosynthetic electron-flow machinery. Photosynthetic activity results from the balance between light absorption and its use. Regulation of light absorption relies on the thermal dissipation in the photosystem II (PSII) outer antenna light harvesting complex II (LHCII). This dynamic regulation of photosynthesis also involves electron transport. Modulation of the efficiency of linear (blue) and cyclic (red) electron flow, and thus ATP synthesis, through the generation of a H^+ gradient is performed. Fd, ferredoxin; FNR, Fd: NADP+ reductase; PQ, plastoquinone. Adapted from (Finazzi *et al.*, 2003)

[10] P- Pigment.

Photorespiration is an important mechanism for protecting PSII from photoinhibition (reviewed in Foyer *et al.*, 2009). In photosynthesis, the fixation of CO_2 is catalyzed by RuBisCO (ribulose-bisphophate carboxylase oxygenase), that catalyzes the carboxylation of its substrate (ribulose-1,5-bisphosphate (RuBP)) to initiate the Calvin cycle; and, RuBisCO also catalyzes the oxygenation of RuBP in the photorespiratory pathway (reviewed in Bauwe *et al.*, 2010). Briefly, oxygenation of RuBP yields two by-products, 2-phosphoglycolate and 3- phosphoglycerate. After, the photorespiratory phosphoglycolate is recycled through a complex series of reactions occurring in chloroplasts, peroxisomes and mitochondria (McClung *et al.*, 2000).

Photosynthesis-related transcripts were significantly down regulated in *O. fusca* labellum (Table 2.6). Ferredoxin NADP oxireductase (FNR, EC. 1.18.1.2) is involved on electron movement on the electron transport chain (ETC) while regenerating NADPH from $NADP^+$, and its lower transcription at 2 DAP may have consisted of an early signal for a possible decrease on electron flux along ETC. In *Arabidopsis* thaliana, the chloroplast-targeted enzyme ferredoxin-$NADP^+$-oxidoreductase (FNR) is present as two isoforms, AtLFNR1 and AtLFNR2. Leaf form FNR (AtLFNR1) has been associated to cyclic electron transfer around photosystem I (PSI), which generates a proton gradient across the thylakoid membrane with ATP production. When FNR1 is inactive, the content of the light-harvesting complexes (LHCI and LHCII) are significantly decreased, as well as chlorophyll content, PSI and PSII proteins contents (Lintala *et al.*, 2007). As a result, FNR is essential for maintaining photosynthesis homeostasis, which is not more needed soon after pollination, a high energy-consuming process. Indeed, several other transcripts were also found to have their abundance decreased at 4 DAP, namely FNR, a chlorophyll a/b binding protein of the light harvesting complex (LHC) I, the photosystem I (PSI) subunit G (PSI-G) and RuBisCO activase (Table 2.6). In petal senescence, a lower expression of genes linked to photosynthesis has been reported as related to N and C mobilization for other developing parts of the plant (van Doorn, 2004; Mishina *et al.*, 2007; Müller *et al.*, 2010). According to Espinoza and co-workers (2007), in leaf senescence, chlorophyll degradation and decrease in photosynthetic activity occur along with decreases in total RNA and protein amounts. Since rapid respiration by young flower tissue is needed to fulfill energy demands for growth and development (Setyadjit *et al.*, 2004; Azad *et al.*, 2008), it was expected to encounter down regulation of respiratory and photosynthetic genes after pollination. Down regulation of RuBisCO activase reflects inactivation of RuBisCO, thus photorespiration is reduced (Lilley *et al.*, 1990). During photosynthesis, light energy is captured by pigments in the light-harvesting complex (LHC) and transferred to the reaction centers (Photosystems I or II) of the thylakoid membrane in green plants. LHC functions as a light receptor that captures and delivers excitation energy to photosystems I and II (Ganeteg *et al.*, 2001 and

references therein). Down regulation in *O.fusca* of a photosystem I subunit G (PSI-G) transcript suggests that photosystem I stability is compromised at 4 days after pollination, since this subunit reflects the abundance of most PSI core proteins. PSI-G plays also an important role in electron transport between plastocyanin and PSI, thus stabilizing the PSI core (Varotto *et al.*, 2002). Up regulation at 4DAP of a transcript coding for a PSII 10kDa (PsbR) protein, one of the 4 nuclear encoded subunits of the oxygen-evolving complex in PSII, was detected in *O. fusca* pollination. Photosystem II is a supramolecular pigment-protein complex embedded in the thylakoid membrane that catalyzes light-induced electron transfer from water to plastoquinone, with concomitant progress of oxygen occurring on the luminal side of PSII (Ferreira *et al.*, 2004; Renger, 2010). The oxygen-evolving complex (OEC) of eukaryotic PSII is composed of three extrinsic nuclear-encoded subunits, PsbO (33 kDa), PsbP (23 kDa), and PsbQ (17 kDa) (Ferreira *et al.*, 2004) and higher plant PSII contains the nuclear-encoded low molecular mass subunits PsbW and PsbR (Suorsa *et al.*, 2006). Roles addressed to PsbR include water oxidation and stabilization of PSII complex, by affecting the properties of both the acceptor and donor side electron transfer reactions (Collet *et al.*, 2003; Suorsa *et al.*, 2006; Allahverdiyeva *et al.*, 2007). Recent studies pointed out for a putative function of PsbR as a docking protein (Suja and Parida, 2008). According to Suorsa and co-workers (2006), PsbR absence decreases the oxygen outcome from thylakoid membranes, suggesting that PsbR is essential for optimization of photosynthetic water splitting and electron transfer in PSII. A drastic decline in activities of PSII, PSI and whole chain electron transport has been reported for several senescing systems, suggesting that the photochemical activity inhibits photosynthesis during senescence (Liu *et al.*, 2006; Zhang *et al.*, 2006; Falqueto *et al.*, 2010). PSII may act also as a sensor for stress (van Rensen and Curwiel, 2000). Accumulation of PsbR transcript at 4DAP may reveal a putative function of PsbR in stabilizing the binding of PsbP and PsbO proteins in the PSII complex crucial for water oxidation.

Down regulation of the mitochondrial enzyme serine hydroxymethyltransferase (SHMT), involved in the photorespiratory pathway of serine (Ser) biosynthesis (Ho and Saito, 2001) was detected at 2 DAP. Being photorespiration a wasteful metabolism releasing CO_2, NH_3 and ATP, important for energy dissipation to prevent photoinhibition (Zhu *et al.*, 2005); this process was expected to be disengaged soon after pollination. A defective mitochondrial SHMT gene have been related to a lethal photorespiratory phenotype (Vool *et al.*, 2006).Yet, 7 different SHMT genes have been described in *Arabidopsis* (Bauwe and Kolukisaoglu, 2003) and functions on photorespiration remains to be fully addressed. Though photorespiratory pathway of Ser synthesis seems to be reduced early after pollination, Ser synthesis is activated by the phosphorylated

pathway at 4 DAP, which is revealed by the up regulation of a phosphoglycerate dehydrogenase protein (PHGDH; EC 1.1.1.95). This enzyme catalyzes the transition of 3-phosphoglycerate into 3-phosphohydroxypyruvate, which is the first and rate-limiting step in the phosphorylated pathway of serine biosynthesis using NAD+/NADH as a cofactor (reviewed in Ho *et al.*, 1999). This pathway has been proposed to be an important Ser supply to non-photosynthetic organs (Muñoz-Bertomeu *et al.*, 2010) and when the photorespiratory rate is low (Ho *et al.*, 1999; Ho and Saito, 2001). Taking the above considerations together, a photorespiration disturbance after pollination is suggested by our data, thus, the non-photorespiratory pathway would guarantee Ser supply to assist the newly protein synthesis 4 days after pollination (4 DAP). Up regulation of a transcript coding for a 3-phosphoglycerate dehydrogenase at 4DAP suggest that when photorespiration rate is low, which is expected when senescence is ongoing, Ser biosynthesis is routed through phosphorylated pathway probably rendering serine as a precursor for several amino acids biosynthesis.

Down regulation of the heme biosynthetic protein protoheme IX farnesyltransferase, similar to the human cytochrome *c* oxidase 10 (COX10), was detected at 2 DAP. In plants, heme is a tetrapyrrole molecule and its synthesis is essential for providing heme groups to cytochromes (Cyts), crucial in the electron transport chain (ETC) as electron carriers located on mitochondrial inner membrane (MIM) (Oborník and Green, 2005; Tanaka and Tanaka, 2007). On the other hand, COX10 is required for COX biogenesis (reviewed in Diaz *et al.*, 2006), being COX the terminal complex of the mitochondrial respiratory chain, complex IV (Kranz *et al.*, 1998; Comelli and Gonzalez, 2009). The COX10 knockout on mammalian cells displayed a respiratory deficiency (Diaz *et al.*, 2006), thus, it is possible that a lowered expression of a heme biosynthetic transcript related to COX10 protein at 2 DAP in the present study is related to a negative impact of pollination on mitochondrial ETC.

Our results suggest that energy processes (i.e. photosynthesis, photorespiration, electron transport chain) in *Ophrys fusca* labellum are early affected by pollination event. Since the flower has fulfilled its biological role, ensuing fertilization, maintenance of such an energy-consuming organ is compromised, and, the outcome of energy depletion may either modulate or be a side-effect of labellum senescence. This hypothesis is in agreement with data on tobacco flower, where gene expression of several proteins involved in respiration is inactivated along with senescence (Müller *et al.*, 2010).

2.4.2.3. cDNA microarray validation through qPCR

cDNA microarrays have the ability to provide an unprecedent capacity for a broad gene expression profiling (Gachon *et al.*, 2004). Since microarray experiments can analyze thousands of genes in one step, quantitative real-time PCR (qPCR) emerged as the most reliable and fast technique for validation of gene expression datasets (Morey *et al.*, 2006). As plants display a high number of multigenic families, cross-hybridization between DNA representatives of gene families on cDNA-based chips may lead to false interpretations (Gachon *et al.*, 2004). Therefore, cDNA microarrays validation through qPCR is crucial. In qPCR, several factors have to be considered (reviewed in Udvardi *et al.*, 2008), being one of the most important the requirement of reference genes validation for each tissue and/or condition studied (Dheda *et al.*, 2005). If reference genes are not validated, normalization may be compromised with influence on the accuracy and incongruency between both techniques (Brunner *et al.*, 2004). In this study, three reference genes were selected from microarray studies based on their stable differential, retrived by RankProduct test, gene expression in both time points assigned (2DAP and 4DAP). Overall, 7 genes (5 % of the differentially expressed genes) were selected according to their expression profiles and functional categories. Expression of these genes was performed by using the same RNA samples that were used for the microarray hybridization experiments. qPCR data showed expression patterns similar to those obtained for microarrays (Fig. 2.15), with exception for class III chitinase transcript, where gene expression obtained from microarray was conflicting with the expression measured by qPCR (Fig. 2.15). This is not unexpected, since one of the limitations of cDNA microarrays relies on the possible cross-hybridization of different transcripts belonging to the same gene family, influencing expression levels (Murphy, 2002).

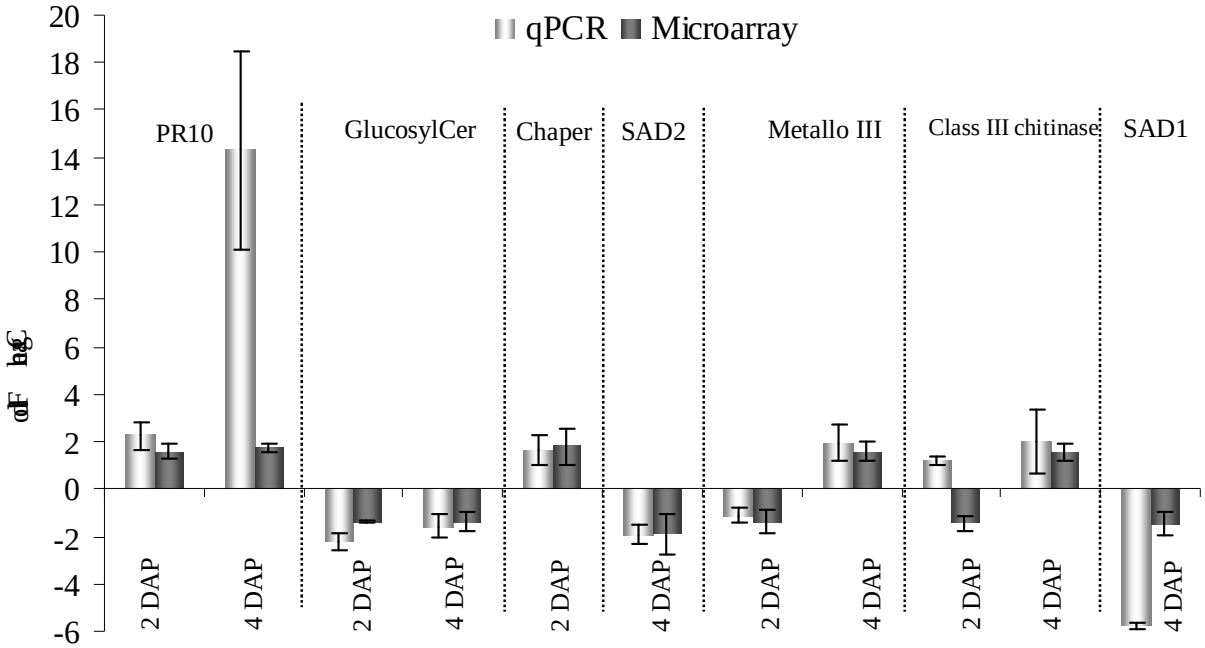

Figure 2.15- Relative expressions comparision of 7 transcripts from cDNA microarray and qPCR results. Transcripts chosen for validation encode for: Pathogenesis- related protein 10c (PR10), GlucosylCer (Glucosylceramidase), Chaperone (Chaper), Stearoyl ACP-desaturase (SAD2), Metallothionein type 3 (Metallo III), Class III chitinase and Stearoyl ACP-desaturase (*Of*up2825-SAD1). Median and Mean Absolute Deviation (MAD) of 5 biological replicates, for both qPCR and microarrays, are presented. Thus, two technical replicates were performed in all qPCR measurements. 40S ribosomal protein, 4-α-glucanotransferase and an oligopeptidase were used as reference genes. DAP: Days after pollination; Fold Change, relative expression of test (labella from pollinated flowers) versus control (labella from unpollinated flowers).

2.5. Conclusions

Flower function relies essentially on attracting pollinators to achieve successful pollination. Upon pollination, metabolic resources of the flower are rapidly mobilized to the developing ovary and flower senescence is undergoing. By removing organs and tissues that are no longer functional, plant preserves its energy resources and eliminates possible sites for pathogens entrance. Another important basis for floral death relies on pollinated flowers removal from the plant or inflorescence so that it does not compete for pollinators with the remaining flowers of the same inflorescence. After pollination, sepals and petals may senesce and either abscise or remain *in situ*. Genes called SAGs appear up regulated during senescence, while photosynthetic genes are actively down regulated during this process (Mishina *et al.*, 2007; Irish, 2009).

The potential complexity of transcriptional and post transcriptional control of senescence can be perceived from the *Ophrys fusca* labellum transcriptome after pollination performed. *Ophrys fusca* labellum transcriptome modulation demonstrates

that pollination triggers events leading to labellum senescence. Expression of stress- and defense-related transcripts at 2DAP (e.g. Lea5 protein, pathogenesis related protein 10, gluthatione *S*- transferase- GST, chaperone, snakins proteins and cyclophilin) suggest a general stress response in the labellum after pollination. The majority of differentially expressed transcripts at 4DAP encode for proteins related to proteolysis, energy and secondary metabolism. Post pollination changes in the labellum suggest an activation of macromolecules breakdown, proteolysis, remobilization of nutrients to other developing tissues and deactivation of the major metabolic pathways (secondary metabolism) and energy-consuming processes (i.e. photosynthesis, photorespiration).

Pollination activates macromolecules breakdown, deactivates major metabolic pathways (secondary metabolism) and remobilizes nutrients to other developing tissues, probably to other flowers from the same inflorescence and/or developing ovule. At the onset of senescence, the majority of down regulated genes include secondary metabolism, transcription and photosynthesis related genes; while another subset of genes, termed senescence associated genes (SAGs), are up regulated. SAGs obtained in *O.fusca* labellum transcriptome analysis, include genes encoding phosphatases, proteases and RNases, which reveal that labellum PCD is triggered by successful pollination, through highly regulated machinery. *Ophrys fusca* pollination gene expression analysis here presented allowed to gather a clear overview of the labellum response upon pollination. Overall, secondary metabolism is unequivocally down regulated following pollination. Soon after pollination (2DAP), genes involved in secondary metabolism pathways are down regulated suggesting that the flower no longer needs its by-products, e.g. regarding floral pigmentation (flavonoid biosynthetic genes). At 4DAP, pollination continues to induce gene repression and a stress- related response may be depicted by the hallmark expression of pathogen and stress-related genes (e.g. PR10c, chitinase, Lea5, cyclophilins). Down regulation of alkaloid biosynthetic genes occurred in *Ophrys fusca* pollination study, and of particular interest is the transcript coding for salutaridino 7-O acetyltransferase, involved in morphine biosynthesis. Despite the fact that *Ophrys fusca* pollination is achieved through sexual deception, it cannot be discarded the possibility of alluring insects through narcotic compound synthesis which, in turn, could increase the chance of flowers to be pollinated, thereby avoiding pollinator learning cues. This is especially important in *Ophrys* pollination system which is characterized by a low visitation rate. In what concerns lipid metabolism, all related transcripts were down regulated at both 2DAP and 4DAP. The following have to be highlighted: transcripts coding for wax biosynthesis (TCER1), fatty acids (FAD2 and SAD), sphingolipids (ceramidase and glucosylceramidase) and sterol metabolism (7- dehydrocholestrol reductase). Down regulation of a gene coding for TCER1, a putative aldehyde decarbonylase, involved in

synthesis of wax long-chain lipids (Fiebig *et al.*, 2000), at 4DAP suggest a post pollination reduction of alkane synthesis, which are important compounds as olfactory mimic cues in *Ophrys* pollination system. Also, cell membranes properties undergo marked changes in membrane phospholipids with loss and increase in the saturation: unsaturation index of fatty acids, namely of PUFAs, which is revealed by the down regulation of FAD2 and SAD transcripts in fatty acids metabolism. The possibility that these desaturases can give rise to a vast array of unsaturated fatty acids, namely alkenes, which are compounds triggering pseudocopulation attempts in the sexual deception *Ophrys* pollination mechanism, cannot be excluded. Thus, the involvement of both desaturases (FAD2 and SAD) in *Ophrys*-pollinator ecological relationship, through the insertion of double bonds at different positions in fatty acid substrates of distinct carbon chain lengths, seems to be a promising challenge that needs to be confirmed. Another class of lipids influenced by pollination is sphingolipids, which are bioactive lipids that regulate plant growth and PCD (Liang *et al.*, 2003). Lipid-derived messengers also interplay with ROS to modulate PCD. In *O.fusca* down regulation of ceramidases at 2DAP and 4DAP (neutral and glucosylceramidase), results on accumulation of free sphingolipid bases and depletion of complex ceramides, followed by H_2O_2 accumulation and subsequent cell death. As a result, it can be considered that massive lipid biosynthesis deactivation, revealed by down regulation of lipid metabolism genes accompanies labellum senescence. The expression of stress-related genes (GST, Lea5, metallothioneins types 2 and 3, chitinases and PR proteins) disclose the PCD event in labellum that is fully recognized 4 days after pollination. Several cysteine proteases, known to be important factors in the attainment of ROS-driven PCD, were up regulated at 4DAP. It is well documented that ethylene and ROS are positive regulators of leaf (Lim *et al.*, 2007) and petal senescence following pollination, namely in the ethylene-sensitive flowers carnation, petunia, tobacco, and orchids (O'Neill, 1997). Thus, ethylene stimulates the expression of SAGs encoding proteins such as cysteine proteases and RNases (Della Mea *et al.*, 2007). Transcripts coding for cysteine proteases, S-like RNases and TRL-14/SAG13 (generally expressed shortly before visible senescence begins) were probably stimulated by post pollination events towards the labellum cell death. Up regulation at 4DAP of transcripts involved in protein synthesis (ribossomal L17 and translation intiation factor) illustrates the requirement of *de novo* protein synthesis in the timely initiation and progression of petal senescence (van Doorn and Woltering, 2008; Bai *et al.*, 2010).

Many of the newly identified orchid genes are presumably involved in modulating the initial signalling events illustrated by stress and pathogen-related genes (e.g. PR10c, cyclophilins, metallothioneins, and chaperone). These include genes that modulate the intensity of the cell death stimulus, participate in the interaction with other signaling molecules, or perceive the cell death signal (down regulation of

ceramidases, HYS1 gene). Other genes relay and amplify the cell death signal (e.g. S-like RNases, cysteine proteases, protein synthesis related transcripts), ultimately to govern global transcriptional reprogramming observed during cell death. As a result of this reprogrammation, sets of genes including proteases and nucleases orchestrate the orderly executed cell suicide and remobilization of resources.

Several transcripts coding for transcription factors involved in floral regulation (SQUAMOSA-binding protein and Flavin-binding Kelch Repeat F-Box1) and on ovule developmental programme (Nam-like protein), may give a small, but yet important, contribute towards the understanding of orchid reproductive biology. Differentially expressed genes coding for expansins up regulated at 4DAP may be a consequence of auxin action, an induced post pollination phenomenon, which is critical for ovary maturation and growth in orchids. Actin cytoskeleton rearrangement induced by cross-pollination is illustrated by expression of transcripts coding for actin and profilin, being also putatively involved in pollen germination.

From the transcriptional study performed on *Ophrys fusca* labellum after pollination, it was verified that an irreversible senescence program on petal senescence is largely independent of environmental factors, unlike leaf senescence which is more closely linked with external stimuli. As a result, once a flower is pollinated or is no longer receptive for pollination purposes, maintenance of this organ is costly in energy requisites. Thus it was not surprising to encounter photosynthetic and secondary metabolism genes down regulated following pollination, both at 2DAP and 4DAP. Senescence, triggered by successful pollination, seems to be regulated by protein degradation and, after, by the newly protein synthesis. After pollination, gene expression seems to drive the labellum to PCD, mainly characterized by a gradual decrease in photosynthetic activity, activation of proteases for large scale proteolysis, pigment depletion (flavonoids/anthocyanins metabolism), modification of the cell wall (expansins, PMEI) and *de novo* protein synthesis required for senescence onset.

This study on *Ophrys fusca* pollination is promising, namely for the understanding of the high regulated process of labellum senescence in orchid pollination biology in general. Future studies on orchids should focus on monitoring the mRNA transcripts of certain genes in order to find specific markers that govern pollination regulatory mechanisms (e.g. scent emission, senescence, pigmentation depletion). Labellum transcriptome analysis here presented accounts for an alternative overview on *Ophrys fusca* peculiar pollination mechanism. Pollination is usually species-specific (Paulus and Gack 1990) providing a mean for reproductive isolation between the intercrossable *Ophrys* species (Schiestl and Ayasse, 2002). Transcripts putatively involved in the biosynthesis of key compounds that trigger pseudocopulation events, such as FAD2 and SAD, may be promising in the evolution of the peculiar pollination mechanism

within *Ophrys*. This approach could provide insights on transcriptional control of biological active compounds involved in *Ophrys* species-specific pollination.

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Chapter 3

METABOLIC PROFILING OF *OPHRYS FUSCA* LABELLUM AFTER POLLINATION

3.1. Abstract

Ophrys has evolved a highly specific pollination system known as sexual deception. Sexually deceptive orchids, like *O. fusca*, attract their pollinators by mimicking the female hymenopteran sex pheromones, thereby deceiving males into attempted mating with the orchid labellum. In order to detect chemical changes after pollination event, a metabolic profiling of in-field *Ophrys fusca* plants was performed using a Gas chromatography- mass spectrometry (GC-MS) analysis. Labella from unpollinated and pollinated flowers were collected 2 (2 DAP) and 4 (4 DAP) days after pollination. Rather than identifying all compounds present in labella extracts, analysis was focused on cuticular hydrocarbons, namely straight chain saturated hydrocarbons (*n*-alkanes) and unsaturated (*n*-alkenes) hydrocarbons with chain lengths 21–29 and with double bond positions 7, 9, 11 or 12. These compounds have previously shown to be responsible for pollinator attraction in *Ophrys* species. A total of 22 compounds were identified including 13 (59 %) different *n*-alkenes. Statistical analysis revealed differences between unpollinated and pollinated labella, both at 2 DAP and 4 DAP. At 2 DAP, alkanes and alkenes are differentially accumulated; whereas at 4 DAP only alkenes displayed differential accumulation. Total amounts of labella extracts do not displayed differences with statistical significance, revealing that compounds production is not immediatelly ceased after pollination. Results presented highlight that post pollination mechanism is not reflected by a significant decrease in compounds production, as opposite to other post pollination systems. This may be related to functions attributed to these cuticular compounds on the flower, namely by preventing water loss. As a consequence, the abrupt cessation of such compounds could cause physiological limitations on the plant (Schiestl and Ayasse, 2001).

Keywords: pollination, sexual deception, GC-MS, *Ophrys fusca*, *n*- alkanes, *n*- alkenes.

3.2. Introduction

3.2.1. Floral scent as a powerful communication channel

Floral scent constitutes an important communication channel between flowering plants, their pollinators and enemies (Raguso, 2008). Plants often attract pollinators with floral displays composed of visual, olfactory, tactile and gustatory stimuli (Raguso, 2008; Leonard *et al.*, 2011a), thus flowers are considered multisensory displays by influencing pollinators behaviour (Leonard *et al.*, 2011b). In flowering plants, scent prime function relies in attracting and guiding pollinators to accomplish pollination, thus ensuring reproductive success (Dudareva and Pichersky, 2000). Further functions have been ascribed to flowers volatile organic compounds (VOCs), namely in defense and protection against abiotic stresses (Pichersky and Gershenzon, 2002; Unsicker *et al.*, 2009). Plants usually emit compounds to defend themselves againts herbivores/florivores and pathogens to ensure survival; and to attract animal pollinators for sexual reproduction (Dudareva and Negre, 2005; Schiestl, 2010). Upon herbivore damage, herbivore-induced volatiles are released as a defensive strategy for attracting carnivorous arthropods (Heil and Bueno, 2007; Bandeili and Müller, 2010). Plants face, thus, a dynamic tension regarding the emission of compounds as an effective defense response to granivores and herbivores/florivores and as attractants to pollinators (van der Meijden, 1996; Knudsen *et al.*, 2006; Raguso, 2009). Floral scents consist of complex blends of compounds, which often belong to distinct chemical classes and are produced following different biosynthetic pathways (Knudsen *et al.*, 2006). Given the complexity of floral scent in terms of number, identity and relative amounts of volatile compounds, a variation at both inter- and intra-specific level may occur (Dudareva and Pichersky, 2000). Thus, such bouquet variations may be at the qualitative level or at proportions of the same compounds (Mant *et al.*, 2005; Schiestl *et al.*, 2010 and references therein). Flower emission of a single compound has been reported namely on modulation of pollinator behaviour (Schiestl *et al.*, 2003; Wiemer *et al.* 2008; Chen *et al.*, 2009).

Pollinators mediate reproductive success and reproductive isolation among plants, namely in Orchidaceae. In orchid pollination, pollinator sharing is generally low thus suggesting strong floral isolation (Schiestl and Schlüter, 2009). Floral isolation is mediated by flower morphology (morphological isolation) and pollinator behaviour (ethological isolation) (reviewed in Schiestl and Schlüter, 2009). Studies in floral color (Aragon and Ackerman 2004; Streinzer *et al.*, 2010) and morphology (Sletvold *et al.*, 2010) have shown that floral traits may be under pollinator-mediated selection. Floral

scent studies have been performed in less extend mainly due to demands on sampling and analysis (Schiestl *et al.*, 2010 and references therein).

3.2.2. Sexual deception in *Ophrys*

3.2.2.1. Flower cues and pollinator's behaviour

Sexually deceptive orchids of the genus *Ophrys* are a prime example of pollination by mimicry in plants (Schiestl, 2005; Schaefer and Ruxton, 2009). Several studies in *Ophrys* floral signals led to the recognition of odour as the major driving cue on pollinator specificity and, consequently, on reproductive isolation within the genus (Paulus and Gack, 1990; Borg-Karlson *et al.*, 1993; Schiestl *et al.*, 1999; Schiestl, 2005; Cozzolino and Scopece, 2008). In 1990, Borg-Karlson provided a comprehensive review of the relationships between orchids of the genus *Ophrys* and their insect pollinators. In this review, the author describes the detailed chemical analyses and field studies conducted on communication between *Ophrys* plants and pollinators. Sexual deception mechanism was first elucidated by Schiestl and co-workers (1999), who showed that specific patterns of cuticular hydrocarbons on the orchid flower are similar to those found in the receptive hymenopteran female of the specific pollinator species. *Ophrys* pollinators are mainly solitary bees (Andrenidae, Anthophoridae, Colletidae, Megachilidae, and Apidae), solitary wasps (Sphecidae and Scoliidae) and occasionally beetles (Scarabaeidae) (Kullenberg, 1961; Borg-Karlson, 1990; Paulus and Gack, 1990).

Compounds involved on sexual deception consist mainly on long-chain fatty acids and their derivates, namely alkanes and alkenes (Borg-Karlson, 1990; Schiestl *et al.*, 1999, 2000). Given that pollinators play a key role in the origin and maintenance of floral variation in natural populations (Rymer *et al.*, 2010), reproductive isolation in sexually deceptive orchids is predicted to be achieved by differences on floral scent (Schiestl *et al.*, 2004; Cozzolino and Scopece, 2008; Peakall *et al.*, 2010).

A sequencial behaviour response of a male hymenopteran attracted by an orchid flower is illustrated (Fig. 3.1). First, the male insect is drawn to the flower by long-range attractants (Fig. 3.1- **Stages 1-2**) and afterwards, the searching-flight becomes more disconcerted (Fig. 3.1- **Stage 3**) and when approaching the flower, visual cues work as secondary stimuli (e.g. labellum colour and shape) (Borg-Karlson, 1990). Once the insect lands on the labellum, tactile cues (e.g. trichomes orientation) may direct the male insect to adopt an adequate position for accomplishing pseudocopulation, i.e. abdominal or cephalic (Fig. 3.1- **Stage 4**, Bergström, 1993). After a failed mating attempt, male pollinator departs from the orchid flower with pollinia attached to its

body and, when visiting another flower the repeated process allows pollinia transfer and, consequently, pollen release.

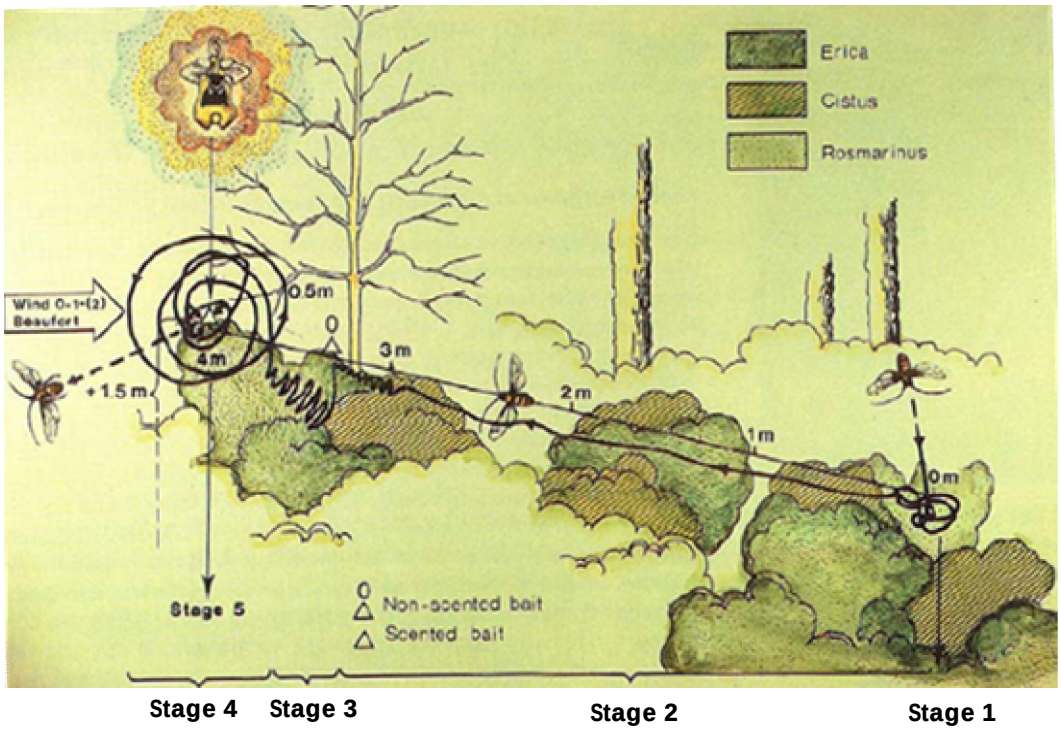


Figure 3.1- Behavioural stages of a male hymenopteran attracted by an *Ophrys* flower. **Stages 1-2**: Olfactory signals emitted by the orchid labellum draws male attention; **Stage 3**: As the insect approaches the flower, the insect searching-flight turns to be more disconcerted, and visual cues may provide a secondary stimulus; **Stage 4**: Pseudocopulation takes place; **Stage 5**: The pollinator insect departs form the *Ophrys* after an unsuccessful mating attempt (Bregström, 1993).

In sexually deceptive orchids, cross-pollination is achieved by mimicking the mating signals of female insects, especially sex pheromones (Schiestl, 2005; Vereecken and Schiestl, 2008) usually of one or few pollinator taxa (Kullenberg, 1961; Nilsson, 1992). These orchids blossom with relative accuracy during the period when young and inexperienced male bees and wasps emerge and make their first exploratory visits (Nilsson, 1992). These orchids exploit, for the imitated insect species, the innate biases of males responding to receptive females, by producing the behaviourally active components of virgin female's sex pheromone (Streinzer *et al.*, 2009). Specificity in pollinator attraction is an hallmark in these orchids as only males of the target species are attracted by the odour bouquet, while unspecific pollinators are not (Streinzer *et al.*, 2009). Although flower odour is determinant for pollinator's attraction, flower morphology does also play an important role. After landing on a flower, morphological cues guide the males into the correct position to take off and/or deposit pollinia. Flowers do not display conspicuous colour signals thereby avoiding accidental attraction (Streinzer *et al.*, 2009; Spaethe *et al.*, 2010), instead, labellum patterns (e.g. colour, shape, pilosity) mimic female insect body parts. Nonetheless, due to the highly

specific olfactory attraction in sexual deception, visual signals are assumed to play a secondary role (Streinzer *et al.*, 2010 and references therein). In fact, it was determined that visual cues displayed by labellum colour in *Ophrys* species were not responsible for increasing pollinator's visiting rates (Vereecken and Schiestl, 2009). A recent study revealed that *Ophrys hedreichii* labellum pattern is involved on the repellent learning during post copulatory behaviour and further used by the orchid as a strategy to increase outcrossing rather than increasing pollinator attraction potential during the flower approach flight (Streinzer *et al.*, 2010).

3.2.2.2. Sexual deception mechanism: a chemical perspective

About one-third of the Orchidaceae species are thought to be deceit pollinated (Cozzolino and Widmer, 2005; Jersáková *et al.*, 2006). The observation that orchid flowers somehow orient flights of male pollinators led to the suggestion that long-range semiochemicals (i.e. chemical compounds which affect insect behaviour- Gullan and Cranston, 2000) are explored by these plants (Cane and Tengö, 1981; Dettner and Liepert, 1994). *Ophrys* genus was the first to be described as being pollinated by sexual deception mechanism (Pouyanne, 1917). From then onwards, several studies have been performed in *Ophrys* in order to unravel flower chemical composition associated to sexual deceit (Kullenberg, 1961; Borg-Karlson, 1990; Schiestl *et al.*, 1999; Schiestl, 2005). Chemical analysis revealed that *Ophrys* flowers produce complex species-specific mixtures of more than 100 compounds, mainly saturated and unsaturated hydrocarbons, aldehydes, alcohols, esters, ketones, and terpenoids, the aromatic compounds being present in minor amounts (Borg-Karlson and Tengö, 1986; Borg-Karlson, 1990). Only a small proportion of those compounds revealed to be actively involved in insect attraction (Ayasse *et al.*, 2000; Schiestl *et al.*, 2000). According to Schiestl and co-workers (1999), in *O. sphegodes* sexual deceptive mechanism flowers have the same compounds and specific patterns of long straight-chain unsaturated (*n*- alkenes) and saturated (*n*- alkanes) hydrocarbons, in relative proportions similar to those found in the sex pheromone of its pollinator species, the solitary bee *Andrena nigroaenea* Kirby. The use of gas chromatography-electroantennographic detection (GC-EAD) allowed the identification of compounds that influence the pollinator behaviour and further GC-MS analysis allowed its chemical structure's elucidation (Ayasse *et al.*, 2003). GC-EAD[11] has been used in compound identification from the complex flower scent perceived by the antennae (e.g. *Ophrys*

[11] Analytical technique in which an insect antenna is used as a parallel detector for compounds separated on a GC column to identify subsets of complex odour blends that show biological activity (Arn *et al.*, 1975).

species- Schiestl *et al.*, 2000; Ayasse *et al.*, 2003; other orchids- Huber *et al.*, 2005; Brodmann *et al.*, 2009). Confirmation of the biological activity using synthetic compounds in field bioassays was the final essential step for confirming the ability on insect behaviour modulation. Schiestl and co-workers (2000) verified that *n*-alkenes emitted by labellum are responsible for the attraction of male pollinators while alkanes exhibited a synergistic effect by increasing the intensity of male responses. Cuticular hydrocarbons (alkanes and alkenes) are part of the plant epicuticular wax preventing water loss (Hadley, 1981; Jetter and Kunst, 2008). Their function on attracting pollinators appears to be an economical floral trait favoured by the natural selection event (Schiestl *et al.*, 1999, 2000). These studies have also shown a higher proportion of the volatile unsaturated hydrocarbons in flowers rather than in leaves (Schiestl *et al.*, 2000), which led to the assumption that scent cuticular compounds in flowers was assigned to a role on pollination, while leaves cuticle may play almost exclusively a function on water loss prevention (Schiestl *et al.*, 2000; Steiger *et al.*, 2010).

Resemblance of the labellum chemical profile of the behaviourally active compounds regarding its model insect is largely responsible for eliciting mating behaviour on male pollinators (Schiestl *et al.*, 1999; Ayasse *et al.*, 2000; Schiestl, 2005; Mant *et al.*, 2005; Vereecken *et al.*, 2010). Such pollinator specificity, guaranteed by a species-specific chemical profile, constitutes an efficient reproductive isolation mechanism among the interfertile *Ophrys* species (Paulus and Gack 1990). Interestingly, Ayasse and co-workers (2003) exposed a special case on *Ophrys* sexual deception mechanism, illustrated by *O. speculum* Link and its pollinator species, the wasp *Campsoscolia ciliata* Fabricius. Despite the fact that *O. speculum* flower odour bouquet consists of identical blends of cuticular hydrocarbons (Erdmann, 1996) as seen in other *Ophrys* species, none of those elicited *C. ciliata* mating behaviour (Ayasse *et al.*, 2003). Instead, in *O. speculum*-*C. ciliata* interaction, pollinator attraction is achieved more or less by a single orchid-released compound, 9-hydroxydecanoic acid, an oxygenated carboxylic acid. Divergency on this interaction regarding other *Ophrys* species was attributed mainly to the pollinator species. In *Andrena*-pollinated *Ophrys* species, species-specific blends of the same hydrocarbons are used to avoid unspecific pollination (Schiestl and Ayasse, 2002). This specific bouquet is crucial since many *Andrena* species live sympatrically with closely related species and heterospecific mating could occur (Westrich, 1989). Instead, a different blend was explored by the *O. speculum* flower, as *C. cilliata* doesn't overlap either temporally nor spatially with other scoliid wasps (Ayasse *et al.*, 2003).

3.2.2.3. Floral odour in reproductive isolation

Since *Ophrys* species are pollinated by one or a few pollinator species, reproductive isolation is achieved by means of chemical mimicry of the sex pheromones of the corresponding pollinator species (Paulus and Gack, 1990; Nilsson, 1992; Ayasse *et al.*, 2011). *Ophrys* species pollinated by the same bee species, independently of their phylogenetic relationship, use similiar odour bouquets for pollinator attraction (Stökl *et al.*, 2005). In sympatric species pollinated by different insect species, e.g. *O. fusca* by *Andrena nigroaenea* and *O. bilunulata* by *A. flavipes*, a similar set of cuticular hydrocarbons is present, although their relative amounts differed significantly (see Schiestl and Ayasse, 2002). In sympatric species pollinated by the same insect species, reproductive isolation is not ensured by the odour bouquet but rather mechanically through the deposition of pollinia in different parts of the insect's body (Borg-Karlson, 1990). For example, *Ophrys fusca* and *O. sphegodes* are both pollinated by males of *Andrena nigroaenea* (Paulus and Gack, 1990), but different pseudocopulation positions prevent hybridization between the two species (abdominal pseudocopulation- *O. fusca*, cephalic pseudocpulation- *O. sphegodes*, for more detail see Chapter 1) (Kullenberg, 1961; Paulus and Gack, 1990). In sexually deceptive orchids, pollinator-driven speciation is directly linked to differences of floral odour bouquets. Orchids with high pollinator specificity mostly rely on pre mating reproductive barriers and have minute post mating isolation mechanisms (Cozzolino and Scopece, 2008). Exceptions have been reported, namely in two pairs of sympatric *Ophrys* species, (*Ophrys chestermanii* and *O. normanii*) displaying similar scents and sharing the pollinator species, in which post pollination isolation factors were attributed in mediating reproductive isolation (Gögler *et al.*, 2009). Another example on the possibility of post pollination factors to work as reproductive barriers was documented by Cortis and co-workers (2009) in a natural hybrid zone on the sympatric species *Ophrys iricolor* and *O. incubacea*.

A shift to a novel pollinator achieved through an odour bouquet modification may represent the main isolation mechanism responsible for *Ophrys* speciation (Cozzolino and Scopece, 2008). According to Vereecken and Schiestl (2010) *Ophrys* hybrids, resulting from two sympatric species *(O. lupercalis* and *O. exaltata*), displayed a novel floral scent and more attractive, than either of the two parents, to a pollinator species not initially involved in the pollination of any of the parent *Ophrys* species. As suggested by Peakall and co-workers (2010) for the sexually deceptive genus *Chiloglottis*, pollinatior-driven speciation may also occur in *Ophrys* system.

Ophrys species are able of attracting alternative pollinators species, leading to the occurrence of natural hybrids (Vereecken and McNeil, 2010). These results reveal

the flexibility of the mimicry system by "overcoming seasonal or annual fluctuations in the local pollinators populations" to guarantee reproductive success (Vereecken and McNeil, 2010). The occurrence of floral odour variation within *Ophrys* species is also an important strategy for preventing insect learning behaviour of the deceptive orchid floral odours (Ayasse *et al.*, 2000). This assumption is based on results by Ayasse and co-workers (2000), according which variation in relative proportions of compounds in flowers at different stems position minimize the learning capacity by insects of the flower bouquet, thus incrementing the possibility of a pollinator to visit different plants in a population. Vereecken and Schiestl (2008) demonstrated that *Ophrys exaltata* presents an imperfect mimick by emitting a different relative proportion of key odour compounds from the sex female pheromones. Instead of reducing flower attractiveness, the orchid flower bouquet was able to attract patrolling males with more success than sexual pheromones emitted by local virgin females of the pollinator species (*Colletes cunicularius*) (Vereecken and Schiestl, 2008).

3.2.3. Metabolomics: a diverse functional tool

Metabolomics aims at identifying the complete set of metabolites or the metabolome of the cell (Joyce and Palsson, 2006; Colquhoun, 2007). The metabolome is composed of hundreds of metabolites. Simultaneous measurement of all components of the metabolome by a single high-throughput method, analogous to those available in transcriptomics (microarrays) and proteomics (2D-PAGE), is not yet available (Bino *et al.*, 2004; Joyce and Palsson, 2006). This is mainly due to metabolites chemical and physical properties and to the high range of concentrations (pM to mM) at which they occur (Hall, 2006; Colquhoun, 2007). The conventional classification of metabolomics proposed by Fiehn (2002) has been accepted worldwide. It encompasses metabolite target analysis, metabolic fingerprinting, metabolic profiling and metabonomics (reviewed in Fiehn, 2002). A comprehensive coverage can only be achieved by using multiparallel complementary extraction and detection technologies with careful experimental design (Saito and Matsuda, 2010). There are several detection strategies that can be used, such as nuclear magnetic resonance (NMR), Fourier transformation infrared spectroscopy (FT-IR), and mass spectrometry (MS) coupled to separation techniques such as high performance liquid chromatography (HPLC), gas chromatography (GC), or capillary electrophoresis (CE) (reviewed in Saito and Matsuda, 2010).

3.2.3.1. Metabolic profiling

Metabolic profiling involves the pre-selection of a set of metabolites or of a specific class of compounds that might participate in a target pathway (Fiehn, 2002; Barderas *et al.*, 2011). These pre-defined metabolites may belong to a class of compounds such as polar lipids, isoprenoids, or carbohydrates, or be selected upstream of members from particular/targeted pathways (Baxter and Borevitz, 2006). With the improved performance of chromatography methods in the late 1960s and early 1970s (improvements on reliability, robustness, selectivity and resolution), peak identification in complex matrices was made possible based purely on retention times. These separation techniques were then coupled to highly sensitive detectors such as flame ionization coupled to gas chromatography (GC-FID) or fluorescence and UV detectors coupled to liquid chromatography (LC) (Fiehn, 2002). The flame ionization detector (FID) is a widely used detector, which is primarily sensitive to hydrocarbons (McWilliam and Dewar, 1958). Generally, GC coupled with a mass spectrometer (MS) detector is the most commonly used strategy for metabolites quantification and identification.

3.2.3.2. Gas Chromatography- Mass Spectrometry (GC-MS)

Gas chromatography (GC), mainly when interfaced with mass spectrometry (MS), is one of the most widely used and powerful method in metabolites identification and quantification. It offers very high chromatographic resolution, yet requires chemical derivatization for many biomolecules, with only volatile chemicals being analysed without derivatization step. Some large and polar metabolites cannot be analysed by GC. Mass spectrometry is used to identify and to quantify metabolites after separation by GC, HPLC (i.e. high-performance liquid chromatography) or CE (i.e. capillary electrophoresis). MS was improved to become as universal as flame ionization, by offering a completely independent method for compound identification and classification when coupled to GC (de Jongh *et al.*, 1969; Roessner, 2007). The analytical procedure can be focused on a smaller number of pre-defined metabolites and sample preparation as well as data acquisition can be focused on the chemical properties of these compounds (Fiehn, 2001). By including compounds with known retention times, shifts in absolute retention times could be checked. In mass spectrometers of most standard GC-MS instruments, compounds exiting the GC column are ionized by electron impact (EI) and the resulting positively charged molecules and molecule fragments are selected according to their mass-to-charge (*m/z*) ratio by entering a mass filter (Fig. 3.2). Total ion chromatograms obtained provide information

on the retention time of each compound and its mass spectrum, consisting on a characteristic ion fragmentation pattern. Detection limits of highly sensitive mass spectrometers are in the picogram range for the full scan mode (scanning ions over a wide molecular range) and may be as low as in the femtogram range (in quadrupole mass filters) in the selected ion monitoring (SIM) mode scanning (Tholl *et al.*, 2006). For automated metabolite identification, reliable information on both retention time and mass spectra is required (Fiehn, 2001). Compounds can be identified through spectral comparisons with authentic compounds from reference spectral libraries such as the NIST (http://www.nist.gov/; National Institute of Standards and Technology, Gaithersburg, USA) or Wiley (http://eu.wiley.com/WileyCDA/WileyTitle/productCd-0470047852,miniSiteCd-STMDB2.html). Although these libraries contain 350 000 entries, most of them include non-biological compounds and lack information on chromatographic behaviour which is essential, in particular, for isomers identification (Wagner *et al.*, 2003). A reference library containing both retention time of these compounds (as determined under the same conditions) and the corresponding mass spectrum can be created (Wagner *et al.*, 2003). Identification by retention time is confirmed by co-chromatography of each standard substance with substances obtained in the plant extract.

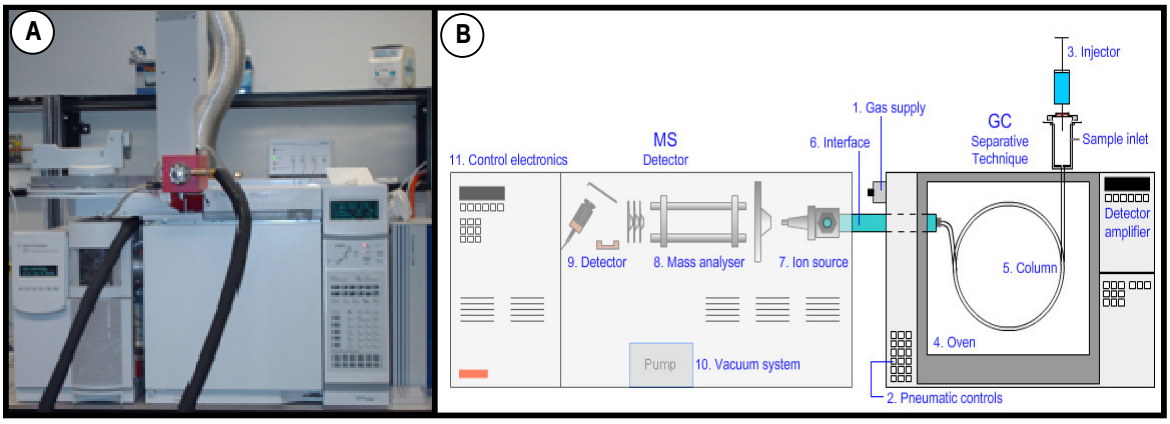


Figure 3.2- Gas chromatography- Mass spectrometry system. (**A**) GC Agilent 6890N system equipped with a mass spectrometer Agilent 5975 Inert XL. Picture by FMonteiro at the Florian Schiestl Laboratory (University of Zurich); (**B**) Schematic diagram of a typical GC-MS system. Gas supply (1) is regulated to the correct pressure/flow by the pneumatic controls (2). In the injector (3), the sample is volatilized and the resulting gas enters the GC column (5), which in turn is temperature controlled in the oven (4). After separation in the GC system, analytes are transported to MS by an interface (6) and, after, products are ionized in the ion source (7) prior to analysis. Further, analytes are separated on the basis of their mass-to-charge ratio (mass analyser- 8) and then ion beam is detected and converted into a usable signal (detector- 9). The final output is an ion signal of the corresponding mass-to-charge ratio of all analytes. Adapted (http://www.chromacademy.com/index.asp)

3.3. Material and methods

3.3.1. Sample collection

Scent collection was made in *Ophrys fusca* plants from a natural population growing in-field as previously reported (Chapter 2- section 2.3.1. Experimental design is presented in 2.3 Material and Methods section). Samples for scent analysis were collected as follows: *O. fusca* labella were cut with a sterile scalpel and immediately sealed in 2 mL PTFE Liner amber glass vials (Agilent Technologies Inc.) containing 1 mL of pentane (Sigma Aldrich) as solvent. Amber PTFE liner vials avoid volatile evaporation and degradation. After 1 h immersion in the solvent, lips were removed and solvent extracts were stored at -20 ºC.

3.3.2. Chemical analysis

Metabolic profiles of 100 *Ophrys fusca* labella extracts were performed using GC-MS facilities at the Florian Schiestl Laboratory, Institute for Systematics Botany, University of Zurich (Switzerland).

3.3.2.1. Quantitative analysis

For quantitative analysis, labella extracts were analysed by gas chromatography with flame ionization detection (GC-FID; Agilent 6890N). Before analysis, 100 µg of *n*-octadecane (purity 99.8%, Fluka, Buchs, Switzerland) was added to samples as internal standard. Briefly, for each sample one vial (Agilent Technologies Inc.) was prepared with 50 µl of *n*-hexane (Merck) to avoid evaporation. After, a microvolume glass insert with bottom spring and polymer feet (Supelco) was added for loading 1 µl of the sample and sealed through a cap with a 8 mm silicone/PTFE septum (BGB Analytik AG). One microliter of each odour sample was injected splitless at 50 ºC (1 min) into the GC, equipped with an AutoSampler 7683 series (Agilent Technologies Inc.), followed by opening the split valve and setting temperature to raise to 300 ºC at a rate of 10 °C min-1. After, the oven was maintained at 300 °C for an additional 15 mins. The GC was equipped with a HP5 column (5% Phenyl Methyl Siloxane, 30 m, 0.32 mm diameter, 0.25 µm film thickness, Agilent Technologies Inc.) with 5 m × 0.53 mm diameter and deactivated retention gap was used. Helium served as carrier gas (6.2 mL. min^{-1}, constant flow mode) and nitrogen was used as make-up gas (4.98 psi). The injector temperature was kept at 300 °C. Chromatogram outputs were recorded and

then analysed by the MSD ChemStation Data Analysis Application E.02.00.493 (Agilent Technologies Inc.) for quantitative and qualitative analysis.

3.3.2.2. Qualitative scent analysis

For compound identification, samples were analysed by gas chromatography with mass selective detection (GC-MS), by using GC parameters as described previously. Briefly, 1 µl of each sample was injected into a GC (Agilent 6890N) coupled with a Mass Spectrometer (Agilent 5975 Inert XL), equipped with the same column used for quantitative analysis. Helium served as the carrier gas (2 ml/min, constant flow mode). The oven was kept at 50 °C (1 min) and then heated to 300 °C at a rate of 10 °C min^{-1}. The transfer line to the MS was heated to 150 °C. The MS ion source was heated to 250 °C and run set to full scan mode. A solvent delay was performed for 3 min and after scan parameters were set for detection within 10- 400 mZ until 8 mins, following which, scan settings were changed to acquire mass at 33- 550 mZ.

3.3.3. Compound identification

GC-FID and GC-MS analysis were done with the same column (HP5), thereby allowing comparison of retention times of the analytes. On a HP-5 column, alkenes elute prior to the corresponding alkanes. Alkenes with the same chain length but different double bond positions display different retention times, so that (Z)-12 elutes first while (Z)-5 elutes last. Rather than identifying all compounds present in extracts, analysis was focused on straight chain saturated hydrocarbons (*n*-alkanes) and unsaturated (*n*-alkenes) hydrocarbons with double bond positions 5, 7, 9, 11 or 12 and of chain lengths 21–29 carbons. These compounds have been shown to be responsible for pollinator attraction in *Ophrys* species (Schiestl, 2005). The isomeric configuration of the alkenes was not determined in this study. The volatiles were analyzed by GC-MS and identification was based on reference compounds injection and comparison of their retention indices, as well as by computerized matching of the acquired mass spectra with those stored in the Wiley and NIST 5.0 mass spectral libraries of the GC-MS data system. Reference compounds were analysed by GC-MS with same parameters used in labellum extracts analysis. A custom-made library was used for compound identification, where compounds involved in *Ophrys*-insect interaction have been deposited and previously identified. Absolute amounts of odour compounds were calculated using the internal standard method (Schomburg, 1990). Further, sampling volumes were used to calculate the absolute amount in micrograms per labellum.

Amounts of individual compounds were divided by the sum of all compounds to calculate relative amounts.

3.3.4. Statistical analysis

Means and standard error (SEM) of absolute (µg per labellum) and relative (%) amounts of all identified compounds were calculated for all solvent extracts. A multivariate analysis of labella extracts with all compounds identified [relative amounts (%)] was performed via a principal component analysis (PCA) (correlation matrix) for all conditions tested (unpollinated and pollinated 2DAP and 4DAP). In order to detect compound differences, a One-Way ANOVA followed by a Fisher's Least Significant Difference (LSD) test as *post-hoc* ($p < 0.05$) was performed when the dataset fitted the conditions of normality. For pairwise comparisons of total absolute amounts, a non-parametric Mann-Whitney *U*-test was performed at $p = 0.025$. Calculations, statistical tests and graphics were carried out using IBM® SPSS® Statistics version 19.0 (SPSS Inc., USA).

3.4. Results and discussion

3.4.1. Descriptive statistics on chemical analysis

Despite the fact that *Ophrys* flowers can produce a bouquet consisting of more than 100 chemical compounds (Borg-Karlson, 1990), metabolic profiling of *Ophrys fusca* labellum was focused on cuticular hydrocarbons (*n*-alkanes and *n*-alkenes) known to trigger behavioural response in male pollinators (Schiestl *et al.*, 1999). To determine changes related to pollination events, labellum extracts were analysed at 2 and 4 days after pollination (DAP). A set of 98 labella extracts were used for further analysis, since two samples were removed from analysis due to contamination with water. From the 22 compounds identified in labella extracts, 9 were *n*-alkanes (41 %) and 13 *n*-alkenes (59 %). The volatile bouquet consisted of straight chain *n*-alkanes with carbon lenghts of 21-31 and straight alkenes with double bonds at positions 7, 9, 11, 12 or 13 (Fig. 3.4- next page). These compounds were identified in both unpollinated and pollinated *Ophrys fusca* labella extracts at 2 DAP and 4 DAP, and their relative and absolute amounts are presented (Tables 3.1 and 3.2- relative amounts, Appendix VI- absolute amounts). Relative amounts of alkanes and alkenes of all conditions tested are shown in Tables 3.1 and 3.2, respectively. An error bar of mean relative amounts for the identified compounds was performed in order to get an overview of compound's variation in the conditions tested (Fig. 3.3).

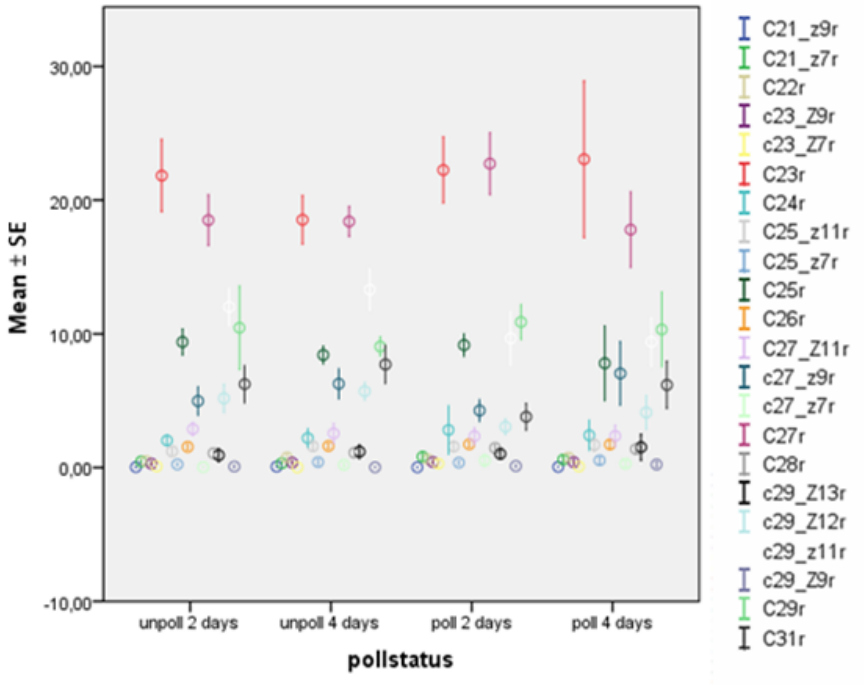
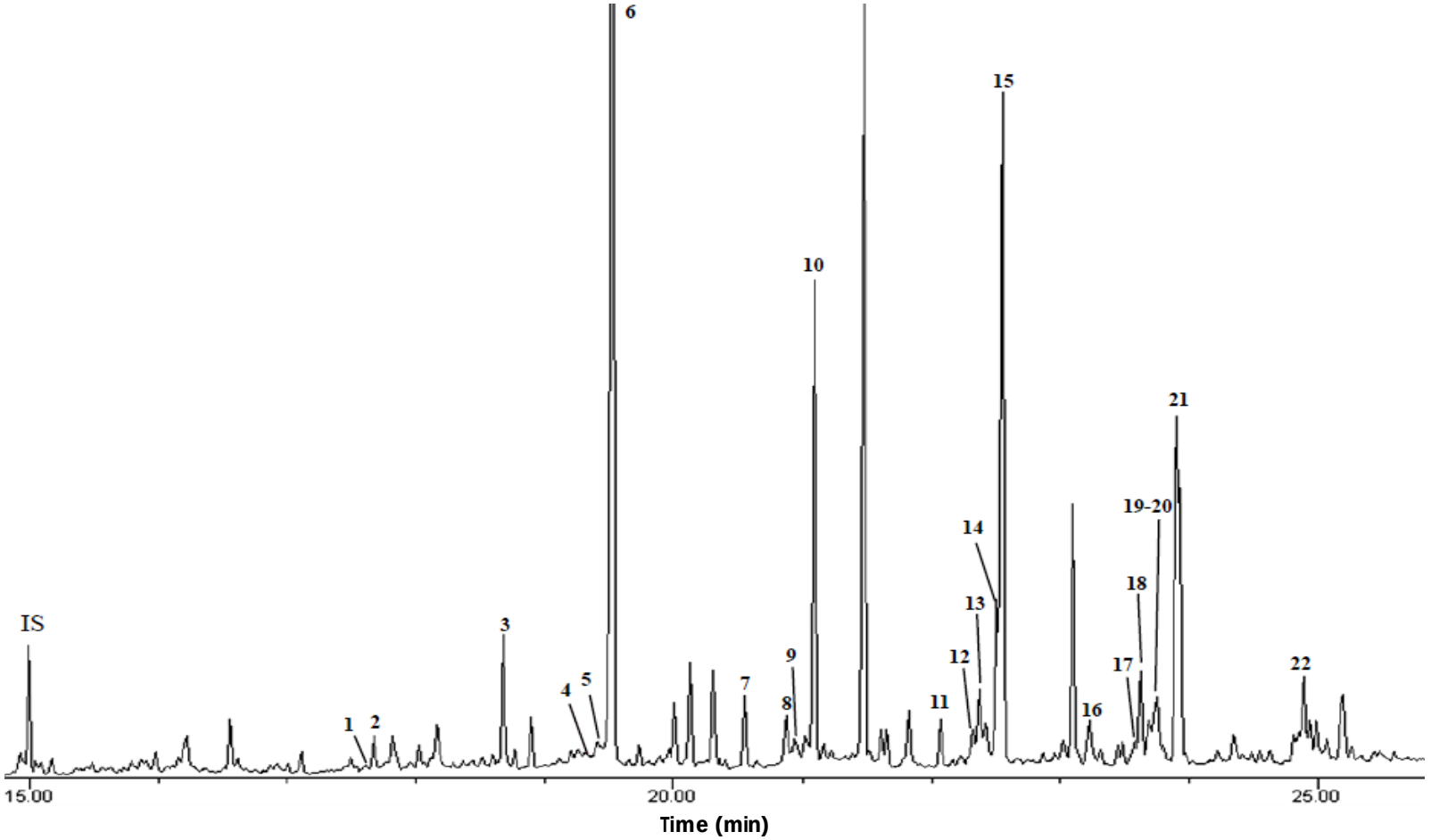


Figure 3.3- Error bar of mean (±SE) relative amounts for all variables (compounds) in unpollinated and pollinated *Ophrys fusca* labellum extracts, 2 and 4 days after pollination.



1- (Z)-9 Heneicosene	**6**- Tricosane	**11**- Hexacosane	**16**- Octacosane	**21**- Nonacosane
2- (Z)-7 Heneicosene	**7**- Tetracosane	**12**- (Z)-11 Heptacosene	**17**- (Z)-13 Nonacosene	**22**- Hentriacontane
3- Docosane	**8**- (Z)-11 Pentacosene	**13**- (Z)-9 Heptacosene	**18**- (Z)-12 Nonacosene	
4- (Z)-9 Tricosene	**9**- (Z)-7 Pentacosene	**14**- (Z)-7 Heptacosene	**19**- (Z)-11 Nonacosene	
5- (Z)-7 Tricosene	**10**- Pentacosane	**15**- Heptacosane	**20**- (Z)-9 Nonacosene	

Figure 3.4- Chromatogram of an *Ophrys fusca* labellum extract focused on the cuticular hydrocarbons (saturated- *n*-alkanes and unsaturated- alkenes) retrieved by a GC-MS analysis. A total of 22 different cuticular hydrocarbons were identified (**1**-**22**). *n*-Octadecane was used as internal standard (**IS**).

Table 3.1. Mean relative amount with standard error [SEM= standard deviation/ √(n)] of *n*-alkanes (straight chain saturated hydrocarbons) in unpollinated and pollinated *Ophrys fusca* labella, 2 and 4 days time point. Compounds are ordered by retention times. Alkane's total amount (µg) is given as mean (±SEM) of total labella sampled.

	2 Days		4 Days	
Alkanes	**Unpollinated (N=25)**	**Pollinated (N=25)**	**Unpollinated (N=24)**	**Pollinated (N=24)**
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Docosane (C22)	0.49 ± 0.07	0.63 ± 0.09	0.75 ± 0.15	0.75 ± 0.11
Tricosane (C23)	21.84 ± 1.32	22.26 ± 1.19	18.53 ± 0.88	20.62 ± 1.47
Tetracosane(C24)	2.03 ± 0.19	1.96 ± 0.17	2.21 ± 0.33	2.42 ± 0.54
Pentacosane (C25)	9.39 ± 0.46	9.16 ± 0.40	8.42 ± 0.32	7.80 ± 1.34
Hexacosane(C26)	1.55 ± 0.09	1.74 ± 0.11	1.62 ± 0.11	1.73 ± 0.15
Heptacosane (C27)	18.51 ± 0.92	22.73 ± 1.13	18.40 ± 0.55	17.80 ± 1.36
Octacosane (C28)	1.07 ± 0.08	1.47 ± 0.12	1.11 ± 0.09	1.39 ± 0.15
Nonacosane (C29)	10.45 ± 0.59	10.89 ± 0.63	9.06 ± 0.35	10.33 ± 1.35
Hentriacontane (C31)	6.24 ± 0.67	3.80 ± 0.49	7.72 ± 0.69	6.18 ± 0.85
SUM (%)	**71**	**74**	**67**	**69**
Mean total amount (µg/labellum)	**4.32 ± 0.27**	**4.02 ± 0.28**	**3.94 ± 0.24**	**4.76 ± 0.67**

Table 3.2. Mean relative amount with standard error [SEM= standard deviation/ √(n)] of *n*-alkenes (unsaturated hydrocarbons) in unpollinated and pollinated *Ophrys fusca* labella, 2 and 4 days time point. Compounds are ordered by retention times. Alkene's total amount (µg) is given as mean (±SEM) of total labella sampled.

	2 Days		4 Days	
Alkenes	**Unpollinated (N=25)**	**Pollinated (N=25)**	**Unpollinated (N=24)**	**Pollinated (N=24)**
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
(Z)-9-Heneicosene	0.04 ± 0.01	0.02 ± 0.008	0.08 ± 0.01	0.05 ± 0.02
(Z)-7-Heneicosene	0.45 ± 0.05	0.74 ± 0.11	0.33 ± 0.06	0.87 ± 0.14
(Z)-9-Tricosene	0.30 ± 0.04	0.44 ± 0.08	0.37 ± 0.03	0.40 ± 0.11
(Z)-7-Tricosene	0.09 ± 0.03	0.30 ± 0.05	0.007 ± 0.001	0.01 ± 0.001
(Z)-11-Pentacosene	1.22 ± 0.11	1.57 ± 0.18	1.59 ± 0.14	1.69 ± 0.24
(Z)-7-Pentacosene	0.24 ± 0.06	0.37 ± 0.07	0.41 ± 0.05	0.48 ± 0.07
(Z)-11-Heptacosene	2.89 ± 0.23	2.35 ± 0.31	2.56 ± 0.16	2.36 ± 0.34
(Z)-9-Heptacosene	4.98 ± 0.51	4.27 ± 0.39	6.27 ± 0.52	7.04 ± 0.36
(Z)-7-Heptacosene	0.04 ± 0.01	0.53 ± 0.04	0.20 ± 0.002	0.29 ± 0.005
(Z)-13-Nonacosene	1.51 ± 0.25	1.03 ± 0.14	1.19 ± 0.22	1.52 ± 0.09
(Z)-12-Nonacosene	5.18 ± 0.50	3.06 ± 0.29	5.71 ± 0.32	4.13 ± 0.49
(Z)-11-Nonacosene	12.02 ± 0.68	9.67 ± 0.68	13.31 ± 0.63	9.40 ± 0.72
(Z)-9-Nonacosene	0.08 ± 0.04	0.12 ± 0.05	0.03 ± 0.002	0.23 ± 0.009
SUM (%)	**29**	**26**	**33**	**31**
Mean total amount (µg/labellum)	**1.74 ± 0.13**	**1.41 ± 0.18**	**1.90 ± 0.15**	**2.13 ± 0.43**

Overall, alkanes are present in high relative proportions accounting for 67-74 % of labellum extracts (Table 3.1) and alkene's proportions ranged from 26 to 33 % (Table 3.2). Alkanes highest relative proportion in labella extracts has been reported in other *Ophrys* species (Ayasse *et al.*, 2000; Schiestl *et al.*, 2000; Mant *et al.*, 2005). A synergistic effect of alkanes, by increasing the intensity of male responses thus incrementing reproductive success, was previously suggested in *Ophrys sphegodes* (Schiestl *et al.*, 2000). The same study pointed out that a blend of synthetic alkanes *per se* was not significantly able at attracting male pollinators.

3.4.2. Chemical changes in *Ophrys fusca* labellum after pollination

3.4.2.1. Discriminating conditions under study

GC-MS data from *Ophrys fusca* labella of unpollinated and pollinated flowers (2 DAP and 4 DAP) were initially analysed using multivariate statistical methods, namely by means of a Principal Component Analysis (PCA). PCA is an unsupervised clustering method that requires no knowledge of the dataset and acts on reducing the dimensionality of multivariate data while preserving most of the variance within it (Goodacre *et al.*, 2000). The principal components (PCs) can be displayed graphically as a score scatter plot, which is useful for observing any grouping in the dataset. Relative proportions of the 22 compounds identified by GC-MS were used for a PCA-based analysis considering 2 and 4 days after pollination (DAP), separately. Graphical representation of two principal components (PCs) is shown in Fig. 3.5 for 2 DAP, and in Fig. 3.6 for 4 DAP dataset.

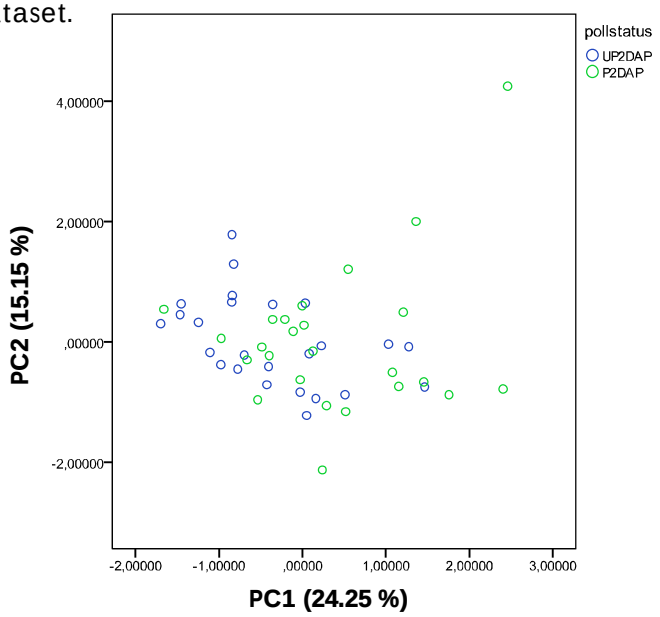


Figure 3.5- Scatter plot of *Ophrys fusca* labella extracts of unpollinated (UP2DAP) and pollinated (P2DAP) flowers at 2DAP by means of a principal component analysis (PCA). PC1- principal component 1; PC2- principal component 2.

From the PCA analysis at 2 DAP, six principal components with an eigenvalue above one, explained 70.8 % of the total matrix variation (data not shown). The first two PCs explained 39.5 % of the total variation (Fig. 3.5), yet no clear differentiation between unpollinated and pollinated data at 2 DAP was observed. At 4 DAP, PCA analysis retrieved nine PCs explaining 82.1 % of total variation (data not shown). The first two principal components only explained a total of 30.8 %, 17.4 % for PC1 and 13.4 % PC2 (Fig. 3.6). Thus, PCA analysis was not able to discriminate any of the conditions tested. Previous studies on *Ophrys sphegodes* labella headspace samples revealed that the same compounds were present in both unpollinated and pollinated flowers. Moreover, variation on quantitative, rather than qualitative, compositions were observed after pollination event (Schiestl and Ayasse, 2001).

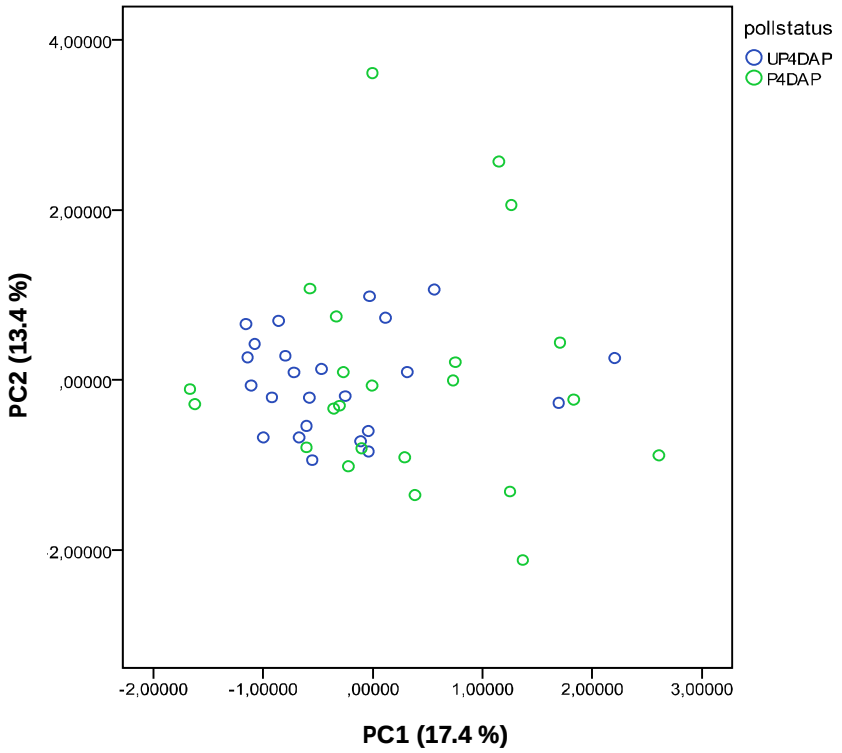


Figure 3.6- Scatter plot of *Ophrys fusca* labella extracts of unpollinated (UP4DAP) and pollinated (P4DAP) flowers at 4DAP by means of a principal component analysis (PCA). PC1- principal component 1; PC2- principal component 2.

3.4.2.2. Odour and pollination event

In most post pollination systems, change and/or cessation of odour production display important functions, namely in saving resources (flower maintenance and odour production are energy-consuming) and in incrementing reproductive success, by reducing attractiveness of pollinated flowers to direct pollinators to unpollinated flowers

of the same inflorescence (Gori, 1983). In order to determine if odour bouquet decreased with pollination event, total absolute amounts of labella extracts in both unpollinated and pollinated flowers at 2 DAP and 4 DAP were calculated (Fig. 3.7).

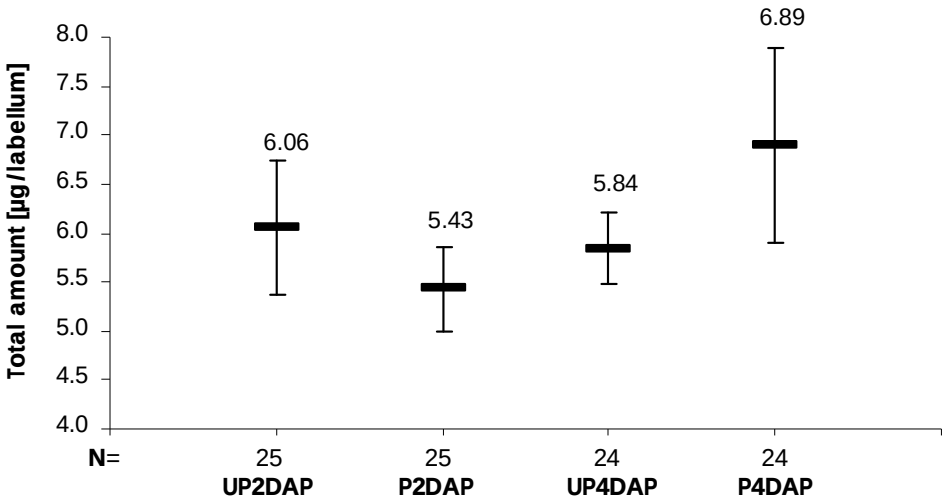


Figure 3.7- Mean total absolute amounts (±SEM) of *Ophrys fusca* labella extracts of unpollinated (UP) and pollinated (P) flowers at 2DAP and 4DAP. N- Sample size.

At 2 DAP, total odour amounts decreased from 6.05± 0.35 µg (unpollinated) to 5.43± 0.42 µg (pollinated), this difference having no statistical significance (Mann-Whitney *U*-test, P>0.025- Appendix VII). At 4 DAP, increase on total odour amounts from 5.84± 0.37 µg (unpollinated) to 6.89± 1.0 µg (pollinated) also revealed no statistical significance (Mann-Whitney *U*-test, P>0.025- Appendix VII). These results are in accordance with previous studies on *Ophrys sphegodes* flower extracts, according which a slight decreased on total amounts were observed, though not being statistically significant (Schiestl and Ayasse, 2001). Maintenance of odour production after pollination has also been reported before (for more detail see Schiestl and Ayasse, 2001). Being pseudocopulation- eliciting odour compounds, part of the plant epicuticular wax important for water loss prevention (Hadlye, 1981; Jetter and Kunst, 2008), change/cessation of these compounds production might not be immediate (Schiestl and Ayasse, 2001).

3.4.2.3. Cuticular hydrocarbons and pollination

Since the elucidation of the chemical basis of the sexual deception mechanism carried out by Schiestl and co-workers (1999), studies on the chemical basis relying *Ophrys* pollination has been focused mainly on *n*-alkanes and *n*-alkenes, which are responsible for displaying copulatory behaviour by male pollinators onto the orchid

labellum (Schiestl *et al.*, 1999; Ayasse *et al.*, 2003; Mant *et al.*, 2005; Stökl *et al.*, 2005). In *Ophrys fusca* labella, an overall study on dynamics of both alkanes and alkenes relative proportion and its correlation with pollination, was conducted. To evaluate if environmental conditions influenced compounds compositions on control labella extracts, variation between unpollinated samples at 2 DAP and 4 DAP was examined (LSD *post-hoc* test- Appendix VIII). No significant variation in odour composition of unpollinated flowers labella extracts was found in the time-course analysed (Appendix IX).

a) Alkanes

In *Ophrys fusca* labella extracts, alkanes composition is dominated by tricosane and heptacosane, and in lower extent by pentacosane and nonacosane (Table 3.1). These compounds were identified previously as being prevalent in *Ophrys fusca* labella extracts (Schiestl and Ayasse, 2002; Stölk *et al.*, 2005). Exception was seen on nonacosane since, to our knowledge, no reports have been published on *Ophrys fusca* labella extracts related to its presence/quantity (Schiestl and Ayasse, 2002; Stölk *et al.*, 2005). At 2 days after pollination, relative proportions of three *n*-alkanes revealed to be statistically significant: heptacosane and octacosane increased and hentriacontane decreased after pollination (Fig. 3.8). Four days after pollination, no differences in alkane relative amounts were observed between unpollinated and pollinated flowers (LSD *post- hoc* test- Appendix VIII and Appendix iX).

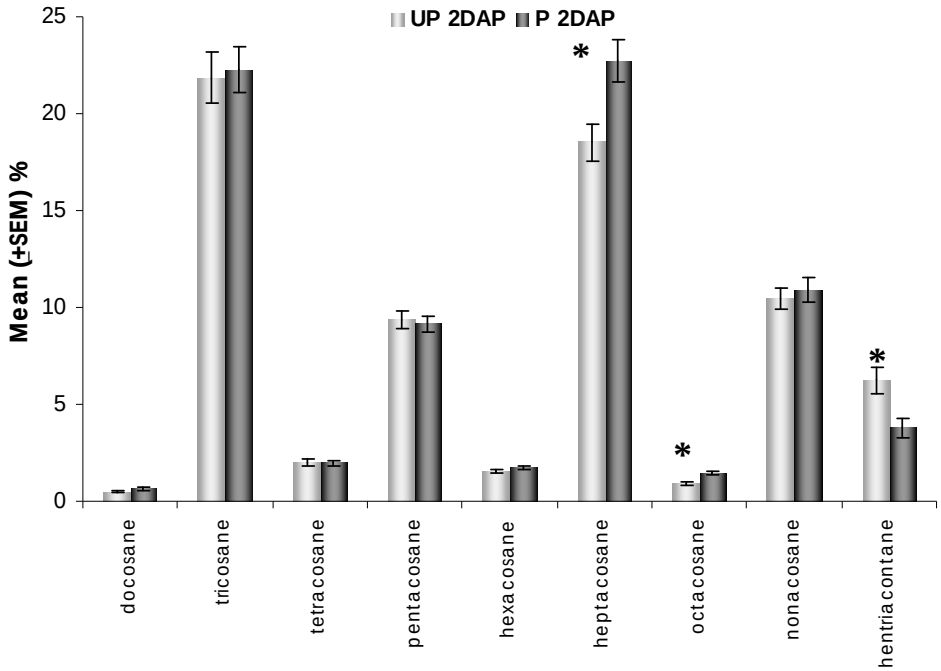


Figure 3.8- Mean relative amounts (±SEM) of *n*-alkanes in *Ophrys fusca* labellum extracts in unpollinated (UP) and pollinated (P) flowers 2 days after pollination (DAP). * P<0.05, LSD *post hoc* test.

b) Alkenes

Alkenes (unsaturated hydrocarbons), although generally less common, were also widespread among the conditions analysed. In *O. fusca* labella extracts the (Z)-12+(Z)-11 alkenes with different chain-lenghts (C21, C23, C25, C27) (Table 3.2) represented 65- 77 % of the total identified alkenes.

At 2 DAP, relative proportions of four alkenes revealed to be statistically significant: (Z)-12+(Z)-11-C29 decreased while (Z)-7 alkenes (C21, C27) increased after pollination (Fig. 3.9; LSD *post-hoc* test- Appendix VIII). At 4 days after pollination, a statistically significant decrease on (Z)-12+(Z)-11-C29 alkenes and increase on (Z)-9-C29 was observed on labella of pollinated flowers (Fig. 3.10; LSD *post-hoc* test- Appendix VIII and Appendix iX).

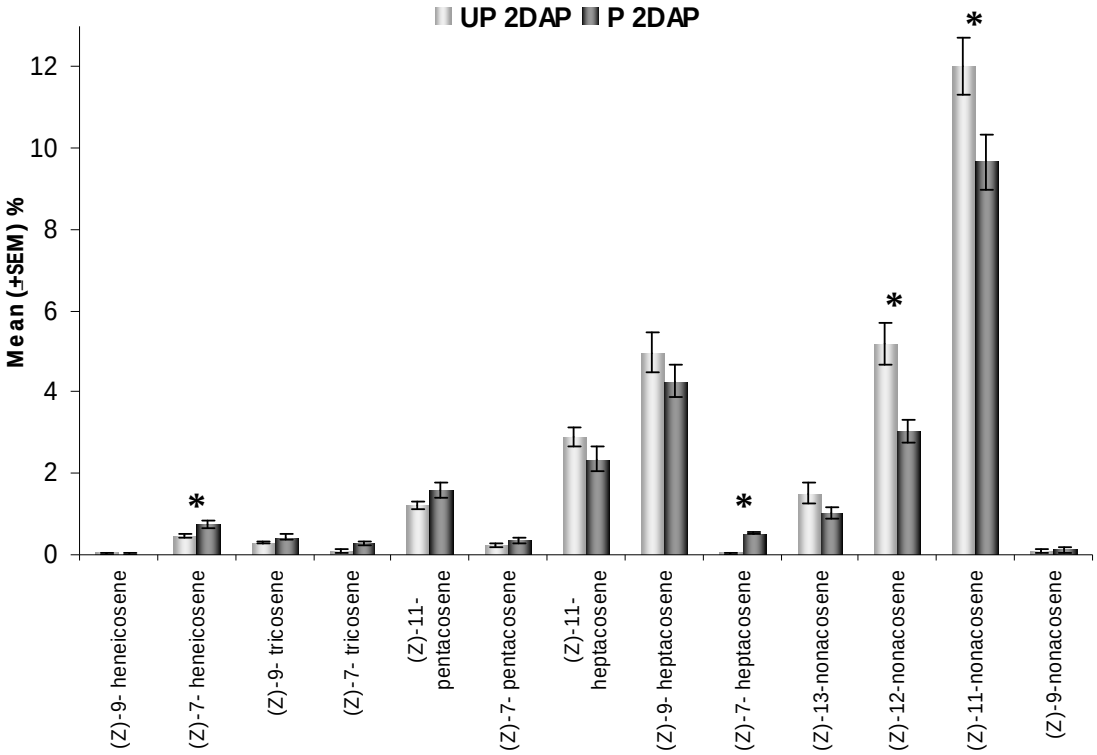


Figure 3.9- Mean relative amounts (±SEM) of *n*-alkenes in *Ophrys fusca* labella extracts in unpollinated (UP) and pollinated (P) flowers 2 days after pollination (DAP).* P<0.05, LSD *post hoc* test.

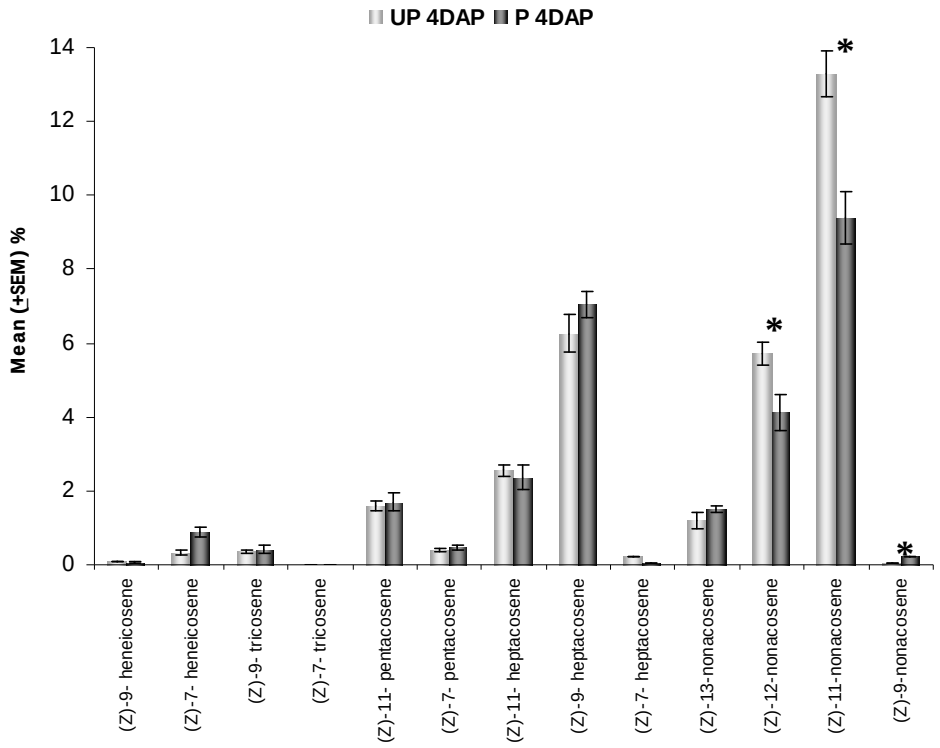


Figure 3.10- Mean relative amounts (±SEM) of *n*-alkenes in *Ophrys fusca* labella extracts in unpollinated (UP) and pollinated (P) flowers 4 days after pollination (DAP). * P<0.05, LSD *post hoc* test.

c) Post pollination mechanism in *Ophrys fusca*

Specific ratios of alkanes and alkenes have been found to be divergent among *Ophrys* species that attract different pollinator species but are identical in species pollinated by the same bee species (Stökl *et al.*, 2005; Cortis *et al.*, 2009). According to Schiestl and co-workers (1999) specific patterns of alkenes play a key role in male pollinators attraction. In a pioneer study on *Ophrys sphegodes*, Schiestl and Ayasse (2001) proposed that the increase on farnesyl hexanoate production in pollinated flowers was responsible for guiding pollinators to unpollinated flowers of the inflorescence, thus having a role as a "flower repulsive compound". *Ophrys sphegodes* and *O. fusca* are both pollinated by males of *Andrena nigroaenea* Kirby (Paulus and Gack, 1990), yet differences on the pseudocopulatory behaviour of the insect prevents hybridization thus maintaining reproductive isolation (Kullenberg, 1961; Paulus and Gack, 1990). No data is available on pollinators of *O. fusca* Portuguese populations. In our study, farnesyl hexanoate was not identified in labella extracts of pollinated flowers. Compounds such as farnesol or farnesyl octanoate identified in *O. lutea* and *O. insectifera* were proposed as displaying a function similar to farnesyl hexanoate (Borg-

Karlson, 1990; Schiestl and Ayasse, 2001). However, in *Ophrys fusca* none of these compounds were found in the samples of pollinated plants.

Probably, the differences in alkene's relative proportions here reported may not reflect a loss of labellum attractiveness after pollination. As suggested by Schiestl and Ayasse (2001), inflorescence attractiveness may be maintaned even after successful pollination of single flowers, thereby increasing reproductive success of the whole inflorescence. Since no farnesyl hexanoate or similar compound was identified after pollination, it remains to be addressed the reasons for the pollinator to select unpollinated flowers rather than pollinated ones in the same inflorescence. Other compounds displaying functions similar to those attributed for farnesyl hexanoate in *O. sphegodes* post pollination mechanism cannot be discarded.

3.4.3. Considerations on *Ophrys fusca* pollinator species

The so-called *Ophrys fusca* -group includes approximately 10 closely related species often hardly distinguishable by morphological traits (Paulus and Gack 1981; Delforge, 1995). In Portugal, a huge variation between *O. fusca* populations is easily observed. Males of the bee *Andrena nigroaenea* have been described as being *O. fusca* pollinator species (Paulus and Gack, 1990), but reports on *Ophrys fusca* in southern Spain pointed out for *Colletes cunicularius* as also a pollinator species (Paulus and Gack, 1981). *Andrena nigroaenea* also pollinates *O. sphegodes*, but cephalic pseudopulation prevents hybridization with *O. fusca* (Kullenberg, 1961; Paulus and Gack, 1990). To our knowledge, *O. fusca* pollinator species is not well characterized in Portugal. Labella extracts of unpollinated flowers represent the species-specific odour profile, since no significant differences were seen on both 2 DAP and 4 DAP (LSD *post-hoc* test- Appendix VIII). Analysis was focused on alkenes with double bonds at positions 7, 9 and 12 (i.e. representing (Z)-11+ (Z)-12 alkenes together) which are known to elicit copulatory behaviour of both *A. nigroaenea* (Schiestl *et al*., 1999) and *C. cunicularius* (Mant *et al.*, 2005) males.

Specificity of an odour signal may reflect the presence of different compounds and/or differences in the relative amounts of a given set of compounds. Analysis of odour bouquet of the sympatric species *O. bilunulata* and *O. fusca*, which are pollinated respectively by *Andrena flavipes* Panzer and by *A. nigroaenea* Kirby, revealed that differences on alkene's patterns were responsible for pollinator specificity (Schiestl and Ayasse, 2002). The (Z)-7-C27 was only found in *O. bilunulata* and (Z)-7-C29 occurred almost exclusively, whereas (Z)-7-C25 was predominantly found in *O. fusca* (for more detail see Schiestl and Ayasse, 2002). Labella extracts composition here reported are in accordance with earlier reports (Schiestl and Ayasse, 2002). Despite the

fact that (Z)-7-C27 was detected only in *O. bilunulata* by Schiestl and Ayasse (2002), these compound was identified in the present study in *O. fusca*, which is in accordance with previous reports (Erdmann, 1996). In *O. fusca* and *O. bilunulata*, different relative proportions of the same odour compounds mediate the pollinator specificity (Schiestl and Ayasse, 2002). *Colletes cunicularius*, a widespread ground nesting solitary bee in Europe, pollinates four different *Ophrys* species, including *O. fusca* in southern Spain (Paulus and Gack, 1990). A study performed by Mant and co-workers (2005) on *O. exaltata* and *Colletes cunicularius*, revealed that (Z)-7 alkenes with different chain lengths are key components of the female sex pheromone of pollinator species. In *O. fusca* under analysis, all three compounds were identified in relative low amounts when compared to (Z)-12+ (Z)-11 alkenes (Appendix IX). In a wider and comprehensive study by Schlüter and co-workers (2011), the alkene's composition of labella for two sympatric species *O. sphegodes* (Fig. 3.11**A**), pollinated by *A. nigroaenea*, and *O. exaltata*, pollinated by *Colletes cunicularius* (Fig. 3.11**B**), were exposed. These species differ mainly in relative proportions of the double bond positions of their major alkenes: *O. sphegodes* produce high levels of 9-alkenes and 12-alkenes (i.e. corresponding to (Z)-11+ (Z)-12 alkenes together), while *O. exaltata* display high levels of 7-alkenes.

In *O. fusca* labella extracts of present study, the most predominant alkenes are those with double bonds at positions 12 followed by the 9-alkenes (Fig. 3.11**C**).

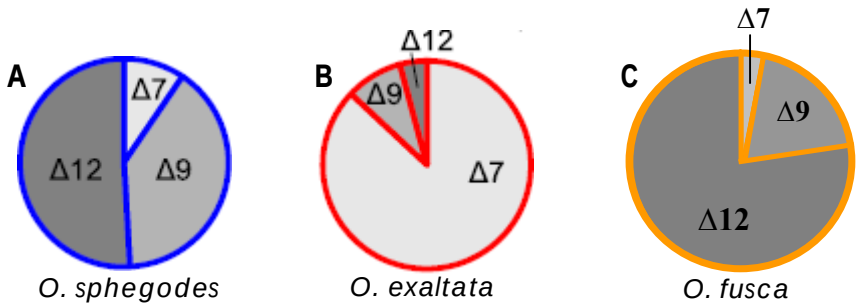


Figure 3.11- Relative proportions of alkenes with double bonds at positions 7, 9 and 12 present in labella extracts:(**A**) *Ophrys sphegodes*, pollinated by *A. nigroaenea* (**B**) *O. exaltata*, pollinated by *C. cunicullarius*. Adapted from (Schlüter *et al.*, 2011); (**C**) *O. fusca* population under study.

Interestingly, the profile of *O. fusca* alkenes does not seem to match any of those exhibited by the other two *Ophrys* species (Fig. 3.11**A**- *O. sphegodes* and **B**- *O. exaltata*). Since the pollinator was not identified during the time-course of this study, the identity of the *O. fusca* pollinator species can only be speculated. *O. fusca* has been described as being pollinated by *A. nigroaenea*, but alkenes proportions does not seem to match to those reported for *O. sphegodes* (Fig. 3.11**A**), also pollinated by the same bee species. Further studies are recommended to unravel differences between

these two species that are pollinated by the same *Andrena* species, but with different pseudocopulation behaviour exhibited by male pollinators (i.e. cephalic- *O. sphegodes* and abdominal- *O. fusca*). In *Andrena*-pollinated *Ophrys* species, males are attracted by species-specific blends of straight chain hydrocarbons, mostly alkenes (Schiestl *et al.*, 1999; Schiestl and Ayasse, 2002; Stökl *et al.*, 2005). Many species of the *O. fusca*-group are pollinated by *Andrena* sp. males (Stökl *et al.*, 2005). Since the composition of *O. fusca* labella extracts (Fig. 3.11**C**) was different from the one reported for *O. sphegodes* (Fig. 3.11**A**), which is *Andrena nigroaenea* pollinated, it cannot be ruled out that a different *Andrena* species may pollinate *O. fusca* population under study. Throughout the time-course of our study, an *Andrena* sp. individual was found trapped on the net disposables covering *O. fusca* plants, and later identified as being a male of the species *Andrena livens* Perez (Schiestl FP, Personal Communication). Being an *Andrena* sp. male, it is possible that this species may be, in fact, a pollinator of *O. fusca*. Up to now, no reports on *Andrena livens* were release as an *Ophrys*-pollinator species and, to confirm this hypothesis, pseudocopulation event should be registered and further GC-EAD analysis performed to determine the behaviourally-active compounds.

3.5. Conclusions

Chemical changes after pollination event were evaluated by a metabolic profiling of in-field *Ophrys fusca* plants by means of a GC-MS analysis. Labella from unpollinated and pollinated flowers were collected 2 (2 DAP) and 4 (4 DAP) days after pollination. Analysis was focused on straight chain saturated hydrocarbons (*n*-alkanes) and unsaturated (*n*-alkenes) hydrocarbons with chain lengths 21–29 and with double bond positions 7, 9, 11 or 12, since they have been shown to be responsible for pollinator attraction in other *Ophrys* species. Variations were depicted in quantitative compositions (e.g. altered proportions of the same compound) rather than in qualitative (i.e. occurrence of a different compound in one of the investigated conditions). The relative proportions of alkenes were the most variable at 2DAP and 4DAP, since various compounds increase or decrease after pollination. Alkanes only varied significantly at 2DAP. No significant decrease in total odour bouquet amounts was verified after pollination, which is in agreement with previous studies (Schiestl and Ayasse, 2001). Our results demonstrate that post pollination mechanism does not rely on an abrupt decrease of odour bouquet, as reported earlier (Schiestl and Ayasse, 2001). This fact may be related to functions attributed to these compounds as part of the desiccation-preventing wax layers on the flowers (Schiestl *et al.*, 1999). The post pollination mechanism by means of an increase emission of flower repelent compound observed in *O. sphegodes* could not be observed in *O. fusca* pollinated samples.

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Chapter 4

FINAL REMARKS

Pollination biology in Orchidaceae has long been intriguing evolutionary biologists and interest dates back to Darwin (Darwin, 1885; Fay and Chase, 2009). The most fascinating aspect of orchid's biology is pollination by deception which is quite common in Orchidaceae (Nilsson, 1992). Food (38 genera) and sexual (18 genera) deception are the most common types of pollination by mimicry, occurring in approximately one-third of the species (Jersáková *et al.*, 2006; Renner, 2006). Sexual deception mechanism was first described in the European *Ophrys* genus (Pouyanne, 1917). In this mechanism, *Ophrys* mimic their pollinators' mating signals and are pollinated by male insects during mating attempts (Schiestl *et al.*, 1999). Labellum or lip is a highly differentiated petal, which provides a landing platform to pollinators (Cozzolino and Widmer, 2005). Flower morphological features such as labellum shape, colour and pilosity which mimic the pollinator female body parts, are also important for drawing male's attention (Schiestl, 2005). Recent studies on orchid pollination focused on mechanisms underlying pollination attraction. In *Ophrys*, these studies have shown that the labellum is the main flower structure triggering copulatory behaviour on male insects (Schiestl *et al.*, 2000; Spaethe *et al.*, 2007; Vereecken and Schiestl, 2008; Stökl *et al.*, 2008a, b, 2009; Vereecken *et al.*, 2010). A general approach on the events following pollination is still lacking. The study here presented intends to contribute for the knowledge on post pollination-regulated mechanisms in the sexual deceptive orchid *Ophrys fusca* Link. To accomplish such goal, two different approaches were assigned: a transcriptional analysis and a metabolic profiling. Transcriptomics and metabolomics were both used to gather insights on the post pollination changes occurring in *Ophrys fusca* labellum. To access pollination-enhanced events, two time points were considered for analysis: 2 and 4 days after pollination (DAP).

Labellum transcriptional analysis allowed probing gene expression modulation of post pollination changes. The first response to pollination appears to be a stress response (2 DAP) and later at 4 DAP, nutrient mobilization occurs and *de novo* protein synthesis is induced for senescence progression. Pollination sets off proteolysis, remobilization of nutrients such as phosphate, carbon and nitrogen from labellum to other developing organs (e.g. flowers from the same inflorescence, ovule and/or ovary) and deactivates energy-consuming processes and major metabolic pathways related to labellum upholding. Soon after pollination (2 DAP), the majority of down regulated

genes in *O. fusca* labellum are involved in secondary metabolism and energy, suggesting that these processes are mainly for ensuring petal vitality and resources until successful pollination. At 4 DAP, *de novo* protein synthesis occurs to guarantee senescence progression to ultimately drive the labellum for irreversible cell death. In *O. fusca*, at 4 DAP, expression of several stress- and pathogen-related transcripts (GST, antimicrobial snakin proteins, Lea5 protein, metallothioneins types 2 and 3, chitinases, PR protein, Cys proteases, RNases), recognized as SAGs in senescence studies, may reveal that until the newly protein synthesis is achieved for senescence progression, labellum transcriptional regulation is mediated by non-specific stress-related pathways. Transcripts identified by microarray analysis reveal pivotal processes associated with secondary metabolism responsible for labellum traits (e.g. pigmentation, compounds emission involved in pollination), proteolysis, stress and defence, and remobilization of nutrients associated with pollination induced-senescence process.

Metabolic profiling in labella extracts was focused on cuticular compounds (alkanes and alkenes), known to trigger the pseudocopulatory behaviour on male pollinators. Results show that post pollination machinery does not rely on an abrupt decrease of odour production, which is in agreement with previous reports (Schiestl and Ayasse, 2001). This fact may be related to functions attributed to compounds involved in *Ophrys*-pollinator interactions, since they are part of the desiccation-preventing wax layers on the flowers and their immediate cessation could cause physiological constraints on the plant (Schiestl *et al.*, 1999). The post pollination mechanism by means of an increased emission of a repellent compound (farnesyl hexanoate) in pollinated flowers described in *O. sphegodes*, was not observed in *O. fusca* labella of pollinated plants. Related compounds were suggested to display similar functions in other *Ophrys* species (Schiestl and Ayasse, 2001) and detection in *O. fusca* remains to be addressed.

Through labellum gene expression analysis, transcripts related to biosynthetic pathways of cuticular compounds, involved in *Ophrys* pollinator attraction, were identified: stearoyl ACP desaturases (SAD). Compounds involved in *Ophrys*-pollinator crosstalk are mainly alkanes and alkenes with chain lenghts 21-29 and double bond at positions 7,9,12 or 13, which are part of the desiccation-preventing wax layer in flowers (Schiestl *et al*., 1999). Alkane and alkene biosynthesis are part of the wax biosynthetic pathway: the first results from decarbonylation in fatty acid elongation system, whereas alkenes formation has been linked to an elongation system including a desaturase (von Wettstein-Knowles, 2007). Expression of two stearoyl ACP- desaturase (SAD) isoforms, SAD1 and SAD2, in two *Ophrys* species (*O. sphegodes and O. exaltata*) has been considered flower-specific (Schlüter *et al.*, 2011). Furthermore, SAD1 revealed to be a non functional desaturase while SAD2 showed a significant association with alkene production with double bonds at 9- and 12-positions (Schlüter *et al.*, 2011). A transcript

coding for a SAD protein in *Ophrys fusca* displayed homology for SAD2 (Chapter 2- Table 2.6). It is tempting to hypothesize that this SAD2 may be responsible for production of 9- and 12- alkenes, the most prevalent in *Ophrys fusca* labellum extracts (see Chapter 3- Fig. 3.11**C**). At four days after pollination, SAD2 down regulation may suggest a reduction of alkene production, possibly of those with double bonds at positions 9 and 12. In fact, labella extracts of pollinated flowers at 4 DAP revealed a significant decrease on (Z)-12-+(Z)-11-nonacosene, but not on 9-alkenes (see Chapter 3- Fig. 3.9). Modulation of the gene expression may be related to a decrease in odour compounds after pollination, which, in turn, could be responsible for the significant decrease of (Z)-12-+(Z)-11-nonacosenes at 4 DAP.

By combining both transcriptional and metabolic profiling analysis to study post pollination events in a sexually deceptive orchid, the work here presented gives an important contribution for the understanding of this peculiar pollination system. Modulation of gene expression after pollination provides insights on the post pollination changes in the labellum revealing a highly regulated mechanism that will lead to irreversible cell death. The custom-made cDNA chip may be useful for performing cross-species hybridization to track differences on transcripts modulation and to disclose the genetic basis underlying sexual deception. Other orchids with distinct pollination strategies (e.g. food deception) can be used as well for prospecting differences on different deceptive mechanisms. Future studies should focus on a post transcriptional approach during post pollination events in this orchid species, thereby allowing the possibility to integrate datasets from different O'mics to unravel the complexity of pollination-enhanced events. Differences in gene expression do not directly correspond to differences at protein expression levels, as they may be governed by post transcriptional and/or translational mechanisms. By performing a proteomic approach, proteins as well as post translational modifications involved in events following pollination could be assigned. A labellum post pollination proteomics analysis would allow tracking enzymes responsible for alkenes' production, thereby giving a more comprehensive walkthrough of their regulation on pollination event. Such observations could adjoin some awareness on the genetic basis of pollinator attraction.

Given the huge amount of information retrieved by transcriptional high throughput studies, a metabolome analysis should be included in future prospects for a global O'mics integration. The integrated output could be used towards the elucidation of the sexual deception mechanism in *Ophrys*.

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APPENDIX I

62 - Semi-natural dry grasslands and scrubland facies

EUNIS Classification: E1.2 Perennial calcareous grassland and basic steppes

6210- Semi-natural dry grasslands and scrubland facies on calcareous substrates (Festuco-Brometalia) (* important orchid sites)

The grasslands of the 6210 habitat are among the most species-rich plant communities in Europe in terms of the number of species they support per unit area. The 6210 habitat type includes a wide range of grasslands communities which are generally assigned to the phytosociological class Festuco-Brometea. This habitat includes dry to semi-dry grasslands and scrubland occurring from the lowland to the mountain level and occurring on calcareous to neutral substrates. The habitat is considered a priority type (6210*) only if it is an important orchid site.

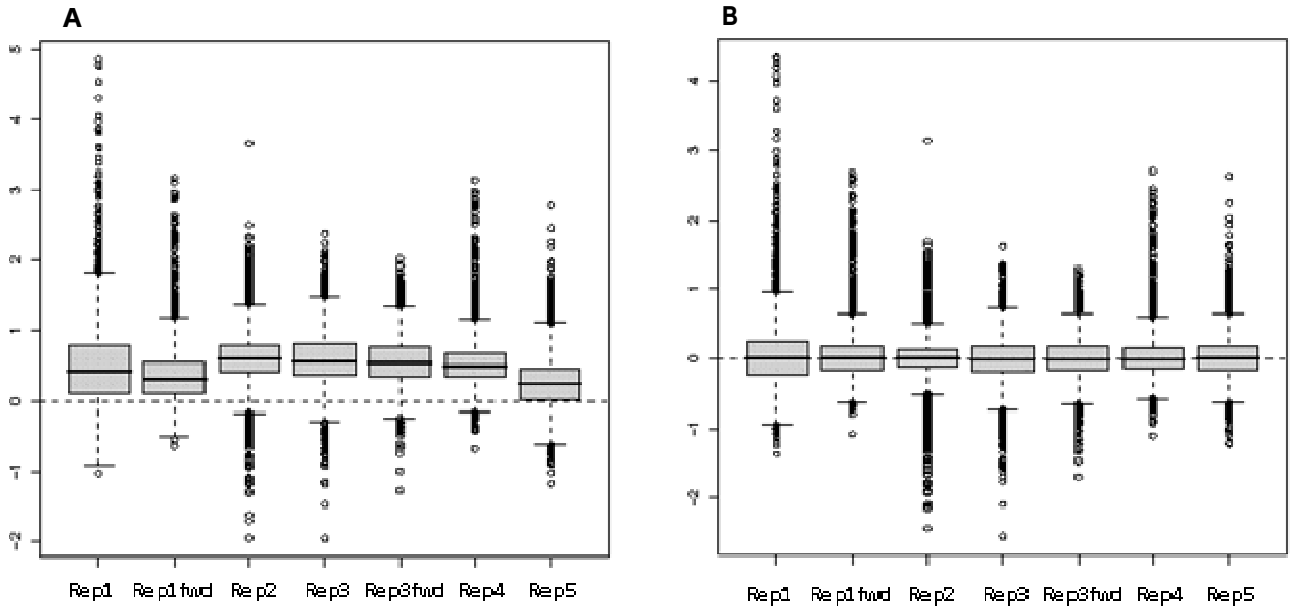
Characteristic species: *Anthyllis vulneraria, Arabis hirsuta, Brachypodium pinnatum, Bromus inermis, Campanula glomerata, Carex caryophyllea, Carlina vulgaris, Centaurea scabiosa, Dianthus carthusianorum, Eryngium campestre, Koeleria pyramidata, Leontodon hispidus, Medicago sativa* ssp. *falcata, Ophrys apifera, O. insectifera, Orchis mascula, O. militaris, O. morio, O. purpurea, O. ustulata, O. mascula, Polygala comosa, Primula veris, Sanguisorba minor, Scabiosa columbaria, Veronica prostrata, V. teucrium*.

In Portugal, 6210 habitat is indicated by the predominance of *Brachypodium phoenicoides* (Class Festuco-Brometea) and presence of the following orchid species: *Ophrys dyris*, *O. fusca*, *O. lutea*, *O. scolopax*, *O. tenthredinifera*, *O. vernixia*, *Orchis collina*, *O. italica*, *O. mascula*, *O. morio*, *O. papilionacea*, among other orchid species.

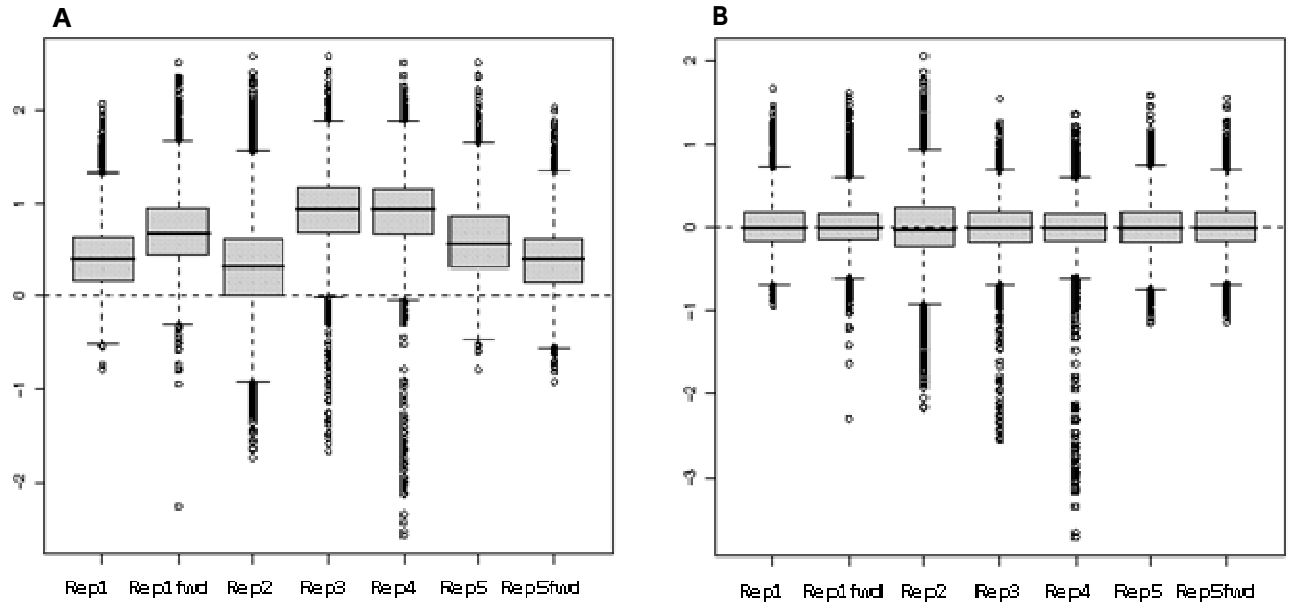
APPENDIX II

Box-plots of the log (base 2) ratio **M** values (log2-transformed intensity ratio between unpollinated and pollinated samples) before (**A**) and after (**B**) print-tip loess normalization are presented for both 2DAP (**1**) and 4DAP (**2**) time points used in gene expression studies. This allows assessing the need for slide scale normalization, a normalization that will ensure the same scale for all arrays. It also permits to assess if the scale of different print-tip groups is comparable.

1. Box-plots in all arrays for 2 DAP hybridizations.

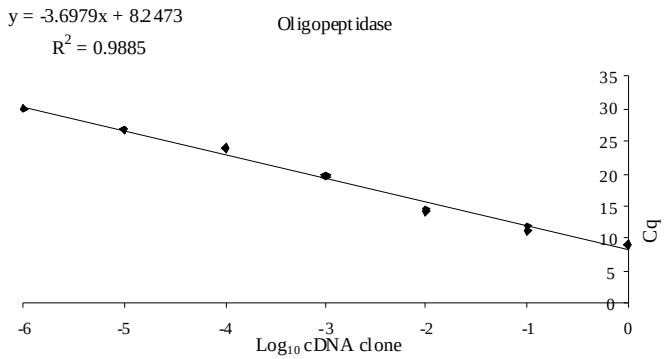


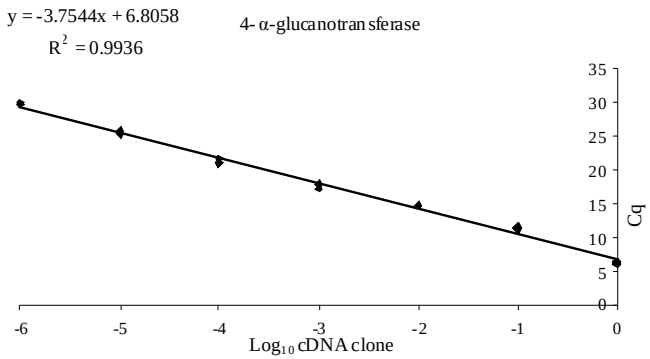
2. Box-plots in all arrays for 4 DAP hybridizations.

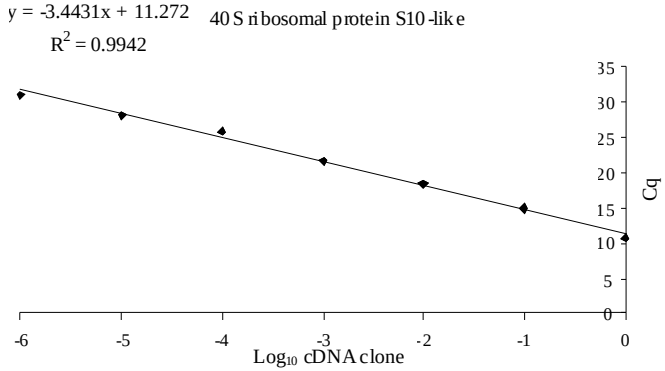


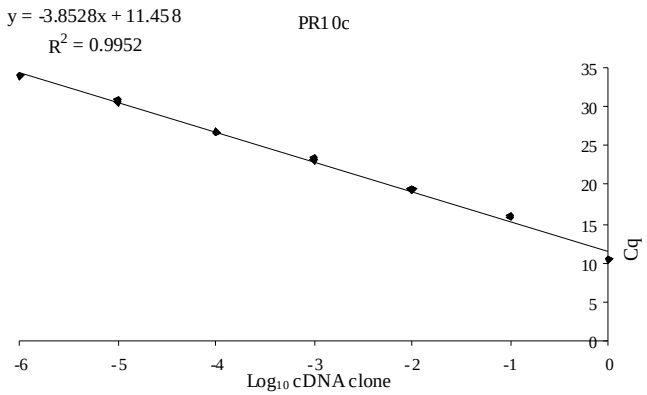
APPENDIX III

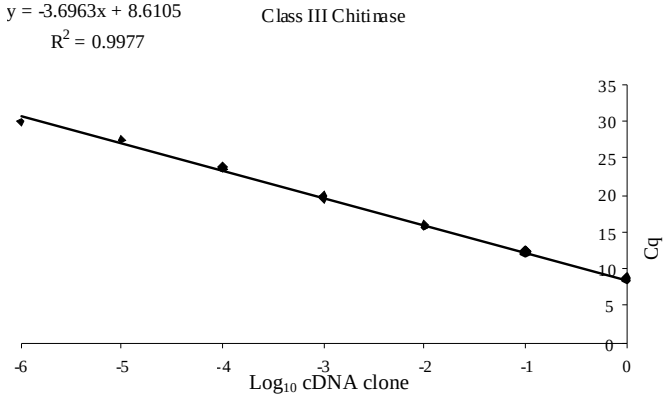
qPCR primer efficiency plots. Median quantification cycle (Cq) values of each set of ten-fold serial dilution plotted against the logarithm of cDNA clone (template) concentration. Reaction efficiency is given by $[10^{(1/-S)}-1] \times 100\%$, where S represents the slope of the linear regression line.

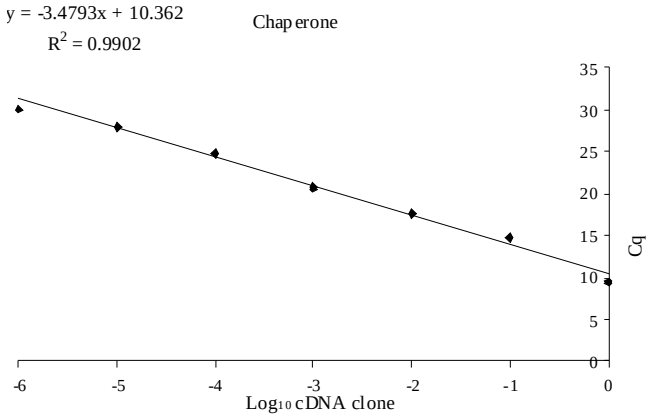






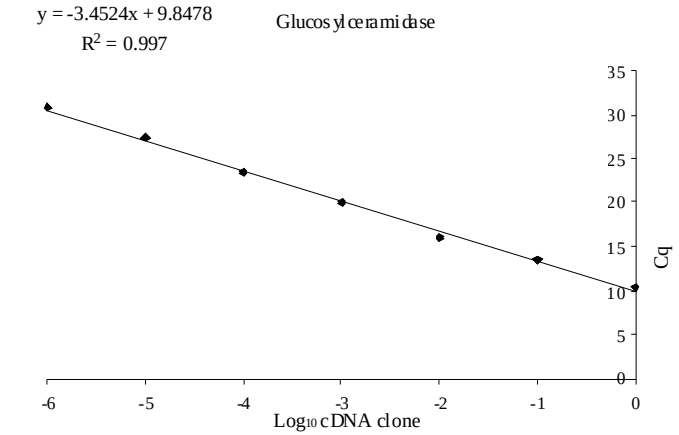


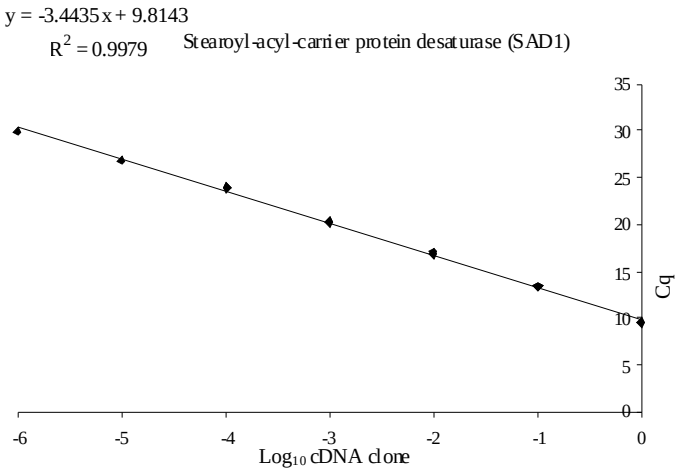


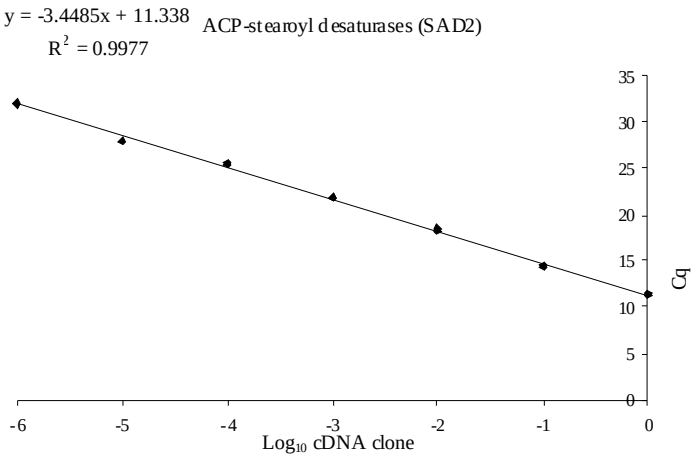


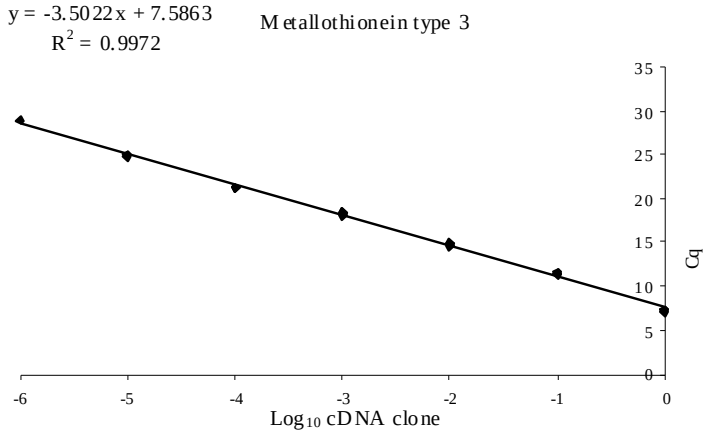
APPENDIX III (CONTINUED)

qPCR primer efficiency plots. Median quantification cycle (Cq) values of each set of ten-fold serial dilution plotted against the logarithm of cDNA clone (template) concentration. Reaction efficiency is given by $[10^{(1/-S)}-1] \times 100\%$, where S represents the slope of the linear regression line.





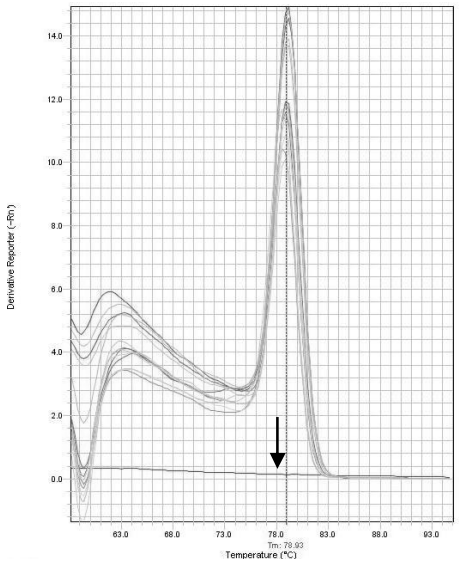




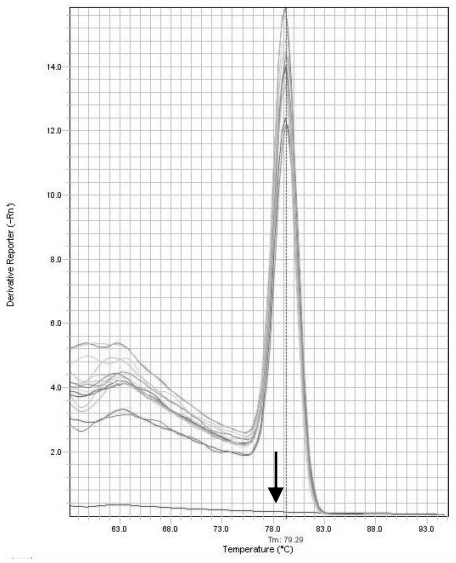
APPENDIX IV

Melting curves depicted from standard curves obtained in the qPCR experiments. Melting curve images were collected using StepOne™ software ver. 2.1 (Applied Biosystems). **Black arrow** indicates negative control melting curve.

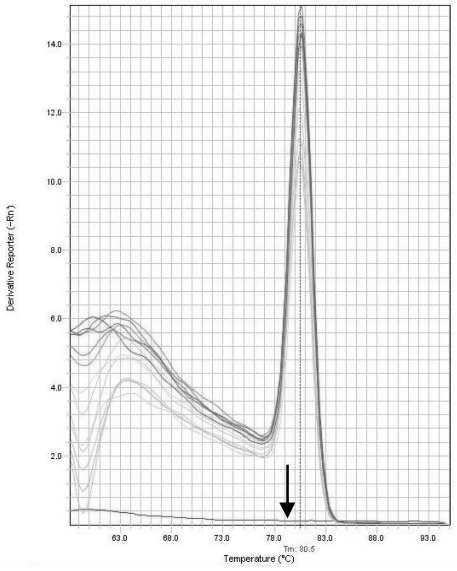
Oligopeptidase



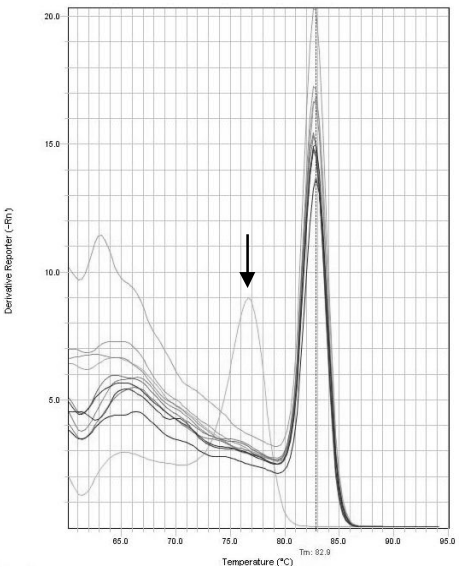
4-α-glucanotransferase



40S ribosomal protein S10-like



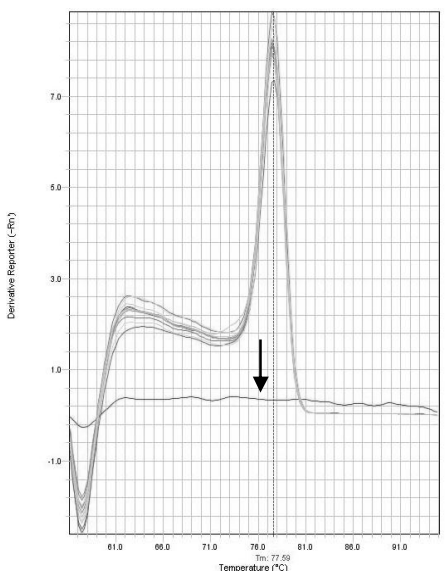
PR10c



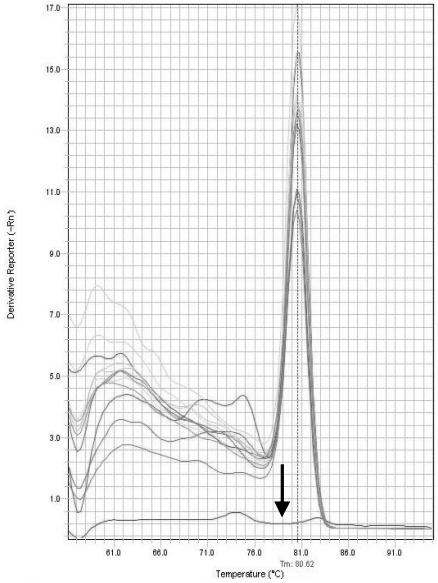
APPENDIX IV (CONTINUED)

Melting curves depicted from standard curves obtained in the qPCR experiments. Melting curve images were collected using StepOne™ software ver. 2.1 (Applied Biosystems). **Black arrow** indicates negative control melting curve.

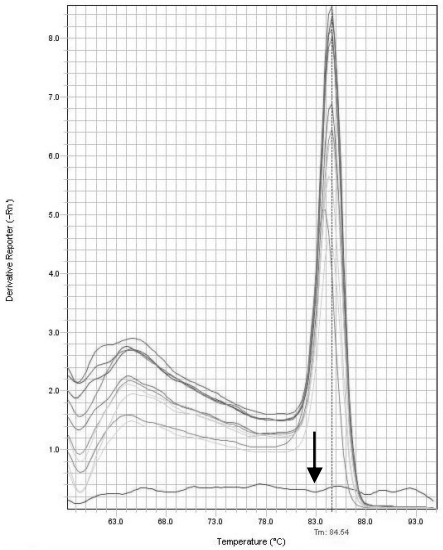
Glucosylceramidase



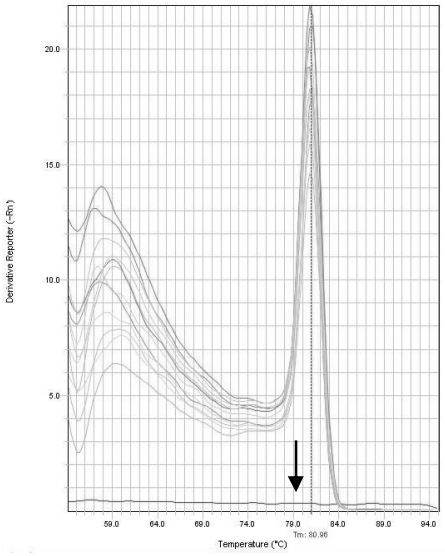
Stearoyl-acyl-carrier protein desaturase (SAD1)



ACP-stearoyl desaturase (SAD2)



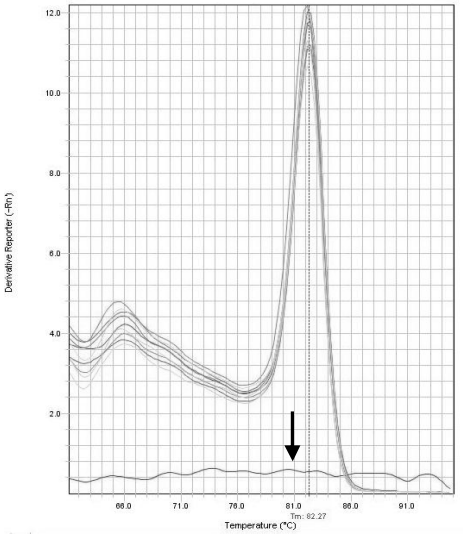
Metallothionein type 3



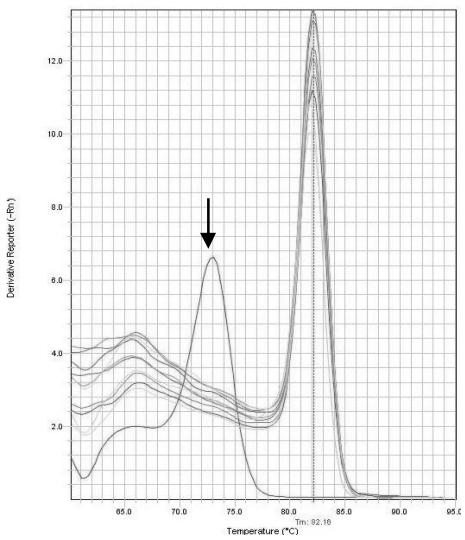
APPENDIX IV (CONTINUED)

Melting curves depicted from standard curves obtained in the qPCR experiments. Melting curve images were collected using StepOne™ software ver. 2.1 (Applied Biosystems). **Black arrow** indicates negative control melting curve.

Class III Chitinase



Chaperone



APPENDIX V

Median fold change (**FC**) calculated from qPCR and microarray data, with mean absolute deviation (**MAD**) indicated. Up regulated values are indicated with positive red values, whereas down regulated are indicated with negative green values. **DAP**-days after pollination.

Target	Time point	Microarrays		qPCR	
		FC	MAD	FC	MAD
Pathogenesis- related protein 10c (PR10)	2DAP	**1.59**	0.31	**2.24**	0.56
	4DAP	**1.74**	0.22	**14.3**	4.21
Glucosylceramide	2DAP	**-1.37**	0.09	**-2.22**	0.40
	4DAP	**-1.4**	0.41	**-1.56**	0.47
Chaperone (Chaper)	2DAP	**1.79**	0.74	**1.70**	0.63
Stearoyl-acyl-carrier protein desaturase (SAD2)	4DAP	**-1.9**	0.84	**-1.91**	0.42
Metallothionein type III (Metallo III)	2DAP	**-1.38**	0.48	**-1.11**	0.30
	4DAP	**1.56**	0.40	**1.96**	0.76
Chitinase A	2DAP	**-1.43**	0.31	**1.21**	0.20
	4DAP	**1.57**	0.38	**1.99**	1.34
Stearoyl-acyl-carrier protein desaturase (SAD1)	4DAP	**-1.49**	0.50	**-5.77**	0.12

APPENDIX VI

Table I: Mean absolute amounts (µg) with standard error [SEM= standard deviation/√(n)]of ***n*-alkanes** (straight chain saturated hydrocarbons), in unpollinated and pollinated *Ophrys fusca* labella at 2 and 4 days after pollination (DAP). Compounds are ordered in retention times. Alkanes total amount (µg) is given as mean (± SEM) of labella sampled.

	2 DAP		**4 DAP**	
Alkanes	**Unpollinated (N=25)**	**Pollinated (N=25)**	**Unpollinated (N=24)**	**Pollinated (N=24)**
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Docosane (C22)	0.029 ± 0.004	0.035 ± 0.06	0.040 ±0.007	0.042 ± 0.007
Tricosane (C23)	1.268 ± 0.077	1.201 ± 0.11	1.080 ± 0.08	1.476 ± 0.282
Tetracosane(C24)	0.124 ± 0.013	0.182 ± 0.09	0.126 ± 0.018	0.149 ± 0.031
Pentacosane (C25)	0.55 ± 0.032	0.482 ± 0.036	0.492 ± 0.036	0.610 ± 0.226
Hexacosane(C26)	0.092 ± 0.006	0.089 ± 0.006	0.092 ± 0.008	0.115 ± 0.022
Heptacosane (C27)	1.11 ± 0.078	1.177 ± 0.072	1.079 ± 0.076	1.086 ± 0.162
Octacosane (C28)	0.064 ± 0.005	0.076 ± 0.008	0.061 ± 0.005	0.100 ± 0.027
Nonacosane (C29)	0.70 ± 0.17	0.563 ± 0.039	0.524 ± 0.034	0.718 ± 0.186
Hentriacontane (C31)	0.38 ± 0.047	0.209 ± 0.028	0.442 ± 0.044	0.461 ± 0.110
SUM (%)	**71**	**74**	**67**	**69**
Mean total amount (µg/labellum)	**4.32 ± 0.27**	**4.02 ± 0.28**	**3.94 ± 0.24**	**4.76 ± 0.67**

APPENDIX VI (CONTINUED)

Table II: Mean absolute amounts with standard error [SEM= standard deviation/√(n)]of **alkenes** (unsaturated hydrocarbons), in unpollinated and pollinated *Ophrys fusca* labella at 2 and 4 days after pollination (DAP). Compounds are ordered in retention times. Alkenes total amount (µg) is given as mean (± SEM) of total labella sampled.

	2 DAP		**4 DAP**	
Alkenes	**Unpollinated (N=25)**	**Pollinated (N=25)**	**Unpollinated (N=24)**	**Pollinated (N=24)**
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
(Z)-9-Heneicosene	0.002 ± 0.001	0.001 ± 0.0006	0.005 ± 0.001	0.003 ± 0.001
(Z)-7-Heneicosene	0.027 ± 0.003	0.042 ± 0.008	0.020 ± 0.004	0.032 ± 0.006
(Z)-9-Tricosene	0.017 ± 0.004	0.021 ± 0.004	0.023 ± 0.005	0.024 ± 0.007
(Z)-7-Tricosene	0.005 ± 0.002	0.018 ± 0.009	0.0006 ± 0.0002	0.006 ± 0.003
(Z)-11-Pentacosene	0.078 ± 0.010	0.090 ± 0.015	0.097 ± 0.012	0.109 ± 0.023
(Z)-7-Pentacosene	0.015 ± 0.003	0.021 ± 0.005	0.022 ± 0.004	0.049 ± 0.021
(Z)-11-Heptacosene	0.180 ± 0.020	0.136 ± 0.029	0.153 ± 0.023	0.170 ± 0.039
(Z)-9-Heptacosene	0.300 ±0.034	0.239 ± 0.031	0.358 ± 0.038	0.541 ± 0.149
(Z)-7-Heptacosene	0.001 ± 0.0004	0.038 ± 0.02	0.009 ± 0.004	0.019 ± 0.009
(Z)-13-Nonacosene	0.065 ± 0.018	0.057 ± 0.012	0.068 ± 0.016	0.145 ± 0.076
(Z)-12-Nonacosene	0.312 ± 0.035	0.170 ± 0.021	0.334 ± 0.30	0.317 ± 0.093
(Z)-11-Nonacosene	0.726 ± 0.055	0.563 ± 0.083	0.802 ± 0.077	0.687 ± 0.136
(Z)-9-Nonacosene	0.003 ± 0.002	0.006 ± 0.002	0.001 ± 0.0003	0.019 ± 0.011
SUM (%)	**29**	**26**	**33**	**31**
Mean total amount (µg/labellum)	**1.74 ± 0.13**	**1.41 ± 0.18**	**1.90 ± 0.15**	**2.13 ± 0.43**

APPENDIX VII

Results of Mann-Whitney *U* test for total amounts in *Ophrys fusca* labella extracts in unpollinated and pollinated flowers time points (2DAP and 4DAP)

Unpoll vs Poll 2DAP		Unpoll vs Poll 4DAP		
	Sum			Sum
Mann-Whitney *U*	239.000	Mann-Whitney *U*		260.000
Wilcoxon W	564.000	Wilcoxon W		536.000
Z	-1.426	Z		-.341
Asymp. Sig. (2-tailed)	0.154	Asymp. Sig. (2-tailed)		0.733

APPENDIX VIII

Statiscally significant compounds resulting from LSD *post-hoc* test (p<0.05) in *Ophrys fusca* labella extracts of unpollinated and pollinated flowers (2DAP and 4DAP)

Timepoint		Compounds
unpollinated/pollinated	**2DAP**	Alkenes: (Z)-7-heneicosene, (Z)-7-heptacosene, (Z)-12+ (Z)-11-nonacosene Alkanes: heptacosane, octacosane, hentriacontane
	4DAP	Alkenes: (Z)-12+ (Z)-11+ (Z)-9- nonacosene
unpollinated 2DAP vs. 4DAP		no variation
pollinated 2DAP vs. 4DAP		Alkene: (Z)-9-heptacosene Alkanes: heptacosane, hentriacontane

APPENDIX IX

Mean relative amounts (±SEM) of alkanes (**A**) and alkenes (**B**) in *Ophrys fusca* labella extracts in unpollinated (UP) flowers 2 days and 4 days after pollination (DAP).