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OLIVE FRUIT FLY (*BACTROCERA OLEAE* ROSSI) – OLIVE TREE INTERACTIONS: STUDY OF PHYSICAL AND CHEMICAL ASPECTS

Tese do 3º Ciclo de Estudos Conducente ao Grau de Doutoramento em Ciências Farmacêuticas especialidade de Nutrição e Química dos Alimentos

Trabalho realizado sob orientação de

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À minha mãe

À Ana

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Abstract

Olive products quality is influenced by diverse factors. Among agronomic ones, pests attack and diseases incidence are probably the most important aspects to consider. Olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) is one of the key-pests of olive tree worldwide, with special importance in the Mediterranean Basin. This pest, besides causing important losses at the production level, causes also a sharp decrease in olive products quality, particularly olive oil.

Differentiated susceptibility of olive cultivars to the attack of olive fly is fully recognized. Indeed, under the same agroclimatic conditions and time period, some cultivars report high levels of attack, while the attack is not so intense on others. Different explanations have been pointed to these differences, but the factors that interfere in such phenomena are yet to be clarified. In this sense, the present work intended to contribute for the clarification of the interconections established between the pest (olive-fly) and the host (olive tree), studying different physical and chemical aspects, of both olive leaves and fruits, that may interfere in the olive fly oviposition in the main olive cultivars of Trás-os-Montes region: cvs. Cobrançosa, Madural, and Verdeal Transmontana. For such, the determination of volatile compounds of olives and leaves (HS-SPME and GC/MS) during olives maturation and their association to pest attack was studied; electroantennographic studies (EAG) based on the action of olive leaves essential oils (EO's), volatiles compounds and recognized semiochemicals were explored over olive fly adults from both sexes with different ages; oviposition assays were implemented to verify the effect of olive cultivar and olives maturation in females oviposition; and physical parameters and chemical composition that may be related to the susceptibility of olive cultivars in field conditions to the attack of olive fly were evaluated.

Generally, olive fly showed higher preference for olives from cv. Verdeal Transmontana, followed by cvs. Madural, and Cobrançosa. The volatile profiles of both leaves and olives from the three cultivars were analyzed during maturation, with a total of 34 and 39 compounds, respectively in olives and leaves, from seven different chemical classes (alcohols, aldehydes, aromatic hydrocarbons, esters, ketones, terpenes, and sesquiterpenes). Cultivar and harvest date influenced the volatile profile both qualitatively and quantitatively. In what concerns to leaves, cv. Cobrançosa emitted higher amounts of volatile compounds (between 519 and 6830 μg.100 g^{-1}). In the three olive cultivars leaves, the main volatiles present in the beginning of maturation were GLV's (green leaf volatiles), while at the end of the study the amount of aromatic hydrocarbons, mainly toluene, was considerable higher in the most susceptible olive cultivars. In olives, the maximum of

volatiles emitted was observed in cv. Verdeal Transmontana, with 324 μg.100 g^{-1} in the beginning of maturation, clearly below leaves emissions. In olives, the GLV's were also the main volatiles, giving place to toluene and sesquiterpenes during maturation. Toluene was correlated with olive fly attack, both in leaves and olives of cvs. Verdeal Transmontana and Madural, while no correlations were established for the attack verified in cv. Cobrançosa. In olives, a positive correlation between the content of α-copaene and infestation was also observed in cvs. Verdeal Transmontana and Madural. These two volatile components are recognized attractants of olive fly and olive fly females oviposition promoters.

In electroantennographic assays (EAG), carried out with EO's from leaves of the three cultivars in study, volatiles compounds from olive tree, and recognized semiochemicals, the response at different concentrations, of both sexes and ages of adults, was tested. EAG response was higher in both sexes with EO's from cv. Cobrançosa, followed by cvs. Madural, and Verdeal Transmontana. An inverse relation between EAG response and infestation of olive fly was verified with EO's, indicating a possible repellent action of olive leaves EO's, especially from cv. Cobrançosa. The EAG signal was influenced by olive cultivar, sex, concentration tested, and adults age. Higher EAG response was registered in males, decreasing the response with adult age. Among the different olive tree volatiles and semiochemicals analyzed, the aldehydes (*E*)-2-hexenal and nonanal were those who caused higher responses, being their signals related to the physiological states of both sexes. Nonanal demonstrated higher EAG response in males between [5-10[days old, while (E)-2-hexenal reported higher EAG response in females in the period of sexual maturity ([5-10[days old) and oviposition (> 10 days old).

In oviposition assays, a considerable high number of ovipositions was verified in olives from cv. Verdeal Transmontana (1073 ovipositions/600 fruits), in opposition to cvs. Cobrançosa (450 ovipositions/600 fruits), and Madural (577 ovipositions/600 fruits). Besides, being the less susceptible olive cultivar, cv. Cobrançosa also reported higher rates of mortality/sterile ovipositions, with only 51% completing their cycle. A higher rate of preference by olives with green, yellow-green and red colorations was also observed, instead of advanced stages of maturation with a black coloration. In survival assays, a significantly higher longevity of males and females emerged from pupae feed from cv. Verdeal Transmontana olives (3.31 and 3.29 days for males and females respectively) was verified, in comparison with cv. Cobrançosa (2.98 and 2.89 days respectively), a fact intrinsically related to the differentiated composition of each cultivar. Data indicates that, besides being less susceptible and causing higher indexes of mortality, the adults

resultant from pupae developed under cv. Cobrançosa olives live less time, being this data of extreme ecological relevance.

In what respect to physical factors, olives with greater volume and less elongation are associated to higher probabilities of attack, a fact observed in cv. Verdeal Transmontana comparatively to cv. Cobrançosa. The color of both leaves and olives is also an important parameter. In leaves, color parameters of down page have not revealed a preponderant effect in oviposition preference. Meanwhile, the higher lightness (*L**) emitted by the upper page of leaves and olives of cv. Verdeal Transmontana during maturation, associated to a considerable late maturation process of this cultivar comparatively to cvs. Cobrançosa and Madural, may stimulate oviposition. The preference of olive fly for olives in less advanced maturation stages, in which olives are green or yellow-green, was also verified. Chemical factors, like fatty acids profile, could internally influence olive fly oviposition, as well as their survival as described previously, once each cultivar possess a characteristic chemical composition.

The results obtained point out for a series of physical and chemical factors that influence olive fly oviposition preference. The aerial parts of olive tree, leaves and olives, exhale volatile components with attractive potential over olive fly females, being their abundance directly correlated with the infestation levels observed in the more susceptible olive cultivars. For the first time, the effect of EO's of olive leaves in the sensibility of olive fly adults was verified, with an inverse relation between cultivar susceptibility and EAG signal. Different volatile components of olive tree have distinct action according to the age and physiological state of olive fly adults. The maturation process, characteristic in each olive cultivar, allied to physical and chemical changes of olives lead to the differentiation of all factors involved in the oviposition preference of olive fly for cv. Verdeal Transmontana, turning cv. Cobrançosa less susceptible.

These achievements constitute important advances into the development of new strategies to manage this pest worldwide.

Resumo

São vários os fatores que influem na qualidade dos produtos do olival. De entre os agronómicos, o ataque de pragas e doenças são provavelmente os principais aspetos a considerar. A mosca-da-azeitona, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) é uma praga-chave da oliveira a nível mundial, com especial importância na Bacia Mediterrânica. Esta praga, além dos importantes prejuízos que provoca ao nível da produção, ocasiona também uma diminuição acentuada da qualidade dos produtos à base de azeitona, com maior incidência no azeite.

É conhecida a suscetibilidade diferenciada ao ataque da mosca-da-azeitona de acordo com a cultivar de oliveira. Assim, nas mesmas condições agroclimáticas, e no mesmo período temporal, algumas cultivares apresentam níveis de ataque elevados, enquanto noutras o ataque não é tão intenso. Têm sido apontadas diversas explicações para a diferente suscetibilidade encontrada, contudo os fatores que interferem nesse fenómeno não se encontram ainda claros. Neste sentido, com o presente trabalho pretendeu-se contribuir para o esclarecimento das relações que se estabelecem entre a praga (mosca-da-azeitona) e o hospedeiro (a oliveira), estudando diferentes aspetos físicos e químicos da folha e fruto da oliveira, que possam interferir ao nível da postura da mosca-da-azeitona nas principais cultivares de oliveira da região de Trás-os-Montes: cvs. Cobrançosa, Madural e Verdeal Transmontana. Para tal, foram conduzidos estudos para determinação de compostos voláteis de folhas e frutos (HS-SPME e GC/MS) durante a maturação dos frutos e estudada a sua relação com o ataque da praga; ensaios de eletroantenografia (EAG) relativamente à ação de óleos essenciais (OE's) das folhas de oliveira, compostos voláteis da oliveira e compostos reconhecidos como semioquímicos sobre os adultos de ambos os sexos com diferentes idades; ensaios de postura para verificar o efeito da cultivar e maturação do fruto na postura das fêmeas; e a avaliação de parâmetros físicos e de composição química que possam estar relacionados com a suscetibilidade em campo das diferentes cultivares ao ataque da mosca-da-azeitona.

Na generalidade das situações, a mosca-da-azeitona mostrou maior preferência por frutos da cv. Verdeal Transmontana, seguida das cvs. Madural e Cobrançosa. O perfil volátil de folhas e frutos das três cultivares foi analisado ao longo da maturação, tendo sido identificados compostos de sete famílias químicas distintas (álcoois, aldeídos, cetonas, ésteres, hidrocarbonetos aromáticos, sesquiterpenos e terpenos) com um total de 34 e 39 compostos, respetivamente nos frutos e folhas. A cultivar e época de colheita influíram no perfil volátil, tanto qualitativa como quantitativamente. No que respeita às folhas, a cv. Cobrançosa emite maior quantidade de compostos voláteis (entre 519 e

6830 µg.100 g^{-1}). Nas três cultivares e no início da maturação, a maioria dos voláteis em folhas são GLV's (green leaf volatiles), sendo que no final do estudo o teor em hidrocarbonetos aromáticos, principalmente tolueno, estava considerável aumentado nas cultivares mais suscetíveis. Nos frutos, o máximo de voláteis emitidos verificou-se na cv. Verdeal Transmontana, com 324 µg.100 g^{-1} no inicio do estudo, bem inferior às emissões das folhas. Nos frutos, os GLV's eram os voláteis maioritários, dando lugar ao tolueno e sesquiterpenos ao longo da maturação. O tolueno mostrou-se correlacionado com o ataque da mosca-da-azeitona, tanto nas folhas como nos frutos das cvs. Verdeal Transmontana e Madural, mas não foram estabelecidas quaisquer correlações com o ataque observado na cv. Cobrançosa. Nos frutos foi também verificada uma correlação positiva entre o teor em α-copaeno e a infestação das cvs. Verdeal Transmontana e Madural. Estes dois componentes voláteis são comprovados atraentes da mosca-da-azeitona e promotores de postura por parte das fêmeas.

Nos ensaios de eletroantenografia (EAG), realizados com OE's de folhas das três cultivares em estudo, compostos voláteis da oliveira e reconhecidos semioquímicos, testou-se a resposta a diferentes concentrações, em ambos os sexos e idades dos adultos. A resposta EAG foi maior em ambos os sexos com OE's de cv. Cobrançosa, seguida das cvs. Madural e Verdeal Transmontana. Verificou-se uma relação inversa entre a resposta EAG e a infestação da mosca-da-azeitona, indicando uma possível ação repelente dos OE's das folhas de oliveira, especialmente de cv. Cobrançosa. O sinal EAG foi influenciado pela cultivar, concentração testada, sexo, e idade dos adultos. A maior resposta EAG foi registada em machos, diminuindo a resposta com a idade dos adultos. Entre os diferentes compostos voláteis da oliveira e semioquímicos analisados, os aldeídos (*E*)-2-hexenal e nonanal foram os que provocaram maior resposta, estando os sinais relacionados com os estados fisiológicos de ambos os sexos. O nonanal demonstrou maior resposta EAG em machos entre os [5-10[dias de idade, enquanto que o (*E*)-2-hexenal reportou maior resposta EAG em fêmeas no período de maturação sexual ([5-10[dias) e postura (> 10 dias).

Em ensaios de postura, verificou-se um número consideravelmente elevado de posturas em frutos da cv. Verdeal Transmontana (1073 posturas/600 frutos) em relação aos das cvs. Cobrançosa (450 posturas/600 frutos) e Madural (577 posturas/600 frutos). Além de ser a cultivar menos suscetível, a cv. Cobrançosa também reportou uma alta taxa de mortalidade/posturas estéreis, com apenas 51% das posturas a completaram o seu ciclo. Observou-se também uma maior taxa de preferência por frutos de colorações verde, verde-amarelado e vermelho, do que estados de maturação mais avançados em que os frutos se encontram com coloração negra. Em ensaios de sobrevivência verificou-

se uma longevidade significativamente maior de machos e fêmeas que emergiram de pupas que se alimentaram de frutos da cv. Verdeal Transmontana (3,31 e 3,29 dias para machos e fêmeas, respetivamente) em relação à cv. Cobrançosa (2,98 e 2,89 dias, respetivamente), facto intrinsecamente relacionado com a composição diferenciada de cada cultivar. Os dados indicam que, além de ser menos suscetível e causar maiores índices de mortalidade, os adultos resultantes de pupas desenvolvidas em frutos da cv. Cobrançosa vivem menos tempo, sendo este um dado ecológico de grande relevância.

No que respeita a fatores físicos, os frutos com maior volume e menor alongamento estão relacionados com maiores probabilidades de ataque, facto observado na cv. Verdeal Transmontana comparativamente com a cv. Cobrançosa. A cor, tanto de folhas como de frutos, é também um parâmetro importante. Nas folhas, os parâmetros de cor da página inferior não revelaram ser preponderantes na preferência à oviposição. No entanto uma maior luminosidade (*L**) emitida pela página superior das folhas e pelos frutos da cv. Verdeal Transmontana ao longo da maturação, fato associado a uma maturação consideravelmente tardia comparativamente com as cvs. Cobrançosa e Madural, estimulará a postura, sendo aqui a cor um fator preponderante. Também foi verificada a preferência da mosca-da-azeitona por frutos com estados de maturação menos avançados, em que os frutos se encontram verdes ou verde-amarelados. Fatores químicos, como o perfil em ácidos gordos, poderão internamente influenciar a postura da mosca-da-azeitona, bem como a sua sobrevivência como referido anteriormente, uma vez que cada cultivar possui um perfil característico.

Os resultados obtidos apontam para uma série de fatores físicos e químicos com influência na preferência de postura da mosca-da-azeitona. As partes aéreas da oliveira, folhas e frutos, exalam componentes voláteis com potencial atrativo sobre as fêmeas da mosca-da-azeitona, sendo a sua abundância diretamente correlacionada com os níveis de infestação observados nas cultivares mais suscetíveis. Pela primeira vez, estudou-se o efeito de OE's de folhas da oliveira na sensibilidade de adultos de mosca-da-azeitona, tendo-se verificado uma relação inversa entre a suscetibilidade das cultivares e o sinal EAG. Diferentes componentes voláteis da oliveira têm ação distinta, de acordo com a idade e estado fisiológico dos adultos da mosca-da-azeitona. O processo de maturação característico em cada cultivar, aliado à coloração e às alterações físicas e químicas dos frutos levam, à diferenciação de todos os fatores envolvidos na preferência da mosca-da-azeitona pela cv. Verdeal Transmontana, tornando a cv. Cobrançosa menos suscetível.

Estas observações constituem importantes avanços no sentido de desenvolver novas estratégias no combate a esta praga em todo o mundo.

Publications and communications resultant from the PhD project

Publications in peer-reviewed journals indexed to Journal Citation Reports da ISI Web of Knowledge:

Malheiro R, Casal S, Baptista P, Pereira JA. A review of *Bactrocera oleae* (Rossi) impact in olive products: from the tree to the table. Submitted. (Chapter 4)

Malheiro R, Casal S, Cunha S, Baptista P, Pereira JA. Olive leaves volatiles along fruit maturation and their possible role in olive fly oviposition preference. Submitted. (Chapter 5)

Malheiro R, Casal S, Cunha S, Baptista P, Pereira JA. Olive volatiles from Portuguese cultivars Cobrançosa, Madural and Verdeal Transmontana: role in oviposition preference of *Bactrocera oleae* (Rossi) (Diptera: Tephritidae). *Plos One* (accepted). (Chapter 6)

Malheiro R, Ortiz A, Casal S, Baptista P, Pereira JA. Electrophysiological response of *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) adults to olive leaves essential oils from different cultivars and olive tree volatiles. Submitted. (Chapter 7)

Malheiro R, Casal S, Pinheiro L, Baptista P, Pereira JA. Influence of olive cultivar and maturation processo in the oviposition preference of *Bactrocera oleae* (Rossi) (Diptera: Tephritidae). Submitted. (Chapter 8)

Malheiro R, Casal S, Baptista P, Pereira JA. Physico-chemical characteristics of *Olea europaea* L. olives and leaves and *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) cultivar oviposition preference. Submitted. (Chapter 9)

Book chapters

Malheiro R, Casal S, Baptista P, Pereira JA 2014. How agronomic factos affects olive oil composition and quality. In: De Leonardis A, editor. Virgin olive oil: Production, Composition, Uses and Benefits for Man. New York: Nova Science Publications, p. 119-141. (Chapter 3)

Proceedings in scientific events

Malheiro R, Ortiz A, Hidalgo F, Casal S, Baptista P, Pereira JA 2014. Electroantennographic response of *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) antenna to olive leaves' essential oils from Portuguese olive cultivars (cvs. Cobrançosa, Madural and Verdeal Transmontana). *IOBC-WPRS Bulletin*, 108, 17-22.

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Acronyms and abbreviations

3,4-DHPEA (hydroxytyrosol)
3,4-DHPEA-EA (Oleuropein aglycone)
3,4-DHPEA-EDA (Dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol)
ANOVA (Analysis of variance)
cv. (cultivar)
$\mathbf{D_{max}}$ (maximum diameter)
$\mathbf{D_{min}}$ (minimum diameter)
DPPH (2,2-diphenyl-1-picrylhydrazil)
DVB/CAR/PDMS (Divinylbenzene/carboxen/polydimethylsiloxane)
EAG (Electroantennography)
EO (Essential oil)
ETc (Evapotranspiration)
EVOO (Extra-virgin olive oil)
FA (Free acidity)
FAME (Fatty acids methyl esters)
FAOSTAT (Statistics Division of Food and Agriculture Organization)
FID (Flame ionization detector)
GC-EAD (Gas chromatography coupled to an electroantennographic detector)
GC/MS (Gas chromatography coupled to a mass spectrometry detector)
GLM (General linear model)
GLV's (Green leaf volatiles)
ha (hectare)
HS-SPME (Head-space solid phase microextraction)
IOC (International Olive Council)
IOOC (International Olive Oil Council)
LOO (Lampante olive oil)
LOX (Lipoxygenase)
MI (Maturation index)
MUFA (Monounsaturated fatty acids)
NIST (National Institute of Standards and Technology)
p-HPEA (tyrosol)
p-HPEA-EDA (Dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol)

PC (Principal component)

PCA (Principal component analysis)

PUFA (Polyunsaturated fatty acids)

PV (Peroxide value)

SFA (Saturated fatty acids)

SPSS (Statistical Package for the Social Sciences)

US$ (United States dollars)

Verdeal T. (Verdeal Transmontana)

VOO (Virgin olive oil)

PART I

Objectives and Introduction

CHAPTER 1.

Objectives

The main objective of this thesis was to study the interactions between olive fly, *Bactrocera oleae* (Rossi), and olive tree, in order to elucidate the factors behind the oviposition preference of this insect to different olive cultivars from Trás-os-Montes region displaying different susceptibilities to this pest: cv. Cobrançosa (less susceptible); cv. Madural (intermediate susceptibility); and cv. Verdeal Transmontana (high susceptibility). To achieve such goal, two main approaches were considered for this study: physical and chemical aspects.

The specific objectives of this thesis were to:

i) Study the volatile composition of olive leaves (Chapter 5) and olives (Chapter 6) during olives maturation and observe possible compounds of interest associated to olive fly infestation levels in the three olive cultivars;

ii) Study the composition of the essential oils from the olive leaves of the three cultivars (Chapter 7);

iii) Study of the influence of olive tree volatiles and olive fly semiochemicals in adults electroantennographic response (Chapter 7);

iv) Study the influence of olive leaves essential oils in the electroantennographic response of olive flies adults antenna (Chapter 7);

v) Verify the oviposition preference of olive fly females according to the olive cultivar and olives maturation stage in oviposition bioassays (Chapter 8);

vi) Study the longevity of adult olive flies according to the olive cultivar in which they developed (Chapter 8);

vii) Study the influence of olives and olive leaves physical parameters (biometrical data and color) in the oviposition preference of olive fly (Chapter 9);

ix) Study of the chemical composition of olive pulp from the three olive cultivars (Chapter 9).

To achieve these objectives, the present PhD thesis is organized in three main parts, each part divided in chapters.

PART I – Introduction and objectives

This part is subdivided in four chapters (Chapters 1 to 4). In the first chapter the primary and secondary objectives are presented as well as the hypothesis tested in this thesis. In the second chapter, an introduction to the problematic of the olive fly, *Bactrocera oleae* (Rossi) in olive crop and several aspects behind its oviposition preference are presented. In the third chapter an overview on how agronomic factors can influence the quality of olive products is explored. In the fourth chapter the impact of olive fly from the field to consumers table, discussing aspects of production, economic issues, quality, composition, and biological properties of olive products, is reviewed in detail.

PART II – Experimental section

This part is subdivided in five chapters (Chapters 5 to 9) corresponding to accepted or submitted papers, which contain the results obtained in this thesis, presented in accordance with the guidelines of each scientific journal. In chapters 5 and 6 the influence of leaves and olives volatile composition in olive fly oviposition preference is discussed. In chapter 7 the effect of leaves essential oils, olive tree volatiles, and olive fly semiochemicals are discussed by electroantennographic assays. In chapter 8, the influence of cultivar and maturation stage in the oviposition of olive fly are presented and discussed. Finally, the roles of physical parameters of leaves and olives as well as chemical aspects behind olive fly preference are discussed in chapter 9.

PART III – General discussion and conclusions

This part is subdivided in two chapters (Chapters 10 and 11). In the first chapter an integrated discussion of all results obtained in the thesis is given. The second chapter is devoted to the conclusions assembled from all the results obtained.

CHAPTER 2.

General introduction

In the last decades, olives (*Olea europaea* L.) production has shown a significant increase both in traditional areas of production – the Mediterranean Basin - as in non-traditional producers in different countries worldwide, like Brazil, China, South Africa, among others. Such expansion and intensification lead to a 226% increase in olives production, from 9 million tons in 1990 to 20.4 million tons in 2013 (FAOSTAT, 2015).

Expansion of olive crop has also lead to dissemination of one of its main pests, the olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) (Figure 2.1A and 2.1B). Olive fly is a monophagous specie, since its larvae feed exclusively on *Olea* species, mainly *O. europaea*. Olive fly females lay their eggs in olive pulp (Figure 2.1C), right beneath the olive surface.

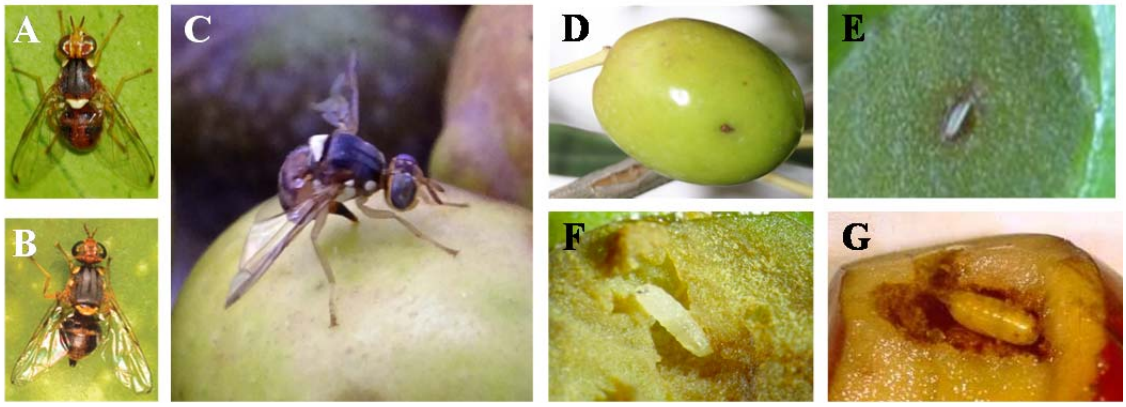

Figure 2.1. Olive flies male (A) and female (B); an olive fly female oviposition attempt (C); oviposition site in olive (D); olive fly egg (E); olive fly larvae (F); olive fly pupae (G).

Under optimal conditions, eggs (Figure 2.1E) can develop in only one day (Tsitsipis, 1977), giving origin to a larvae that immediately start to feed on olive pulp (Figure 2.1F). These larvae will grow and pass through three instars (L1, L2 and L3), creating galleries inside olives and destroying the tissues on their path. At optimal conditions, larvae may take eight days to reach their next stage, transforming into pupae (Figure 1G) that will emerge as olive fly adults (Figure 2.1A and 2.1B), varying this period from nine days, at optimal conditions, to 49 days (Neuenschwander and Michelakis, 1979).

Olive fly cause serious damages to olive crop, reducing olives production yield while simultaneously degrading olive products composition, quality, and properties (Pereira et al., 2004). In respect to olives production, Bueno and Jones (2002) predicted an average annual loss of about 15% due generally to pests and diseases incidence. One third of these losses are exclusively attributed to olive fly, causing an estimated economic impact of 800 million US$ a year (Nardi et al., 2006). The current crop season (2014/15) is a good example of how olive fly, together with climatic conditions, can impact olive productivity in Europe. The lowest production of extra-virgin olive oil (EVOO) since the beginning of XXI century is forecasted in this season (Figure 2.2).

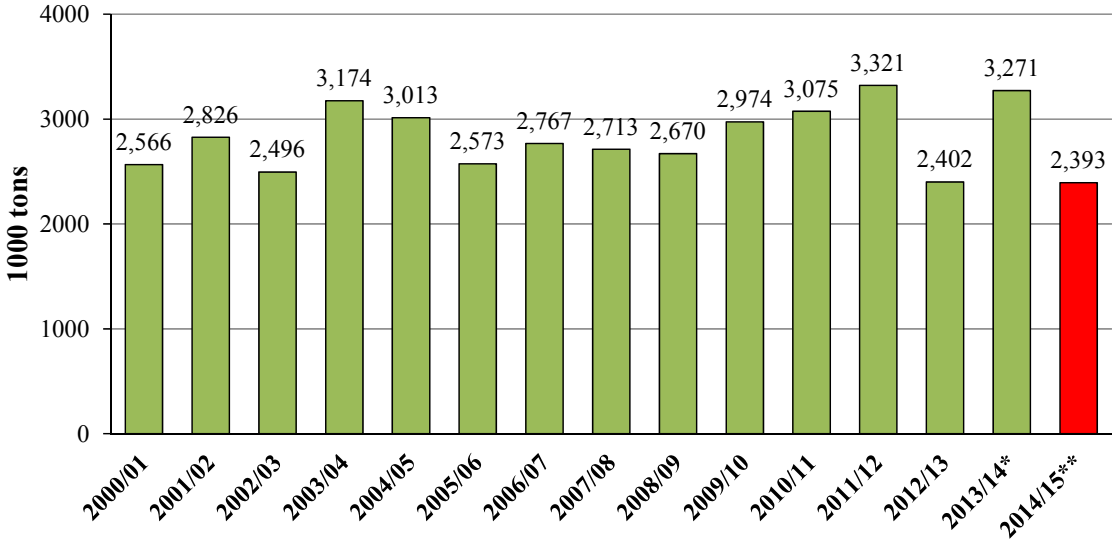

Figure 2.2. Extra-virgin olive oil production since the beginning of XXI century (*estimated data; **forecast; IOC, 2014a).

Extra-virgin olive oil production decreased 27% comparatively to the 2013/14 crop season (IOC, 2014a). Part of this loss is related to climatic conditions (hot summer in Spain, affecting flowering season and reducing the production in 54%) and olive fly infestation in Italy that, together with an excessively wet summer, reduced Italian production by 34% (IOC, 2014a). According to the main olive growing producers associations in Portugal, a similar situation was observed in our country, with a reduction between 10 to 20% due to olive fly attack.

Olive fly infestation affects all links of the olive chain, ending on consumers' pockets, since olive oil prices in international markets raised significantly in only one year period. The drastic reduction in olives production, where olive fly has a considerable share, leads to the reduction of production and quality of olive products, mainly olive oil. Olive oil prices were severely affected. Prices in Chania (Greece), Jaén (Spain) and Bari

(Italy), three of the main olive oil producing regions worldwide, increased considerably in one year (IOC, 2014b). From February 2014 to February 2015, 1 kg of EVOO raised from 2.5 € to 3 € in Chania. In Jaén the prices raised from 2 € to 3.2 €, while in Bari 1 kg of EVOO increased from 3.1 € to 6 € (IOC, 2014b). Therefore, imposed by climatic changes and actions of olive fly, a constant increase of olive oil prices is expected worldwide for the current year.

Independently of the damages caused by olive fly, some olive cultivars report less susceptibility to this dipteran comparatively to others, under the same agronomic and edaphoclimatic conditions. All over the world this phenomena is reported and observations are systematic over the years. Even in years with low amounts of fruits and high olive fly populations, some cultivars remain comparatively less susceptible then others (Gonçalves et al., 2012). In Greece, in the region of Chalkidiki, cv. Chondroia Chalkidikis reports infestation levels above 75%, while cv. Koroneiki reported only 26% (Navrozidis et al., 2007). In Calabria (Southern Italy), 55% of olives from cv. Giarraffa were infested while only 19% of those from cv. Tondra near dolce were affected (Iannota et al., 2007). In Jordan, cv. Nabali is attacked in considerably low levels (< 6%) while in cvs. Rase'e, Shami, and Santia more than half of the fruits were infested (Al-Zaghal and Mustafa, 1987). According to Al-Salti et al. (2011) cv. Alkhudairi is apparently a resistant olive cultivar to olive fly (0% infestation) in Homs region (Syria), while cv. Aldeibli report an infestation level of 24%. In the North-Western region of Syria other cultivars are more heavily infested: cv. Djilt between 30-40% and cv. Ziety with 57% of the fruits infested (Saour and Makee, 2004). In Turkey (Izmir region) cv. Çilli report an infestation level higher than 60%, in opposite position is cv. Ayvalik with levels below 40% (Gümusay et al., 1990). In the USA, namely California, cv. Mission reports infestation levels around 50-60% while a group of other cultivars (Arbequina, Frantoio, Koroneiki, and Leccino) report infestations below 5% (Burrack and Zalom, 2008).

In Portugal, in Trás-os-Montes region, the second most important olives producing region in the country, the three most representative olive cultivars also report oviposition preference of olive fly females: olive fly has a higher preference for cvs. Verdeal Transmontana and Madural, while cv. Cobrançosa reports considerably lower infestation levels each year (Gonçalves et al., 2012; Malheiro et al., 2014; Rodrigues et al., 2014).

The interaction between pests and plants, in this particular case *Bactrocera oleae* – *Olea europaea*, is an important step to understand the cues and factors behind olive fly preference regarding olive cultivar. Three aspects are considered to influence the oviposition preference: physical, chemical and molecular aspects.

Several works were carried out in order to study the interaction between olive fly and distinct olive cultivars with different susceptibility degrees. Regarding physical factors, so far, olives volume and elongation are important parameters in olive fly preference. Higher volumes and lower elongation increase the odds of infestation by olive fly (Rizzo *et al*., 2012). Epidermis physical characteristics were also revealed by Gonçalves *et al*. (2012) as crucial aspects during oviposition, being hardness and elasticity, among others, aspects that influence a successful oviposition. Olives color was also pointed out to influence olive fly females preference (Vlahov, 1992; Iannota and Scalercio, 2012) with a dual action, attracting when olives are green, and deceiving females when olives are darker.

In what concern to chemical aspects, the chemical composition of olives could intervene in olive fly oviposition preference. These aspects come from outside and inside olives. From the outside, olives epicuticular waxes profile and amounts could reduce significantly olive fly oviposition (Kombargi *et al*., 1998), and they are specific according to olive cultivar (Guinda *et al*., 2010) and influenced by maturation (Peragón, 2013). According to Aluja and Mangan (2008), volatiles are important in order to find a host to oviposit. In fact, an array of volatiles, like toluene, ammonia, styrene, ethylbenzene, α-pinene and α-copaene proved to be attractants of olive flies (Scarpati *et al*., 1993; Scarpati *et al*., 1996; Alfonso *et al*., 2014), while (*E*)-2-hexenal proved to be a highly repellent volatile to olive fly (Scarpati *et al*., 1993). Nonanal, a minor component of the sexual pheromone of olive fly (Botsi *et al*., 1995), and exhaled from different attractive traps with food sources, elicited high response in both males and females of olive fly (Seris, 2011). The chemical composition of the olive pulp, inside olives, is also an important aspect during oviposition. Phenolic compounds, for instance, can trigger a series of internal defense mechanisms against olive fly aggression, by enzymatic reactions (Sivakumar *et al*., 2007; Spadafora *et al*., 2008) and driven by molecular aspects (Koudounas *et al*., 2015). These mechanisms could be related to high rates of mortality of eggs and larvae of olive fly inside olives, since oleuropein, the main phenolic compounds in olives at early maturation stages (Sousa *et al*., 2014), suffer hydrolysis, by the action of β-glucosidase, forming highly toxic molecules inside olives (Spadafora *et al*., 2008).

Nevertheless, these kind of studies focused only in olives, never considering olive leaves as potential actors in the olive fly oviposition preference, but their presence could not be disregarded by olive flies in the time to choose in which host to oviposit.

The study of the interactions between olive fly and olive trees, aiming for the aspects behind olive fly oviposition preference may provide important information in order

to improve the control of olive fly population by new strategies based on aspects that mediate olive fly preference. In the next chapters the state of the art of these aspects will be reviewed, and the study of physical and chemical aspects in cvs. Cobrançosa, Madural and Verdeal Transmontana will be presented.

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CHAPTER 3.

How agronomic factors affects olive oil composition and quality

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Abstract

Olive oil is one of the most popular vegetable oils worldwide but several factors might affect its quality and composition, from the tree to the spoon. Olive oil quality and composition is mainly influenced by olive fruit characteristics, and therefore all aspects that influence their development have a crucial effect on olive products. Those factors include the selection of olive cultivar, its cultivation, degree of crop intensification and production systems, agricultural practices, including irrigation and fertilization, olive pests and diseases management, all these factors clearly defining the composition of olive fruits and the inherent quality and properties of olive products.

In the last decades, huge modifications in olive tree cultivation have been observed, related essentially with two great factors: development of olive cultivations in new producing areas and crop intensification in traditional producing areas. Generally, most agronomic factors, including crop density, farming system, irrigation and fertilization, have no substantial effects on fresh olive oil quality parameters and classification. Nevertheless, a considerable incidence of olive pests and diseases can easily take fresh

olive oils to the lampante category. In opposition, all agronomic factors seem to influence olive oil composition. Antioxidants are the main affected components, with a crucial effect on olive oil sensorial attributes, bioactive and nutritional properties, as well as its oxidative stability.

In present chapter the influence of diverse agronomic factors on olive fruits and olive oils production, composition and quality, is reviewed and discussed, giving special importance to olive farming-systems, fertilization and irrigation, as well as the incidence of olive pests and diseases.

Keywords: olive oil; agronomic factors; composition; quality; olive-farming systems; fertilization; irrigation; olive pests and diseases

Introduction

Olive oil is a premium vegetable oil that, contrary to the majority of commercial vegetable oils, can be consumed in its crude form, without refining, maintaining and preserving its composition and potentially all associated beneficial properties. Olive oil is the 9th most produced vegetable oil worldwide [1] and it is gaining importance and relevance comparatively to other vegetable oils. Indeed, in the last two decades both production and consumption figures increased considerably (Figure 3.1).

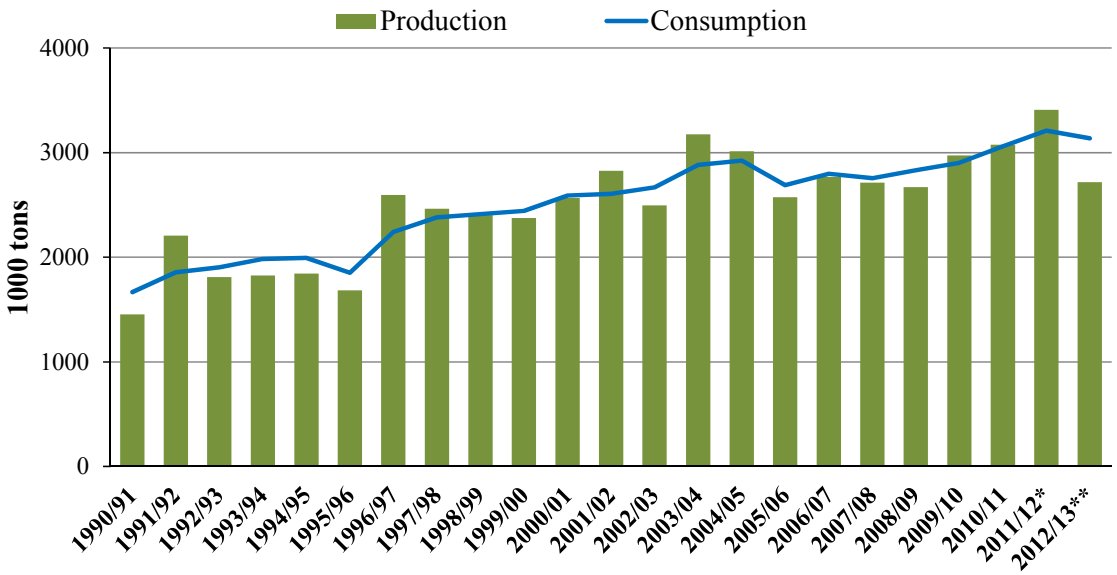

Figure 3.1. Worldwide olive oil production and consumption data (1000 tons) since 1990/91 [2, 3]. *Provisional data; **Predicted data.

Since the early 90's, olive oil production increased about 50% while its consumption increased 65% [2, 3]. Such increase is not only associated to global population growth, but mostly with the popularity that this vegetable oil gained over the last decades. Originally restricted almost to the Mediterranean region, olive orchards are now planted in regions where olives were absent or had a small representation. In particular, its cultivation is increasing in America (in the North mainly in California; Central America, mainly in Mexico; and in South America in Argentina, Chile and Brazil), South Africa and Australia. Olive oil is also being introduced in new potential markets, namely in Brazil, China and Russia, which also foment the consumption of this premium vegetable oil.

The inclusion of olive oil and related olive products in the daily diet is associated to innumerous health benefits, a direct consequence of its characteristic chemical composition. Among those components, monounsaturated fatty acids, pigments (chlorophylls and carotenoids with β-carotene as a very active component), tocopherols (mainly α-tocopherol), sterols (mainly β-sitosterol), and diverse phenolic compounds (hydroxytyrosol; tyrosol; oleuropein and derivatives) are recognized as the most active compounds (Figure 3.2).

In respect to major components, fatty acids, olive oil is rich in monounsaturated fatty acids (MUFA), mainly oleic acid ($C_{18:1}$), and has reduced amounts of both saturated (SFA) and polyunsaturated fatty acids (PUFA) but provides adequate amounts of essential fatty acids (linoleic and linolenic acids) [4]. This entails a high antiatherosclerotic potential, and a lower risk of cardiovascular diseases [5].

Concerning pigments, β-carotene is one of the most abundant carotenoids in olive oils [6] and, together with lutein, has antioxidant properties [7], inhibiting olive oil photoxidation by acting as singlet oxygen quenchers [8].

Tocopherols, especially α-tocopherol, display a dual activity since they are antioxidant compounds and exert a vitaminic action (vitamin E) contributing considerably to olive oils stability [9, 10] and consumer's health. Additionally, ingestion of α-tocopherol appears to have a preventive effect on the some of the most important diseases of the modern society, as cancer and Alzheimer [11, 12].

Olive oil phenolic compounds, also with recognized antioxidant capacity, are associated to innumerous healthy properties and benefits to consumers' health. There are several reports and literature revisions that highlight the *in vitro* [13-15] and *in vivo* [16-18] activities of these compounds and their pharmacological properties [19]. Phenolic compounds also have a marked influence on the olive oil sensorial attributes, particularly

the spicy, astringent and pungent sensations, as well as on the olive oil global quality and stability.

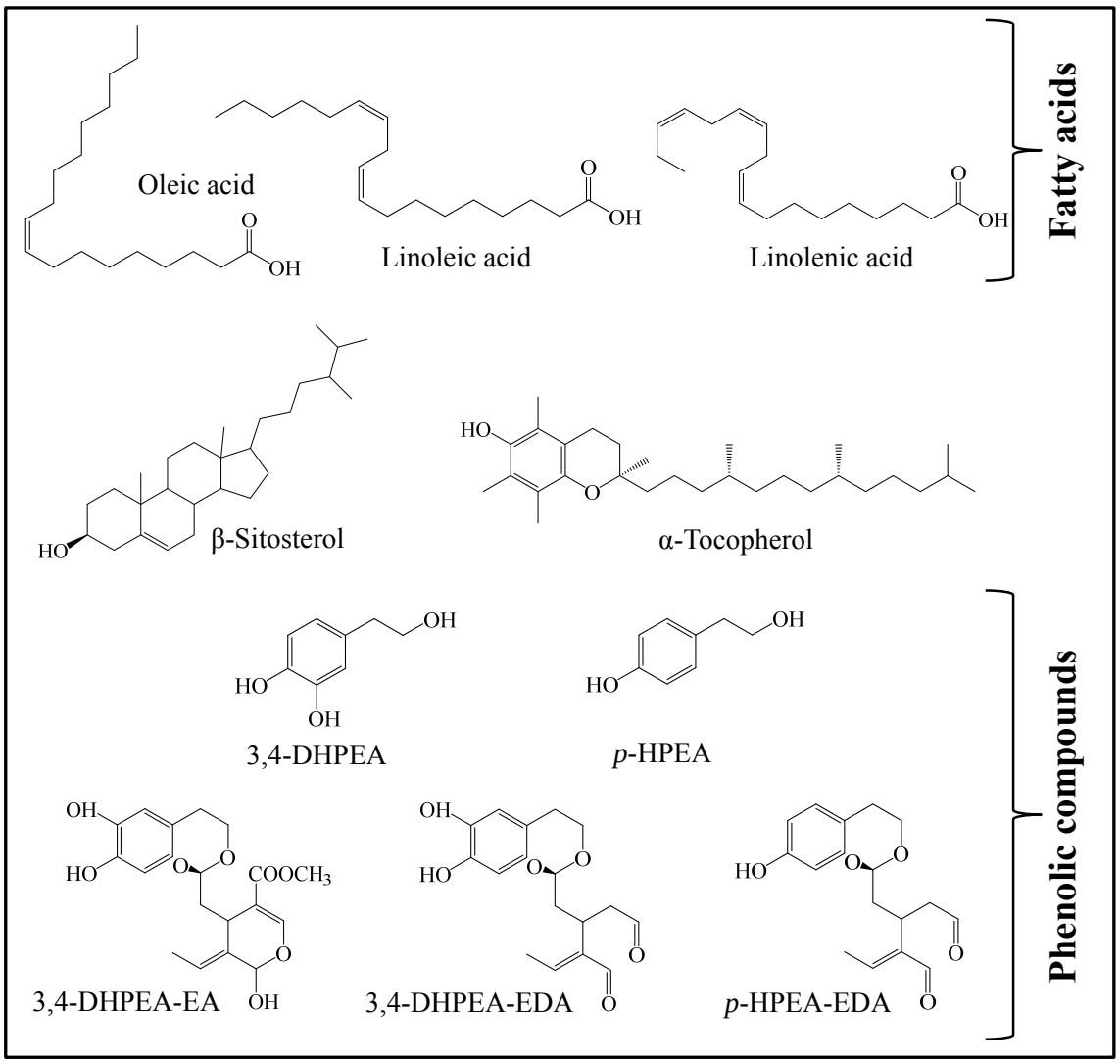

Figure 3.2. Olive oil components with nutritional and bioactive potential. (3,4-DHPEA – 3,4-dihydroxyphenylethanol (hydroxytyrosol); ρ-HPEA – ρ-hydroxyphenylethanol (tyrosol); 3,4-DHPEA-EA – oleuropein aglycon; 3,4-DHPEA-EDA – Dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol; ρ-HPEA-EDA – Dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol).

Olive oil composition, however, is the results of a diversified array of agronomic and technological factors. On this chapter, the impact of the application of fertilizers in olive orchards, the new planting systems linked to irrigation methods, together with the incidence of olive pests and diseases on the production, on the olive oil composition and quality will be reviewed.

Planting Systems and Irrigation

Olive trees are cultivated in three main olive-farming systems: i) traditional/low density olive groves (Figure 3.3A); ii) intensive olive groves (Figure 3.3B); iii) and high density or super intensive olive groves, also known as hedgerow olive orchards (Figure 3C) [20].

Figure 3.3. Different olive-farming systems. A – Traditional/low density olive grove; B – intensive olive grove; C – super intensive or hedgerow olive grove.

Traditional olive groves have usually 100 to 200 trees ha^{-1} (Figure 3.3A), are rain fed and, in some cases, harvest is still manual. These groves are usually composed by large and old trees, with several decades and, in some cases, even centenary olive trees, making mechanical harvest difficult. In some regions this kind of olive-farming system is being gradually abandoned or replaced by the two other more modern olive-farming systems. Intensive plantations are mainly irrigated, have between 200 and 550 olive trees ha^{-1}, and harvest is usually made mechanically (Figure 3.B). In hedgerow olive orchards olives are exclusively collected by mechanical means to turn harvest as profitable as possible, being the olive trees irrigated following deficit irrigation programmes. These kind of olive orchards contain more than 1500 olive tree ha^{-1} (Figure 3.3C) [21]. Additionally, olive groves can be classified according to their production system: conventional olive groves and organic olive groves.

Planting system and olive oils quality and composition

Studies comparing the influence of different olive-farming systems on the olive oil composition and quality are scarce. These studies are difficult to implement since many variables interfere in the outline, starting from olive varieties, irrigation regimes, soil composition and the standardization of olive trees density. Therefore, most studies on olive-farming systems focus mainly in trials to test the adaptation of different olive cultivars

to high density olive orchards, irrigation and fertilization optimization, as well as to observe production yields and the quality and composition of the obtained olive oil from specific olive cultivars.

By studying low density plantations (from 51 to 156 trees ha^{-1}) in traditional olive groves of cv. Chemlali, Guerfel et al. [22] verified that planting density is a key aspect to be considered for the quality and composition of olive oils in arid areas. These authors verified that tree densities from 100 to 156 trees ha^{-1} result in olive oils with higher oxidative stability, as observed in Figure 3.4. Authors verified higher content of chlorophylls and carotenoids, oleic acid and total phenols in densities of 100 trees per ha, contributing to the observed effects, but the specific coefficients of extinction at 232 and 270 nm (K_{232} and K_{270} respectively) increased, eventually threatening extra-virgin olive oil classification.

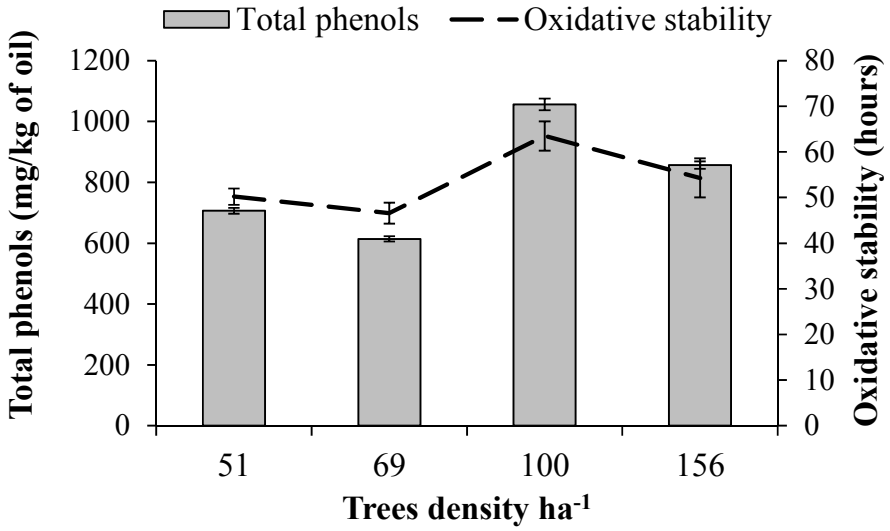

Figure 3.4. Oxidative stability (hours) and total phenols content of olive oils from cv. Chemlali cultivated in traditional olive groves under different olive tree densities (data updated from [22].

Regarding intensive olive groves, adaptation of cv. Arbequina in different planting densities (from 179 to 385 trees ha^{-1}) was studied by Tous et al. [23]. These authors verified that, at full production, an average of more than 5000 kg of olives per ha were obtained, with the highest economic return with 312 trees ha^{-1}. The same group also studied adaptation of different olive cultivars (Arbequina-i 18, Arbosana, Canetera, Joanenca, Koroneiki, and Fs-17) to super intensive olive orchards with a plantation density near 2500 trees ha^{-1} [24]. The results clearly demonstrated that Arbequina-i 18 is the most adapted to this olive-farming system, producing nearly 24000 kg of olives per ha,

followed by Arbosana and Canetera [24]. According to these authors, an increased plant density is mainly affected by light competition: being cultivated closely light competition is high, reducing crop yield [23].

In high density olive orchards (1250 trees ha^{-1}) Allalout et al. [25] studied olive oil quality and composition of several olive cultivars (Arbequina, Arbequina-i 18, Arbosana, and Koroneiki). The authors verified that Arbequina was the cultivar with lower oil content, while in the extreme opposite was Koroneiki. Nevertheless, all cultivars reported good quality indices, being classified as extra-virgin olive oils.

Plant density seems to have a crucial effect on olive oil yield but it appears to have no influence in the olive oils quality. Meanwhile, some aspects need to be taken in consideration because if plant densities are extremely high it can affect plants physiological development. The competition for soil mineral nutrients as well as for light exposure can influence negatively the production of olive fruits, affecting yield, and possibly having repercussions at quality and composition of the obtained olive products. Furthermore high density olive-farming plantations are irrigated, which also influence productivity, quality and composition of olive oil, as we discuss ahead in this chapter.

Organic vs. conventional olive oil: quality and composition

Organic agriculture is increasing in all crops worldwide, mainly due to consumers' awareness about pesticides residues in food products. Indeed, conventional agriculture, due to the application of phytosanitary products, leads to pesticide residues in olives [26], passing them to olive products [27, 28]. In 2010, nearly half a million ha were already dedicated to organic olives production [29]. A consumers' satisfaction study in Greece demonstrated that 78% of consumers were satisfied with organic olive oil, describing its beneficial health aspects as the most competitive advantage [30].

Comparing productivities of conventional and organic olive groves, higher productions are usually observed in conventional groves. Ninfali et al. [31] verified that both Leccino and Frantoio olive varieties produce more in conventional groves (5514 and 4721 kg ha^{-1}, respectively) than in organic ones (4125 and 3494 kg ha^{-1}). The same authors also verified higher pest incidence in organic olive groves (10%) than in conventional ones (4%).

In which respect to olive oil quality from organic and conventional olive groves, data reported in literature is sometimes contradictory. Gutiérrez et al. [32] reported that organic olive oils possess higher quality when compared to conventional ones, while

Ninfali et al. [31] were unable to found the same trend during a 3-year consecutive study. In the study performed by Gutiérrez et al. [32] the olive oil from cv. Picual displayed better results in all quality indices evaluated (free acidity, peroxide value and K_{232}). The differences found between both cultural practices were more pronounced with olives maturation, with higher degradation in conventional olive oils. Sensorial evaluation was also better scored in organic olive oil than in conventional ones, in particular the positive attributes green fruit, mature fruit, bitter and spicy [32]. In which respect to Frantoio and Leccino olive cultivars [31], the same quality parameters were not consistent since in some years conventional olive oils display significant higher quality, and in other years the same trend is not observed. In the sensorial evaluation, no marked differences were observed between both agricultural practices in cv. Frantoio. However, conventional olive oils from cv. Leccino were fruitier, pungent, bitter, and exhaled higher cut grass sensations than olive oils extracted from organic olive fruits [31].

Regarding olive oils composition, and according to Gutiérrez et al. [32], organic olive oils are richer in antioxidant compounds, such as α-tocopherol, *o*-diphenols and phenolic compounds, while Ninfali et al. [31] did not found significant differences in the same components when the three years of study were compared. The same authors' found no differences in the antioxidant activity displayed by organic and conventional olive oils while organic cv. Picual olive oils were reported as having significantly higher oxidative resistance than conventional olive oils, partially attributed by the high content of antioxidant compounds, by Gutiérrez et al. [32]. Sterolic fractions of both organic and conventional olive oils were similar, while the fatty acids profiles differ mainly in oleic and linoleic acids contents. Oleic acid content was higher in organic olive oils while linoleic acid content was superior in conventional olive oils [32, 33].

Irrigation and olive oils quality and composition

Irrigation is already a common practice in intensive, super intensive or hedgerow olive orchards and, in minor proportion, in traditional olive groves. It is clear that irrigation practices increment considerably olive fruits as well as olive oil production yields [34-41]. But do irrigation practices influence olive oils quality and composition? One approach to context this question could be given in Figure 3.5, which schematizes the general effect of olive trees irrigation on quality, composition, stability and bioactivity of olive oils.

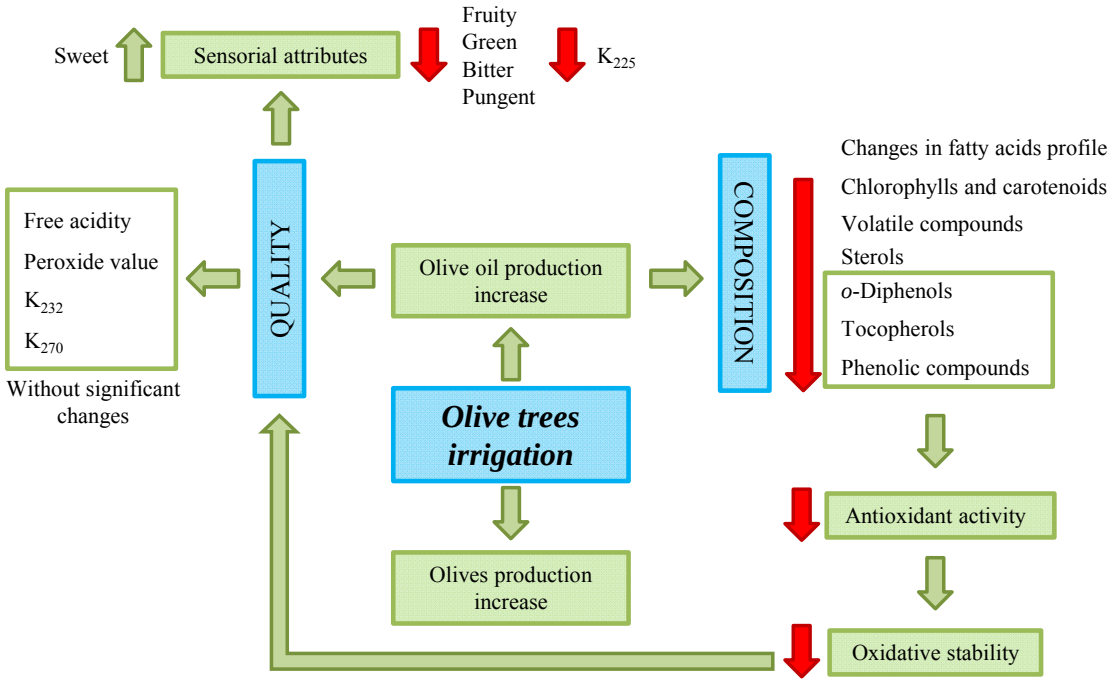

Figure 3.5. Global impact of irrigation practices on olives and olive oil productivity, quality and composition.

As illustrated in Figure 3.5, olive trees irrigation considerably affects olive oils composition, sensorial component and, in minor extension, olive oils quality. Starting from quality, several studies highlight slight increases in free acidity and higher peroxide formation in olive oils extracted from irrigated olives, but without statistical meaning [37, 41-43]. Regarding specific extinction coefficients at 232 and 270 nm (K_{232} and K_{270}, respectively) some studies report a slight decrease in these quality parameters while others report no significant differences with different irrigation regimes. However, olive oil classification is not endangered by irrigation practices since all olive oils continue within the legal limits of extra-virgin olive oils [44]. Regarding sensorial characteristics, olive oils extracted from irrigated olives are less fruity, pungent, and bitter, and the higher the quantity of water applied to olive trees, the lower the score of those positive attributes [41-45]. By contrast, the same studies report that olive oil becomes sweeter due to the lower grade of bitterness and pungency of the oils obtained from irrigated trees. The bitterness index (K_{225}) is in accordance to these observations, being negatively correlated with the amount of water applied to olive trees, a fact also linked to the phenolic composition of olive oils. Almost all studies which conducted trials with different irrigation regimes determined the phenolic content of the extracted olive oils. The results are clear and concise: irrigation reduces drastically the phenolic compounds in olive oils [35, 39, 41-45]. These results were also corroborated by Romero et al. [46] and Servili et al. [47] that

verified richer qualitative and quantitative profiles of phenolic compounds in olives from different regulated deficit irrigation programs. Besides phenolic compounds, chlorophylls, carotenoids, tocopherols [43], sterolic fraction [41], and volatile composition [41, 47] are reduced in olive oils extracted from irrigated olives. Changes in the fatty acids profile were also observed, generally with higher contents of oleic acid being reported in olive oils from irrigated trees and higher linoleic content in olive oils from non-irrigated olive orchards [43, 47].

The changes inflicted by irrigation practices in the composition of olive oils interfere with their bioactivity, reducing the antioxidant potential, since olive oils from irrigated olives lose important antioxidant compounds, including phenolics, *o*-diphenols and tocopherols. This loss of antioxidant potential by olive oils is critical, affecting their stability and resistance to oxidation.

Indeed, oxidative stability loss is directly correlated with the amount of water applied in the irrigation regimes, with lower oxidation resistance as water increases. Berenguer et al. [42] reported oxidative stability losses between 10% and 71% in Arbequina olive oils with an irrigation treatment of 140% and 104% of ETc (olive tree evapotranspiration), respectively. Other authors reported, for the same olive cultivar, losses between 17% [43] and 27% [46] in studies of linear irrigation strategies and deficit irrigation strategies, respectively. Gómez-Rico et al. [44] also reported oxidative stability losses between 4% and 36% in Cornicabra olive oils.

Overall, olive oils extracted from irrigated olive fruits display good quality after extraction, allowing them to be classified as extra-virgin olive oils. However, since olive oil composition is severely affected, particularly being depleted of antioxidant compounds, their storage stability could be reduced. This is a very important aspect since, once stored and bottled, olive oils from irrigated olives may pass through preservation deficiencies and may arrive to consumers already degraded and rancid due to autoxidation [48]. Furthermore, these aspects may raise authentication concerns, since labelled extra-virgin olive oils may arrive to consumers with sensorial defects, being the product inconsistent with the denomination labelled.

Fertilization

One of the goals of olive trees fertilization is improving and increasing olives production and yields. In each campaign, substantial amounts of mineral nutrients are lost in olive groves due to soil lixiviation and erosion, irrigation, tillage practices, fruits removal,

branches removal by pruning practices, and natural drop of olive leaves. Therefore, it is essential to provide to the crop the necessary mineral nutrients in order to assure adequate growth and yields in the following years. The most modern olive-farming systems with intensive production foment an increased consumption of mineral nutrients, needed to ensure a proper production. According to Rodrigues et al. [49], olive tree responds markedly to the application of nitrogen (N), mainly in low fertility soils. Also, the application of potassium (K) leads to an increase in olive trees growth and olives yield in fertilized olive groves. Indeed, 60% of K is located in olive fruits [50], which are annually removed with the harvest, being its reposition essential. Phosphorous (P) is another mineral nutrient applied to olive trees, being essential to root growth and plant tissues. Boron (B) and magnesium (Mg) are other two mineral nutrients applied to olive trees [51, 52].

Nevertheless the application of fertilizers is not free of consequences to olive production and the quality and composition of the obtained olive oils. The most related cases are nitrogen over-fertilization [53], as excessive doses reduce flowering, flower quality and ovule longevity [54] as well as fruit set, reducing fruit load [50] and consequently also olive oil production. Excess of nitrogen has also influence in olive oils quality and composition [55, 56] as described in the next section of the present chapter. Therefore, a proper diagnostic of olive tree mineral status is essential, including foliar diagnosis [57], as well as an optimization in the application of fertilizers according to the olive tree needs [58].

Fertilization and olive oil quality and composition

Most studies regarding fertilization effects in olive oils quality seem to be in agreement as to no major interferences are observed. A good example is that the application of different doses of N-P-K (4N-1P-3K complex fertilizer) in olive orchards submitted to irrigation did not cause quality changes in olive oils from cv. Manzanilla de Sevilla [59]. Quality parameters (free acidity, peroxide value, K_{232} and K_{270}) remained low with the increasing dose of fertilizers. However, these observations do not extendable to the olive oil composition, with differences observed at the molecular level. The main components affected are the phenolic compounds [55, 56, 59, 60]. According to the different treatments applied to olive trees in different years, cv. Manzanilla olive oils lost between 16 and 32% of phenolic compounds comparatively to control treatments without application of fertilizers [59, 60]. Regarding conditions of N over-fertilization, olive oils from

cv. Picual lost between 16 and 51% of phenolic compounds [55, 56]. With a considerable decrease in phenolic compounds, several aspects of olive oil are expectedly affected: being strong antioxidant compounds, olive oil becomes less protected from oxidative agents. This hypothesis was confirmed in the above mentioned studies, since the oxidative stability in both olive oils from cvs. Manzanilla and Picual was considerably reduced [55, 56, 59, 60]. Another aspect directly related with the reduction of phenolic compounds is the olive oil natural bitterness (measured by K_{225}). K_{225} parameter reduces with fertilization, therefore the sensorial score is compromised, with positive attributes like bitterness, spicy and pungent sensations being considerably reduced in olive oils due to phenolics loss.

Tocopherols (mainly α-tocopherol) and pigments (chlorophylls and carotenoids) appear to have an opposite tendency. Nitrogen application significantly increases the amounts of α-tocopherol in olive oils, while for β- and γ-tocopherols no significant changes are observed [56]. Fernández-Escobar et al. [56] also verified an increment in chlorophylls and carotenoids with increasing N doses, but without statistical meaning. Regarding fatty acids composition of olive oils, the changes observed due to N application appears to be related to olive cultivar as only merely small changes were observed in cv. Picual [56] but some important modifications were reported in cv. Manzanilla olive oils with different fertilization treatments [59]. The PUFA fraction increased significantly with the application of higher doses of N-P-K, mainly due to the increase in linoleic acid, while MUFA decreased significantly due to the reduction in oleic acid representation. These observations were accompanied during two consecutive years, which corroborate the influence of fertilization in the fatty acids profile of olive oils.

In a long term, just as discussed in the section “Irrigation and olive oils quality and composition” of the present chapter, the changes inflicted by fertilization in olive oils composition may be critical for its preservation. Oxidative stability is reduced due to antioxidant compounds loss, and the unsaturation degree increases, which turn them more prone to oxidation.

Olive Pests and Diseases

Olive pests and diseases are responsible for serious crop losses and quality degradation of olive products, including olive oil. According to Bueno and Jones [61] 15% of olives production is lost each year due to pest incidence. Nowadays, due to the increase of olives cultivation and their expansion in new areas of the globe, olive pests

spread across olive producing areas, raising damages. On this chapter we will focus on two of the most important olive pests: the olive fruit fly (*Bactrocera oleae* (Rossi); Diptera: Tephritidae), and the olive moth (*Prays oleae* Bern.; Lepidoptera, Plutellidae), and in two of the main diseases that affect olive tree: the olive anthracnose (*Colletotrichum* sp.) and verticillium wilt (*Verticillium dahliae* Kleb.) (Figure 3.6).

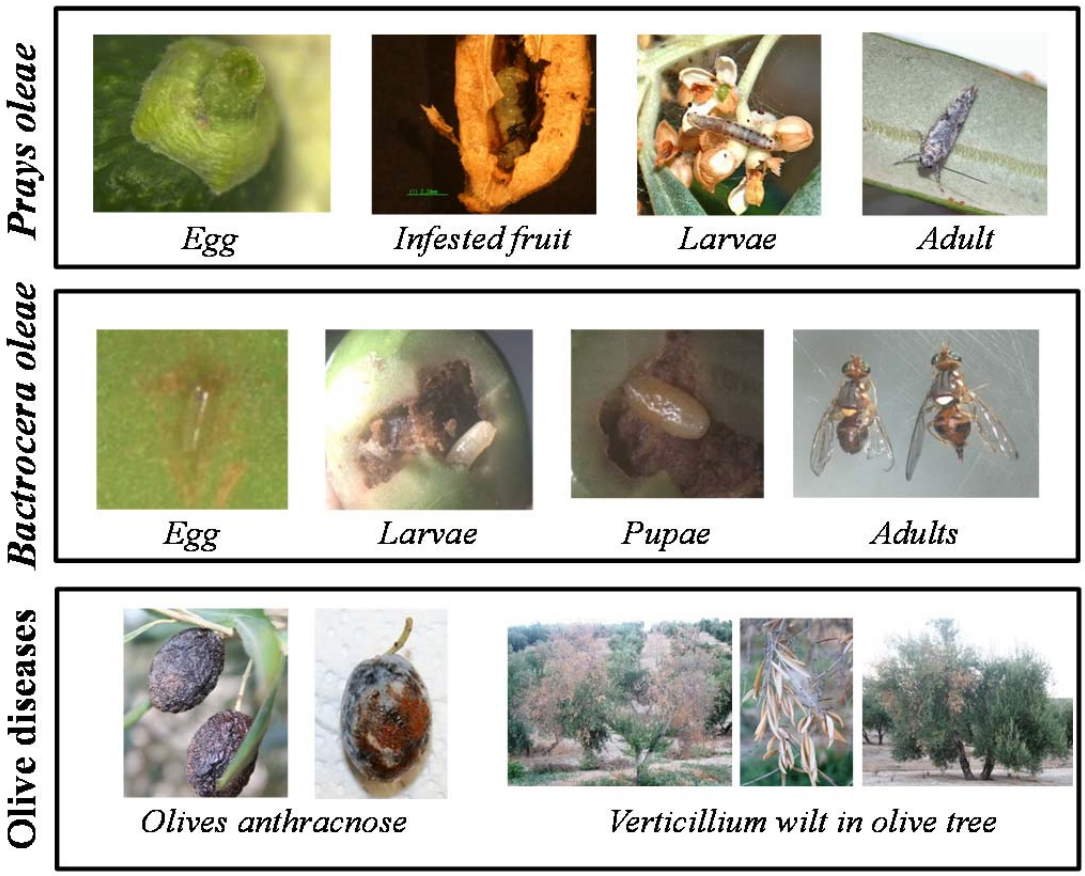

Figure 3.6. Main olive pests and diseases that affect *Olea europaea* L..

Olives production losses caused by olive pests and diseases

Olive moth has important economic effects on olives production, affecting therefore olive oil quality and amounts. Olive moth cause high levels of fruit drop due to burrowing into fruits in the early stages of development. The larvae of antophagous generation feed on the seed inside olive stone and then exit the fruit, increasing fruit drop, mainly in the month of September, but also during fruit maturation. In a long-term study, Ramos et al. [62] verified the consequences of different levels of incidence of olive moth in southern Spanish olive groves. These authors observed that high levels of olive moth attack (more

than 40% of fruit drop) occurs approximately every three years and cause an average loss of 131792 tons of olives. These losses entailed an economic damage of about 79.5 million € at that time. With moderate levels of attack (average of 29% of fruit drop), 50332 tons of olives were lost, with a damage of 30.4 million €. With low level of attack (average of 10% of fruit drop) 18623 tons of olives were lost with an economic prejudice of 11.2 million € [62]. These results highlight the importance and threat that this olive pest represents to olive crop.

Even regarding olives production losses, after olive moth attack, olives are susceptible to olive fly infestation. The adult female of this dipteran lay her eggs on olive fruits, and the larvae feed on olive pulp, consuming from 3 to 20% of pulp according to the olive variety [63, 64], and creating galleries inside the fruit. When the larvae are ready to pupate, that can occur inside the fruit or in the soil, larvae opens an exit hole in fruit for larvae or adults leave. Similarly to what happens with olive moth, olive fly infestation also causes fruit drop from the tree but in lower amounts comparatively to olive moth, as witnessed by Bento et al. [65]. In no chemical sprayed olive groves, these authors report a fruit drop of about 19% of olives per tree. The most critical data was checked when authors assessed the infestation level of olives at the harvest moment, and verified that 84% of the fruits were infested.

Concerning olive diseases, anthracnose is considered the most destructive disease worldwide in olive crop. Firstly reported in Portugal [66] this disease rapidly spread to all olive producing areas in the globe. Olive anthracnose is caused by several causal agents, including *Colletotrichum acutatum*, more prevalent and aggressive than other species, as example *C. gloeosporioides* [67, 68]. Anthracnose causes fruits dehydration, fruit rot and mummification [69] as observed in Figure 6. This pest also aids in the spread and entrance of the causal agent in olive fruits. In years when *B. oleae* populations and infestation levels are high, olive anthracnose can cause up to 100% of fruit production losses [70, 71]. According to Moral et al. [72], only in Spain, about 70 million € are lost each year due to anthracnose prevalence.

Other disease with global importance is verticillium wilt. This is a vascular disease caused by the soil-borne fungus *Verticillium dahliae* Kleb. [73]. Similarly to olive anthracnose, verticillium wilt can be found in almost all olive producing regions in the world (Table 3.1).

Table 3.1. Verticillium wilt incidence in olive groves and disease incidence in olive trees cultivated in different olive producing countries.

Country	Incidence in olive orchards	Disease incidence in olive trees	References
Algeria	90%	12%	[74]
Greece	-	2-3%	[75]
Israel	-	12-50%	[76]
Italy	6.2-35.8%	-	[77]
Morroco	60%	10-30%	[78]
Spain	39.5%	-	[79]
Syria	-	0.85-4.5%	[80]
Turkey	35%	3.1%	[81]

This is a very dangerous disease since it can attack partially the trees or it can kill the entire olive tree. Another worrying aspect about this disease is the high infestation levels recorded at nurseries (about 50%), as witnessed in Italian olive nurseries by Nigro et al. [77]. Its symptoms are partial leave defoliations, inflorescences necrosis and branches dieback. This disease influence considerably olive trees development and growth, as well as fruit production affecting considerably olive oil production.

Olive pests and diseases on olive oil quality

Studies regarding the effect of olive moth on olive oil quality are inexistent, once attacked fruits drop to the soil and this fruits are not recommended to be used for oil extraction. However, literature highlights accordingly the nefarious effect of olive fruit fly infestation in olive oil quality. Olive oils quality degradation is directly proportional to the amount of olive fruits infested by olive fly and mainly olive fruits with exit holes [82-85]. Two main chemical processes occur in infested olives, hydrolysis and oxidation. During its development, olive fly larvae consume considerable amounts of olive pulp, creating galleries in the fruit. This consumption and tissue destruction lead to enzymatic reactions between lipases and triglycerides, increasing the amounts of free fatty acids in the pulp and therefore olive oils free acidity (FA). By other hand olive pulp become oxidized, by the entrance of exogenous elements as air, cold/heat, water and several types of microorganisms, mainly bacteria and fungi that provoke fruit rot, through the hole created in the fruits. Olive oils extracted from infested olives report higher peroxide value (PV), due to the compounds formed during primary oxidation, mainly hydroperoxides [48] when compared with olive oils extracted with healthy fruits. Others quality parameters that allow monitoring oxidation occurrence also report higher values in olive oils extracted from

infested fruits: K_{232} and K_{270} (which measure respectively the presence of primary and secondary products of oxidation).

As already mentioned, exit holes created by olive fly are an infectious window, since many pathogenic agents enter by those sites. *Camarosporium dalmaticum* (Thüm.) Zachos & Tzav.-Klon, and *Botryosphaeria dothidea* are some examples of pathogenic agents that cause fruit rot and that are correlated with olive fly infestation [86, 87]. *C. acutatum* incidence increase in years of high olive fly populations and infestation levels. Olive oil extracted from olives with anthracnose reports lower quality compared to healthy olives and lower quality than olives infested by olive fly [88]. Furthermore, an increase in bacteria, yeasts and moulds is observed in olives infested by pests leading to an increase in the free acidity of olive oils especially if olives are stored prior to oil's extraction [89].

Sensorial characterization is a very important component of olive oils quality and classification. Regarding sensorial component, olive oils from infested fruits by pests and diseases have lower fruity, green, bitter and pungent sensorial attributes and increased defects are noted, mainly fusty, musty, winey, grubby and many times rancid [83, 84, 90]. Many defects arise from degradation and fermentative processes of olives. The perceived sensorial component is mainly affected by the changes observed in the volatile compounds released by the olive oil. In fact, olive oils from fruits infested by pests and diseases report lower green and cut-grass sensations due to loss in (*E*)-2-hexenal, one of the main volatiles responsible for those notes [91, 92], and one of the most abundant volatile compounds in olive oils [93]. The increase in fusty and musty defects is due to microbial contamination of the olive fruits and winey defect due to fermentative processes that release high amounts of alcohols (methanol, ethanol and isoamylic) [94] and acetic acid [95].

The conjunction of chemical degradation due to pests and diseases incidence together with the sensorial component gives us an idea of the changes inflicted in olive oils and the overall quality that they display. Olive oil quality is severely affected and fresh olive oils cannot be classified as extra-virgin olive oils, being considered virgin, or even lampante olive oils [96, 97] due to both quality and sensorial defects.

Olive pests and diseases on olive oil composition, stability and bioactivity

Olive oil minor components are directly implicated in the olive oil quality and its properties as well. A very important factor that enhances the deleterious effects of pests and diseases in olive oils composition is the maturation process. Several authors studied

the composition of olive oils extracted from infested fruits (mainly olive fly) and they highlight the fact that mature olives are characterized by a poorer composition in several olive oil components than greener ones and the intrinsically related quality is also severely affected [83, 84, 95]. Some of the most affected compounds include the fatty acids, mainly unsaturated fatty acids, sterols (β-sitosterol), tocopherols (α-tocopherol) and, in great extent, phenolic compounds (Figure 3.2).

In this section we will focus mainly in olive fly due to the scarce and many times inexistent information about the impact of olive moth, anthracnose and verticillium wilt in the composition of olive oils. Therefore, and starting by olive oil pigments, both chlorophylls and carotenoids are severely affected by olive fly infestation. Olive oils extracted from green cv. Chemlali olive fruits lost about 73.8% and 39.2% of chlorophylls and carotenoids, respectively, when 100% of the fruits are infested [84]. Similar remarks were observed by Tamendjari et al. [95] when studying Algerian olive cultivars. This is an aspect that not only influences the composition of olive oil, but can easily have repercussions at the consumers' preference, since olive oils become less green and more yellow, an aspect which isn't appreciated by regular consumers.

Regarding fatty acids profile, the information collected from literature is not consistent. Some authors [84, 85, 95] studied the effect of olive fly in fatty acids of olive oils without noticeable t changes in the profile . However, by studying three Algerian olive cultivars (cvs. Chemlal, Azzeradj and Bouchouk), Tamendjari et al. [97] established negative correlations between olive infestation level and unsaturated fatty acids, mainly oleic and linoleic acids. The same authors report a positive correlation with saturated fatty acids according to the infestation level of olive fly. These data highlights the oxidative processes suffered by the olive fruits during infestation, which leads to a lower amount of unsaturated fatty acids in the olive oil. Furthermore, unsaturated fatty acids are proner to oxidative processes [98] than saturated ones.

Sterolic fraction (mainly β-sitosterol) and the triterpenic alcohol uvaol are affected by olive fly infestation [99]. These compounds have their content reduced with olive fly infestation level.

Besides being important minor components of olive oil, tocopherols are scarcely studied regarding the effect of pests and diseases incidence on their contents in olive oil. When studying Portuguese olive cultivars (cvs. Cobrançosa, Madural and Verdeal Transmontana), Pereira et al. [85] verified that the β-tocopherol and γ-tocopherol content in the olive oils was not affected by the infestation level. However, α-tocopherol content decreased with the olive fly infestation, with a characteristic response according to the olive cultivar, as witnessed in Figure 3.7.

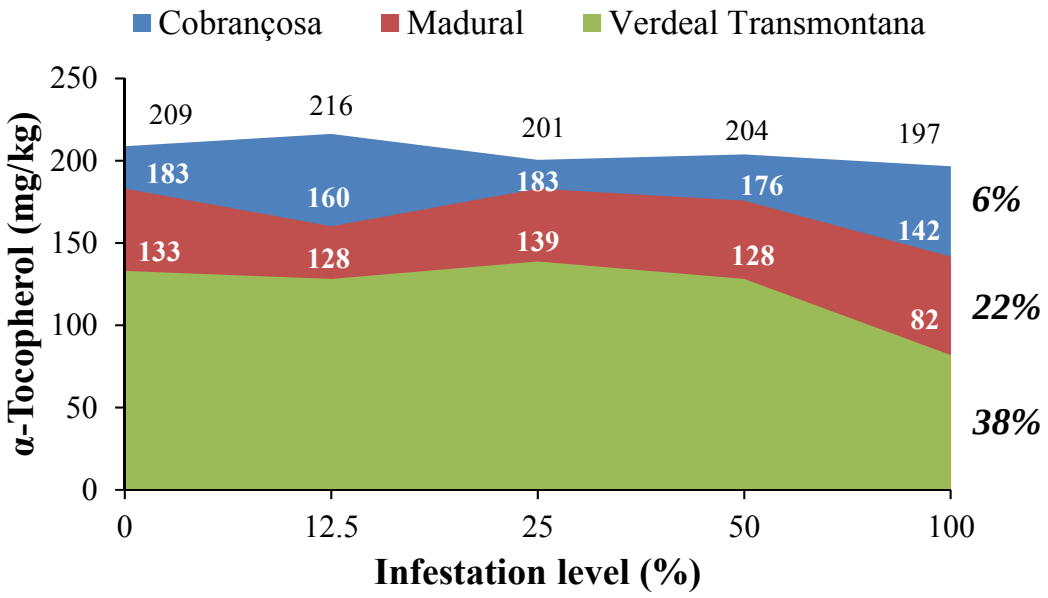

Figure 3.7. Changes recorded in α-tocopherol amounts in olive oils from cvs. Cobrançosa, Madural and Verdeal Transmontana extracted from olive fruits with different infestation levels (in bold and italic is presented the percentage of loss of α-tocopherol between 0 and 100% infestation level). Data from [85].

Cv. Cobrançosa was the one that lost lower amounts of α-tocopherol, only 6% at 100% infestation level. Meanwhile, cvs. Madural and Verdeal Transmontana reported losses of about 22 and 38%, respectively, a lost that clearly compromises the composition and quality of the olive oils obtained from these olive cultivars. The trend observed in Figure 7 for α-tocopherol is the same observed for total tocopherols, since α-tocopherol is the most abundant tocopherol in olive oils. Therefore, olive fly influences the amount of a powerful antioxidant compound with impact on the olive oil stability and therefore, as already mentioned, in the quality of olive oils.

In which concerns to phenolic compounds, literature is consistent: olive oils extracted from olive fly infested fruits are poor in phenolic compounds [82-84, 97]. Gucci et al. [82] verified that all phenolic compounds represented in Figure 2, namely hydroxytyrosol, tyrosol, 3,4-DHPEA-EA, 3,4-DHPEA-EDA, and ρ-HPEA-EDA, are negatively correlated with the percentage of olive fruits with exit holes. Only (+)-pinoresinol and (+)-1-acetoxypinoresinol maintain their contents with the increasing percentage of exit holes [82]. Regarding total phenols content, Gucci et al. [82] report a loss of about 75% of phenolic compounds when comparing cv. Frantoio olive oils from healthy olives and those extracted from olive fruits with 100% exit holes. Similar results were verified in other olive varieties: 21% and 50% respectively in Croatian olive cultivars

Istarska bjelica and Buža [83]; 83% in Tunisian olive cultivar Chemlali [84]; and between 60-68% in Algerian olive cultivars Azzeradj, Chemlal and Bouchouk [97]. The loss of phenolic compounds is induced by the olive fly larvae. Part of the phenolic compounds lost are ingested by the larvae during its development and pulp consumption, while other part of phenols are oxidized in the olive fruit and degraded by microorganisms and enzymes present in the fruit or that contaminate the fruit after adult emergence.

With all the described changes suffered by olive fruits components due to olive fly infestation, the oxidative stability of olive oils as well as its bioactivity are compromised. According to Gómez-Caravaca et al. [100] and Mraicha et al. [84], olive oil antioxidant properties are reduced with olive fly infestation, as reported in Figure 3.8A.

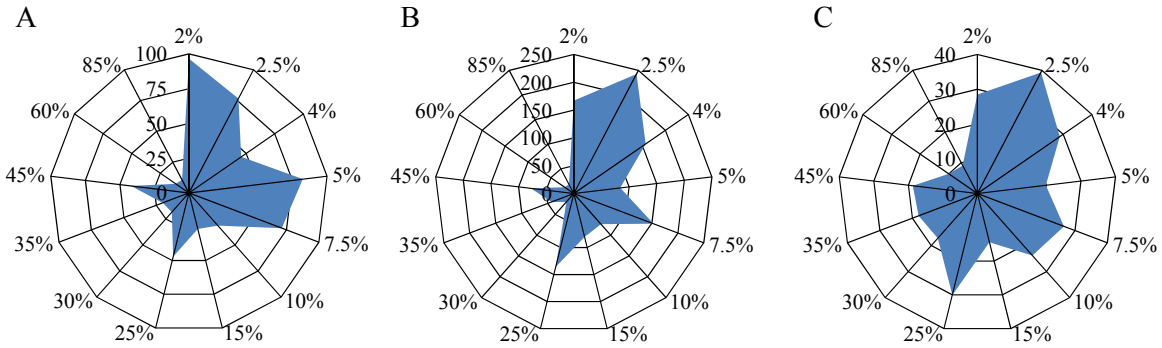

Figure 3.8. Antioxidant activity (Fig. 8A) (µg quercetin/mL of extract), phenolic content (sum of simple phenols, lignans and secoiridoids; Fig. 8B) (mg/kg of oil) and oxidative stability (Fig. 8C) (hours), of olive oils extracted from olive fruits with different infestation levels. Data updated from [100].

Antioxidant potential is reduced (Figure 3.8A) due to the loss of antioxidant compounds as it is the case of tocopherols, namely α-tocopherol (Figure 3.7), and, in higher extent, in the content of phenolic compounds as already witnessed and reported in Figure 3.8B. Olive oils extracted from olives with an infestation level below 30% report nearly three times more phenolic compounds than olive oils extracted from olives above the mentioned infestation level [100]. This scenario leads, in a final stage, to loss of oxidative stability by olive oils. Higher infestation levels are related with lower resistance to oxidation (Figure 3.8C) since lower amounts of antioxidants are available to protect fatty acids from oxidative agents, specially unsaturated ones, therefore the quality of the obtained olive oils is reduced. Below 30% of olive fly infestation, the oxidative stability is comparatively 10 hours higher than from olive oils extracted from olives with an infestation level superior than 30% [100].

Concluding Remarks

Agronomic factors influence considerably olive oils quality and composition. While irrigation and fertilization applied in proper amounts, according to the plant needs, does not affect olive oils quality significantly, it considerably affects olive oil composition. For both factors this entails important issues regarding olive oils stability and preservation during storage, reducing significantly olive oils shelf-life. Furthermore, a diversified array of functions and health properties are lost in olive oils from over-irrigated and over-fertilized olives, due to the considerable reduction in antioxidant compounds (mainly phenols, *o*-diphenols, α-tocopherol).

Regarding olive pests and diseases, olive oil is primarily affected on the economic field, since significant losses are entailed each year in olive fruits production. Olive oils quality and composition is significantly changed by olive fly, a fact that leads to reduced quality, and stability. Olive fly actions are so severe that olive oils are frequently classified as lampante olive oils.

Therefore, proper optimization of irrigation and fertilization programs need to be carried out according to the olive varieties, the geographical region, climate conditions, soil properties and most of all, plant status and needs. Regarding olive pests diseases, effective control programs and phytosanitary programs need to be implemented to ensure olive oils quality, composition, stability and properties.

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CHAPTER 4.

A review of *Bactrocera oleae* (Rossi) impact in olive products: from the tree to the table

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Abstract

Olive oil is a recognized premium vegetable oil. It's inherent chemical composition and sensory attributes make it highly appreciated worldwide. Nevertheless, olive oil quality and composition is severely compromised by several agricultural and technological parameters, among which olive pests play a key factor, particularly the olive fly *Bactrocera oleae* (Rossi) (Diptera: Tephritidae). This pest reveals oviposition preferences according to the olive cultivar grown around the world, being the cause for severe economic damages each year. The extent of such losses goes from the field and tree itself to the consumers because the composition and properties of the olive products are also strongly affected. The damages caused by olive fly, seen by an economic perspective, as well as their influence in olive products classification, quality, composition, stability, nutritional, bioactive and functional properties are discussed in the present paper.

Keywords: *Bactrocera oleae*; olive crop; economic losses; olive products; quality, composition.

Introduction

Bactrocera oleae (Rossi) (Diptera: Tephritidae), known as the olive fruit fly, or simply as olive fly, is a monophagous frugivorous pest since it feeds exclusively from *Olea* species. Among *Olea* species, *O. verrucosa*, *O. chrysophylla*, *O. ferruginea* and, more universally, *O. europaea* trees are attacked by this dipteran (Daane and Johnson, 2010). Adult females of *B. oleae* lay their eggs inside olives, right beneath olive epicarp, proving direct access to food to larvae just after egg emergence. In laboratory conditions, a single female can lay about 10 to 40 eggs a day and between 200 and 500 eggs in a lifetime (Zalom *et al*., 2009), while in nature a single female can oviposit about 12 eggs a day and about 200-250 eggs in a lifetime (Mavragani-Tsipidou, 2002). Oviposition sites are easily detected at naked eye, since a brown spot appears after egg oviposition. Olive fly eggs do not develop below 7.5-10 ºC and are unviable above 30-32 ºC (Tsitsipis, 1977). Under optimal conditions, eggs can develop in 1 day, giving origin to larvae that feed on olive mesocarp, creating galleries inside the fruit as larvae moves. Larvae are classified according to their developing stage as L1, L2 and L3. For larvae development, temperatures cannot be set below 10-12.5 ºC nor above 30-32 ºC (Tsitsipis, 1977). Under optimal conditions, larvae of *B. oleae* can fully develop in 8 days, with an interval from 8 to 37 days, consuming from 45 to 150 mg of olive pulp (Neuenschwander and Michelakis, 1978; Neuenschwander and Michelakis, 1979; Tsitsipis, 1977). Once ready for pupae formation, larvae from *B. oleae* open an exit hole in the olive epicarp and either escape from the fruit to pupate in the soil or pupates inside the fruit and open an exit hole for the adult. Under optimal conditions pupae can develop in 9 days, ranging from 9 to 49 days (Neuenschwander and Michelakis, 1979; Tsitsipis, 1977). Contrarily to larvae, adults have diversified sources of nutrients for their diet, feeding on insect honeydews, nectar and pollens from the available plants, fruits exudates, as well as in bird feces, bacteria and yeasts (Daane and Johnson, 2010). The number of olive fly generations per crop varies according to different factors: geographical region, agronomic and climatic conditions, quality of the fruits, among others. In Middle East three to five generations can be observed while in Europe two to five generations of this pest can be developed.

The magnitude of damages caused by *B. oleae* in olive crop is substantial (Figure 4.1), justifying the application of control measures.

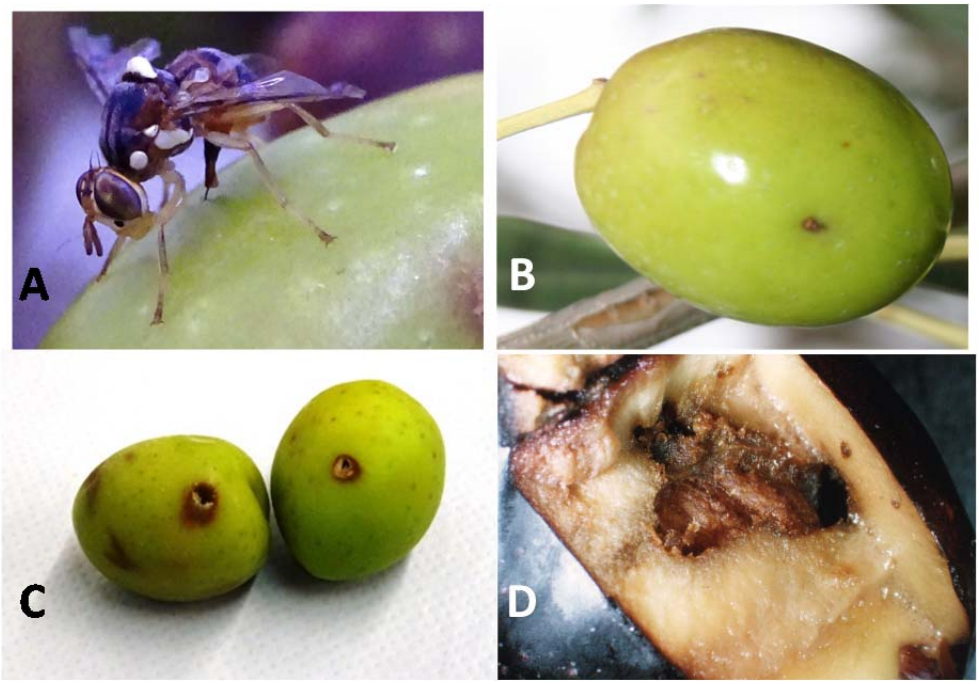

Figure 4.1. Olive fly impact in olives: olive fly ovipositing (A); olive fly oviposition site (B); exit holes in olives caused by olive fly larvae (C); damages caused in olive pulp by olive fly larvae (D).

Conventionally, pesticide sprays are the most common strategy, with negative impacts on environment and consumers health once pesticide residues could remain in olive products (Amvrazi and Albanis, 2009). Nevertheless, more friendly production schemes are increasing, making use of cultural, biotechnical, physical and biological control strategies. However many of these strategies are insufficient to control *B. oleae* populations and infestation levels.

Despite all efforts to control olive fly, this is still one of the most destructive and hazardous pests to olive crop, causing severe and irreparable losses (Figure 4.2).

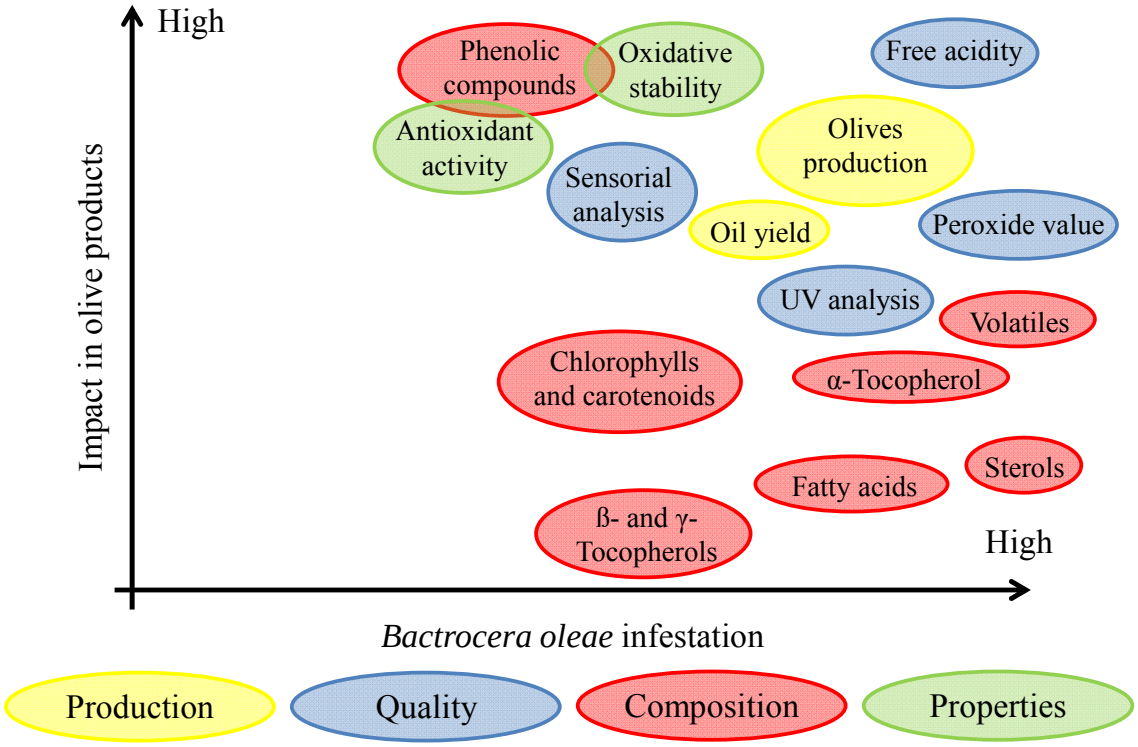

Figure 4.2. Olive fly infestation: major chemical and physical characteristics contributing to olive products quality loss.

The variation observed in *B. oleae* damages between years, regions and olive cultivars are important factors that can be taken in consideration for olive fly control programs.

Cultivar susceptibility

Despite being a key pest in olive crop, some cultivars are less susceptible towards *B. oleae* adult females. Such lower susceptibility is geographically specific, and according to the available olive cultivars in each region. Table 4.1 reports the infestation levels of different olive cultivars from different geographical origins around the Mediterranean Basin and California.

Table 4.1. Susceptibility of olive cultivars from different countries where *B. oleae* is present.

Country	Region	Cultivars	Infestation level (%)*	Reference
Croatia	-	Buža	17	Koprivnjak *et al*. (2010)
		Istarska bjelica	21	
Greece	Agios Mammas, Chalkidiki	Chondrolia Chalkidikis	75.4	Navrozidis *et al*. (2007)
		Kalamon	53.6	
		Koroneiki	25.6	
		Lianolia	27.2	
Italy	-	Bardhi Tirana	8.5	Iannota *et al*. (2001)
		Carboncella di Pianacce	9.5	
		Carolea	22.5	
		Cassanese	24.5	
		Cucco	26.5	
		Dritta di Moscufo	11.0	
		Gentile di Chienti	9.5	
		Giarraffa	23.5	
		Intosso	31.0	
		Kalinjot	23.0	
		Kokermadh i Berat	10.0	
		Leccino	20.0	
		Maiatica di Ferrandina	12.5	
		Mixan	11.5	
		Nocellara del Belice	23.0	
		Nociara	9.5	
		Picholine	24.0	
		Santa Caterina	23.5	
	Calabria (Southern Italy)	Bardhi Tirana	28.3	Iannota *et al*. (2007)
		Carboncella di Pianacce	44.7	
		Carolea	53.3	
		Cassanese	36.7	
		Gentile di Chienti	48.8	
		Giarraffa	55.0	
		Nocellara del Belice	43.5	
		Nociara	49.7	
		Picholine	46.5	
		Tondra nera dolce	19.2	
Jordan	-	Ascolano	< 50	Al-Zaghal and Mustafa (1987)
		Nabali	< 6	
		Nasohi	< 30	
		Rase'e	> 50	
		Kheli	< 30	
		Shami	> 50	
		Santa	> 50	
Montenegro	Bar	Žutica	70-99.4	Perović and Hrnčić (2013)
Portugal	Mirandela	Cobrançosa	58.3	Gonçalves *et al*. (2012)
		Madural	88.2	
		Verdeal Transmontana	70.0	
Syria	Homs	Aldeibli	24	Al-Salti *et al*. (2011)
		Alkhudairi	0	
	North-Western Syria	Djilt	30-40	Saour and Makee (2004)
		Ziety	57.4	
Turkey	Izmir	Ayvalik	< 40	Gümusay *et al*. (1990)
		Çakir	50 – 60	
		Çilli	> 60	
		Domat	40 – 50	
		Memecik	> 60	

Table 4.1. (continued).

U.S.A.	California	Arbequina	< 5	Burrack and Zalom (2008)
		Frantoio	< 5	
		Koroneiki	< 5	
		Leccino	< 5	
		Manzanillo	15-20	
		Mission	50-60	
		Sevillano	20-30	

*when available, data from the last harvest assessed in each study.

It can be observed that under the same geographical conditions, some of cultivars are preferred by the olive fly to oviposite. This information should be taken in account in the design of new olive plantations (Iannota *et al*., 2007).

In order to reveal the mechanisms of choice of *B. oleae* females to oviposit in different olive cultivars, studies on the interactions between the olive tree and olive fly have increased in the last years. Three group factors are being currently exploited in order to achieve plausible answers for the displayed variable susceptibility: physical, chemical and molecular aspects. Each factor must not be accounted separately and independently, since the answer to the olive fly preferences seems to reside within the interaction and correlation of the three mentioned factors.

Physical factors

Fruit size, weight, and volume, fruit epicarp parameters (break force, elasticity, firmness, and break energy), and fruit colour are among the most studied physical factors. Neuenschwander *et al*. (1985) were amongst the pioneers in the study of olive cultivars susceptibility. These authors verified that olive flies prefer bigger fruits, justifying why some table olive cultivars are firstly attacked than oil cultivars, usually with smaller fruits. This fact is even interesting since *B. oleae* larvae are more protected inside bigger fruits from the action of some of its natural enemies. Other fact is that olive fly prefers greener comparatively to ripened fruits (Vlahov, 1992). The dark coloration of ripe fruits could confuse female flies in drupe recognition, reducing infestation levels in olives with advanced maturity stages (Iannota and Scalercio, 2012). This fact was also confirmed by Katsoyannos and Kouloussis (2001) who observed different attractiveness of fly adults according the traps coloration. As to the epicarp physical properties, the lower skin elasticity and higher skin firmness of cv. Verdeal Transmontana, was pointed out as an important factor of its high susceptibility in comparison to other Portuguese cultivars

(Gonçalves *et al*., 2012). The combination of these parameters facilitates oviposition of *B. oleae* females, since the epicarp is more firm facilitating skin penetration.

Chemical factors

The composition of olives may also give some answers to cultivar susceptibility, as expressed in Table 4.1. Olives composition can exert protective effects against fly attack from both outside and inside the fruits. Olives' surface, particularly its composition in natural waxes, appears to exert a repellent action towards *B. oleae* females. Olives' waxes are cultivar dependent and the application of waxes solutions in olives nearly cut by half *B. oleae* oviposition comparatively to control olives in Greek cultivars (Kombargi *et al*., 1998). Scarpati *et al*. (1996) found ammonia and styrene in olives and leaves surface, two olive fly attractants, produced mainly from the metabolism of microbal flora and therefore dependent to the epiphytic community.

Volatile compounds emitted by olive tree (fruits and leaves) seems to possess a particular importance in the olive fly host selection for oviposition (Aluja and Mangan, 2008) being perceived by *B. oleae* antenna sensilla (Liscia *et al*., 2013). This could justify different cultivars susceptibilities to olive fly once each cultivar emits a different array of volatile compounds (Malheiro *et al*., 2013). On the other side, the bacterial community of olive fruit, like *Pseudomonas putida*, could also emit volatiles receptive to the olive flies palps sensilla (Liscia *et al*., 2013). In oviposition tests Scarpati *et al*. (1993) revealed that (*E*)-2-hexenal acts as repellent while toluene and ethylbenzene, both volatiles emitted by the olive leaves, act as oviposition promoters.

The interaction between phenolic compounds and enzymes is reported as defense mechanisms to phytophagous insect attacks. In particular, β-glucosidase induces the cleavage of oleuropein, the main phenolic compound in drupes and olive leaves (Pereira *et al*., 2007; Vinha *et al*., 2005), producing highly reactive aldehyde molecules. Oleuropein enzymatic cleavage is higher in the less susceptible cultivar (Spadafora *et al*., 2008) and the reaction was still observed 1 hour after fly injury. β-glucosidase increases significantly its activity in attacked fruits, with a maximum activity after 20 minutes of oviposition (Spadafora *et al*., 2008) in both susceptible and less susceptible cultivars. Other studies highlight that β-glucosidase activity is higher in uninfected fruits than in infected fruits, revealing a possible olive drupe defense mechanism even before a possible insect attack (Sivakumar *et al*., 2007).

Molecular factors

The study of up-regulated genes expression resulting from the attack of *B. oleae* and its association with olive cultivars susceptibility are scarce. However, such studies could give important answers to the developed defense mechanisms of olive drupes to the olive fly attack, and the differences reported in different cultivars.

Corrado *et al*. (2012) were able to identify 196 expressed sequence tags and 26 protein spots from olives with galleries created by *B. oleae* larvae. These authors found that some molecular processes were affected by olive fly, such as stress response, phytohormone signaling, transcriptional control and primary metabolism. Furthermore these scientists, by cloning the full-length coding sequences of two genes (Oe-chitinase I and Oe-PR27), verified that these genes were wound-inducible genes activated due to *B. oleae* actions. The expression levels of these genes were considerable higher in the galleries created by *B. oleae* than in puncture fruits. Meanwhile, comparing uninfected fruits with those with stings, the expression of both genes was also considerably higher in fruits with stings (Corrado *et al*., 2012). In other works from the same authors, 28% of the expressed sequence tags were classified as possessing hydrolase activity and capability to hydrolyse *O*-glycosyl compounds, a mechanism which involve phenolic compounds and is believed to be an important defense mechanism. Other 26% expressed sequence tags were related with cation binding properties (Imperato *et al*., 2012).

World distribution of *Bactrocera oleae*

Bactrocera oleae is a key pest of *Olea europaea* L. worldwide. This insect is present in most areas where olives are cultivated for commercial purposes. Olive fly is widely distributed in the Mediterranean basin, where approximately 98% of the estimated worldwide 9.8 million ha of olive trees are cultivated (Ponti *et al*., 2009). In Europe, *B. oleae* is widely distributed in Greece and in the Crete Island, in Italy and in the islands of Sicily and Sardinia, and in the Iberian Peninsula (Figure 4.3). It is also present in Azores Islands, Balearic Islands, Cyprus, France, Malta, Switzerland, Turkey, and in Balkans region: mainly in Croatia, Montenegro, and Slovenia.

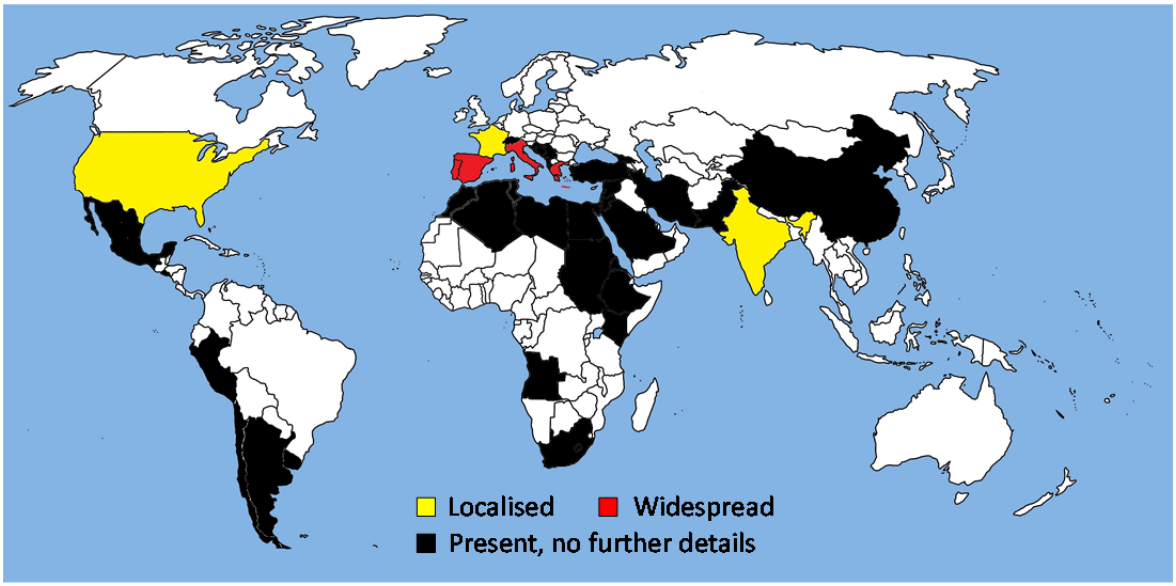

Figure 4.3. World distribution of *Bactrocera oleae* (Rossi).

The African continent is mainly affected in the Northern part, near to the Mediterranean Sea: Algeria, Egypt, Libya, Morocco and Tunisia are heavily affected by *B. oleae*. This pest is also present in Central Africa, mainly in Angola, Eritrea, Ethiopia, Kenya, and Sudan. South Africa and Canary Islands also report *B. oleae* presence (CABI, 2014).

In Asia, the Middle-East region is the most affected, being *B. oleae* present in Israel, Jordan, Lebanon, Saudi Arabia and Syria. *B. oleae* is also present in China, India, and an endemic specie of this pest was reported in Pakistan, *B. oleae* var *asiatica* (Nardi *et al*., 2006; Nardi *et al*., 2010).

In North America, *B. oleae* was accidentally introduced in 1998 in California, more specifically in Los Angeles Basin (Burrack and Zalom, 2008; Zalom *et al*., 2008). Since that time, *B. oleae* spread through the region, being present in all growing region of the state by 2004. The pest also spread to Mexico, occupying Central America.

In South America, *B. oleae* presence in Argentina, Chile, Peru and Uruguay is already a reality (Figure 4.3) (Augustinos *et al*., 2002) due to introduction of olives cultivation or production intensification. So far, Australia is one of the few areas in the world where olives are cultivated and well established for commercial purposes, without *B. oleae* infestation.

Economic importance

Olive trees cultivation is increasing and spreading to new areas of the globe and olive fly is causing increasingly damages each year. Bueno and Jones (2002) predicted that the direct consequence of pests could at least reduce 15% of olives production worldwide. The same authors estimated, in 2002, that such production losses could cost about 800 million US dollars (US$). Furthermore, each year farmers spent about 100 million US$ to control pests' populations (Bueno and Jones, 2002).

If we consider 15% of production loss, predicted according to the most recent data (Bueno and Jones, 2002), more than 3.6 million tons of olives were lost in 2013, considering an effective production of 20,344,342 tons (FAOSTAT, 2015). Regarding olive products, for the 2013/2014 campaign, provisional data point out to the production of 3.27 million tons of olive oil (IOC, 2014a) and 2.60 million tons of table olives (IOC, 2014b). Such data indicate that about 580,000 tons of olive oil and 458,000 tons of table olives were lost due to olive pests, olive fly included. Concerning olive oil, if we consider the medium price in the international market in the beginning of January 2015 (≈ 2800€/ton, POOLred, 2015) a reduction of 1,624 million € could be estimated. Regarding to table olives, probably the reduction was less than the one observed to olive oil once the fruits could be used for oil extraction.

A good example of the importance to maintain olive fly control programs is the case of Greece, where *B. oleae* is a key pest. In the entire country about 97 million olive trees are included in control programs (bait sprays), estimated in 30 million €/year (about 0.31€ per tree) bringing economic benefits of about 550 million €/year to Hellenic olive industrial sector (Kalaitzaki *et al*., 2014).

Economic losses due to *B. oleae* affect the entire olive chain. In the first level are the olive farmers that have to handle with productivity losses due to fruit drop, rot fruits, and lower income in table olives and olive oil production. Industrials are also affected due to low quality of fruits for table olives and olive oil extraction, leading to low quality products which influence their classification with a consequent lower income. The consequences end at the consumer's table as table olives infested with *B. oleae* are rejected at the sensorial level by consumers and olive oil quality is reduced drastically, influencing also its nutritional and beneficial properties (detailed characterization in section 5).

Loss of production

Production losses begin immediately after larvae emergence from the eggs. Only after 9 days from egg emergence each larvae of *B. oleae* consumes about 56.4 mg of olive pulp (Neuenschwander and Michelakis, 1978). If we consider pulp consumption from egg emergence until the formation of pupae, the data is specific according to the cultivar, varying from 44.8 mg (cv. Tsounati) to 147.8 mg (cv. Koroneiki) (Neuenschwander and Michelakis, 1978). Some authors consider the pulp consumption to vary between 10% and 15% of the fruits pulp weight (Kyriadikis and Dourou, 2002; Tamendjari *et al*., 2009a). A direct consequence of pulp consumption is that the connection between the olive fruits and the olive tree becomes very fragile. The easier detachment from the tree increases with the biological cycle of the pest, being observed lower attachment in olives with older exit holes (Neuenschwander and Michelakis, 1981). Such fact leads to an abnormal increase of fruits drop in years of high population densities and infestation levels. In Portugal a fruit drop of about 19%, associated to a loss of about 4.6 kg/tree, was recorded in 1995. At harvest moment 84% of the fruits were infested, in a total of 20.3 kg of olives infested/tree (Bento *et al*., 1999). In Italy, in years with low infestation levels, between 0.1 and 0.2 kg of olives per tree fall down due to *B. oleae* action at the beginning of the optimum harvest period. In years with high infestation levels, nearly 1.1 kg of olives was lost per tree (Petacchi *et al*., 1994). In California olives yield losses in olive orchards without pest control inputs vary between 22.4% (cv. Koroneiki) and 53.1% (cv. Leccino) in oil olives and from 8.8% (cv. Mission) to 100% (cv. Manzanillo) in table olives cultivars (Cobourn *et al*., 2009). Translating the production losses to economic losses, for the represented data in California, and considering that oil olives are sold for 450 US$ per ton (COOC, 2008), farmers' income reduced between 100 and 239 US$ per ton of harvested olives due to *B. oleae* infestation. The case is even more serious if we consider table olives cultivars. In this case, olives for table olives processing are sold according to their size and cultivar, varying between 350 and 1210 US$/ton, which means that farmers income could drop to 1210 US$ per ton in the case of cvs. Manzanillo and Mission olives (COOC, 2008), if we consider a loss of 100%.

Once *B. oleae* larvae leave the fruit, further consequences are to come for olive fruit. *Bactrocera oleae* larvae can pupate inside or outside the fruit. In both cases exit holes are opened in the fruits epidermis and galleries are created within the fruit mesocarp. Together with other physical wounds (oviposition sites), exit holes and galleries are open windows to infectious and phytopathogenic agents. Olive anthracnose is a good example. This disease can cause up to 100% of fruit production loss in high susceptible

olive cultivars in years where *B. oleae* populations and infestation levels are high (Moral *et al*., 2008; Moral and Trapero, 2009), causing fruit rot and mummification. *Camarosporium dalmaticum* (Thüm.) Zachos & Tzav.-Klon and *Botryosphaeria dothidea* are others pathogenic microorganisms associated to *B. oleae* infestation, causing fruit rot and mummification (Iannota *et al*., 2007; Iannota *et al*., 2012; Latinović *et al*., 2013). Olive fly infestation also influences the microflora present in the olive paste for olive oil extraction, causing quality losses, mainly in free acidity and peroxide values (Torres-Vila *et al*., 2003; Torbati *et al*., 2014).

Olive products

As discussed in the earlier section, *B. oleae* attack causes severe economic losses to olive producers. This fact is mainly correlated to olives production. However, olives and their derived products (mainly table olives and olive oil) are greatly affected as well. In this section, olives and their resulting products are reviewed concerning the influence of *B. oleae* attack in their classification, quality, composition and inherent properties, also related with indirect losses by producers.

Table olives

Table olives are the most popular agro-fermented food product worldwide (Malheiro *et al*., 2012). Since 1990 table olives production increased worldwide nearly 270%, from 0.95 million tons to 2.51 million tons predicted in 2013/2014 (IOC, 2014b). Regarding the same period, table olives consumption increased proportionally, from 0.96 million tons in 1990/91 to 2.54 million tons in 2013/14 (IOC, 2014c). Olives infested with *B. oleae* cannot be used for table olives processing (Kailis and Harris, 2007).

Table olives can be classified in three trade categories: i) Extra or Fancy; ii) First, 1st, Choice, or Select; and iii) Second, 2nd, or Standard (IOOC, 2004; Codex Stan 66, 1981). In order to be classified in each category olives may respect a maximum limit of defects according to their style preparation and commercial presentation, as presented in Table 4.2.

Table 4.2. Maximum tolerances allowed in the three table olives trade categories, according to their preparation style and presentation (Codex Stan 66, 1981; IOOC, 2004).

	Extra category			First Category			Second category		
Defects	Green olives	Olives darkened by oxidation	Olives turning colour and black olives	Green olives	Olives darkened by oxidation	Olives turning colour and black olives	Green olives	Olives darkened by oxidation	Olives turning colour and black olives
Stoned or stuffed olives*									
Stones or stone fragments	1	1	2	1	1	2	1	1	2
Broken fruit	3	3	3	5	5	5	7	7	7
Defective stuffing:									
Place-packed	1	1	1	2	2	2	-	-	-
Random-packed	3	3	3	5	5	5	7	7	7
Whole olives, stoned or stuffed*									
Blemish fruit	4	4	6	6	6	8	10	6	12
Mutilated fruit	2	2	3	4	4	6	8	8	10
Shrivelled fruit	2	2	4	3	3	6	6	6	10
Abnormal fruit	4	4	6	6	6	8	10	10	12
Abnormal colour	4	4	6	6	6	8	10	10	12
Stems	3	3	3	5	5	5	6	6	6
Cumulative maximum of tolerances for these defects	12	12	12	17	17	17	22	22	22
Harmless extraneous material**	1	1	1	1	1	1	1	1	1

*Maximum tolerance as % of fruit; **Maximum tolerance as units per kg or fraction.

Stings of *B. oleae* or exit holes caused by larvae increase the percentage of olives defects, therefore declassifying table olives. The main defects related with *B. oleae* infestation are the blemish and mutilated fruits. On the other hand, the galleries created by larvae cause frequently deformities in the fruits, turning them abnormal. Another aspect that needs particular attention is the sensorial analysis of table olives. As well as for olive oil, the sensorial analysis of table olives is well established by an international standard (COI/OT/MO No 1/Rev.2; IOC, 2011). In the case of table olives infested with *B. oleae*, a negative attribute may be perceived, musty, due to the contamination of the olives and moulds development. Furthermore, the kinaesthetic sensations are severely affected, since the hardness, fibrousness and crunchiness of the table olives is considerably reduced due to pulp consumption by *B. oleae* larvae.

Olive oil

Olive oil is a well-recognized vegetable oil for its sensorial characteristics, nutritional and beneficial healthy properties. Such characteristics and properties are leading to the spread of olives cultivation worldwide with a continuous increased production, of about 77% in the last two decades (IOC 2014a). Such production increment was a demand due to the increase in consumption (53% in the last 20 years; IOC 2014d), being this vegetable oil gradually introduced in new markets (Brazil, China, Russia).

The healthy properties of olive oil are intrinsically associated with its chemical composition and minor components: fatty acids, due to being rich in monounsaturated and essential fatty acids; phenolic compounds; and sterols (Covas *et al*., 2006). Meanwhile, *B. oleae* infestation affects olive oil production, quality, composition, as well as the inherent properties related to the minor compounds as detailed in the next sections. While fruits infested with *B. oleae* are not used for table olives processing, in the case of olive oil extraction, many times, high levels of infested fruits are present.

Oil yield

Since *B. oleae* larvae hatch from fertile eggs until they pupate inside the fruit or abandon the olive to pupate in the soil, the larvae feeds exclusively from the pulp. Therefore, olive oils extracted from infected olives report a lower yield than healthy olive fruits. Olive oil yield losses vary between 11.5% and 14.9%, with higher losses verified in

mature olives (Tamendjari *et al*., 2004). These oil yield losses depend on the cultivar, reporting cvs. Chemlal, Bouchouk, and Azzeradj respectively, 49, 57.5, and 63 kg of oil per ton (Tamendjari *et al*., 2009a).

Therefore the presence of *B. oleae* in olive fruits is directly related with a decrease in olive oil production due to pulp consumption by this dipteran, and is also a serious economic damage to producers.

Bactrocera oleae and olive oil quality

Sensorial defects of olive oils extracted from infested olives might declassify olive oils. Chemically, olive oil's quality is also depreciated and can declassify olive oils extracted from infested olives similarly. In Table 4.3 is reported the influence of *B. oleae* in the quality parameters of olive oil in several olive cultivars from different olive producing countries.

Table 4.3. Influence of *B. oleae* infestation (healthy and totally infested fruits, when available data) in quality parameters of olive oils from different olive cultivars around the Mediterranean basin (*values in bold and italic exceed the maximum legal values for EVOO).

Country	Olive cultivar	Infestation level (%)	FA	PV	K_{232}	K_{270}	Olive oil classification[a]	References
Algeria	Azzeradj	0	0.26	8.0	1.56	0.18	EVOO	Tamendjari *et al*. (2009a)
		100	***1.05****	17.3	2.09	***0.29***	LOO	Tamendjari *et al*. (2011)
		0	0.32	8.2	2.20	0.16	EVOO	
		100	***1.25***	-	2.31	***0.26***	LOO	
	Bouchouk	0	0.53	5.8	1.92	0.12	EVOO	
		100	***1.32***	10.0	2.32	0.19	VOO	
	Chemlal	0	0.21	5.4	1.74	0.08	EVOO	
		100	0.80	10.5	***2.70***	0.13	LOO	
		0	0.22	10.9	1.95	0.10	EVOO	
		100	***0.92***	16.8	2.47	0.15	VOO	
Croatia	Buža	0	0.09	2.1	1.50	0.10	EVOO	Koprivnjak *et al*. (2010)
		100	0.19	8.2	***3.94***	***0.31***	LOO	
	Istarska bjelica	0	0.24	0.8	1.53	0.16	EVOO	
		100	0.43	2.7	1.62	0.16	EVOO	
Greece	Koroneiki	0	0.14	4	0.97	0.07	EVOO	Kyriakidis and Dourou (2002)
		100	0.20	9	1.16	0.12	EVOO	
Italy	Coratina	0	0.31	3.0	1.49	0.10	EVOO	Angerosa *et al*. (1992)
		100	0.80	12.3	1.95	0.13	EVOO	Gómez-Caravaca *et al*. (2008)
	Dritta	5	0.5	10.2	-	-	-	Gucci *et al*. (2012)
		35	***1.2***	19.0	-	-	-	
	Gentile	5	0.4	7.9	-	-	-	
		15	0.6	9.8	-	-	-	
	Intosso	2.5	0.2	5.9	-	-	-	
		15	***0.9***	9.1	-	-	-	
	Leccino	2.5	0.3	9.9	-	-	-	
		30	***1.0***	12.1	-	-	-	

	Nebbio	0	0.40	3.8	1.54	0.10	EVOO	
		100	***1.15***	12.0	1.88	0.15	VOO	
	Frantoio	0	0.1	2.3	-	-	-	
		100	0.3	5.2	-	-	-	
Portugal	Cobrançosa	0	0.33	11.4	2.41	***0.24***	VOO	Pereira *et al*. (2004)
		100	0.32	16.6	2.43	0.22	EVOO	
	Madural	0	0.23	14.7	***2.66***	0.18	VOO	
		100	0.31	14.9	2.49	0.21	EVOO	
	Verdeal Transmontana	0	0.28	19.1	1.57	0.17	EVOO	
		100	0.53	***35.1***	1.53	0.17	LOO	
Tunisia	Chemlali	0	0.52	-	1.98	0.14	EVOO	Mraicha *et al*. (2010)
		100	***3.41***	-	***3.06***	***0.23***	LOO	
EU[b] EVOO	**-**	**-**	**≤ 0.8**	**≤ 20**	**≤ 2.50**	**≤ 0.22**	**-**	

[a]Olive oil categories: EVOO – extra-virgin olive oil; VOO – virgin olive oil; LOO – Lampante olive oil; according to the Commission Commission Regulation (EEC) No 2568/91 for the quality parameters considered;
[b]Legal maximum values for extra-virgin olive oils, according to the Commission Regulation (EEC) No 2568/91.

In the next points the influence of *B. oleae* in the quality parameters of olive oils will be discussed.

Free acidity (FA)

Olive oils extracted from olives infested by *B. oleae* have a considerable increase in the percentage of free fatty acids in (Table 4.3). Gómez-Caravaca *et al*. (2008) studied different olive oils from different olive cultivars, different geographical proveniences, different extraction systems, extracted from olive fruits with different infestation levels (from 2 to 85%) and concluded that around 20% of the oils could not be considered EVOO (FA > 0.8%, (ECC, 1991). Furthermore, 6% of the oils would be classified as lampante olive oils (FA > 2%) (Gómez-Caravaca *et al*., 2008), all extracted from higher than 60% of infested olives. In Portuguese olive oils a marked effect was observed between cultivars, since no increased acidity was observed in 100% infested cv. Cobrançosa, medium effects were reported in cv. Madural (FA = 0.23% and 0.31% in healthy and 100% infested fruits, respectively), and a high influence was observed in cv. Verdeal Transmontana (FA = 0.28% and 0.53% in healthy and 100% infested fruits, respectively) (Pereira *et al*., 2004). An increase of 0.1% in FA was verified as a result of a combination of maturation and infestation level (Koprivnjak *et al*., 2010), while some authors report FA values seven times higher comparing olive oils extracted from 100% infested fruits and from healthy fruits (from 0.52% to 3.41%) (Mraicha *et al*., 2010).

During larval development, pulp consumption destroys several tissues in the olive fruit. Such destruction leads to lipolytic reactions between lipases and triacylglycerols, arising therefore the amount of free fatty acids in the olive oils. Furthermore, FA values increase with olive fruits storage prior olive oil extraction due to the prolonged interaction time between lypases and their substrate (Pereira *et al*., 2002). Since *B. oleae* infestation also increments the microbial community in olives (Torres-Vila *et al*., 2003), the combination of storage and *B. oleae* infestation may cause unprecedented increases in FA values due to fermentative processes and enzymatic activity. As witnessed by several authors, the increase in FA values caused by *B. oleae* presence in olive fruits can easily declassify olive oils from EVOO category, and in some circumstances classify them as lampante olive oils.

Peroxide value (PV)

Regarding the peroxidation of olive oils extracted from infested fruits, the results found in literature are concise and precise: *B. oleae* infestation rise PV independently of the olive cultivar, geographical origin and extraction system (Bendini et al., 2008; Gucci *et al*., 2012; Kyriakidis and Dourou, 2002; Pereira *et al*., 2004; Tamendjari *et al*., 2004; Tamendjari *et al*., 2009b), causing the oxidation and chemical degradation of the oils.

Until 10% of infestation, PV do not suffer significant changes (Bendini *et al*., 2008). Meanwhile, peroxides formation is positively correlated with the percentage of exit holes verified in olives (Gucci *et al*., 2012). In crop years with high population densities and infestation rates, PV could easily increase and influence the classification of olive oils as EVOO's (Table 3). The combination of infestation level (60%), storage and light exposure induces a clear increase in PV of five folds comparatively to control olive oils (Gucci *et al*., 2012). In Portuguese cultivars (cvs. Cobrançosa, Madural and Verdeal Transmontana), with an adjusted infestation level of 30%, Pereira *et al*. (2002) observed an increase in PV of olive oils extracted from olives stored during 7 days. However, after 14 days of storage, PV generally decreased in the three cultivars, which means that secondary oxidation reactions were already being conducted. In the first stages of oxidation hydroperoxydes are formed from unsaturated fatty acids. However, hydroperoxides are very unstable molecules and a secondary oxidation takes place, decomposing themselves in aldehydes, alcohols, ketones, acids, dimmers, trimers, polymers and cyclic compounds (Malheiro *et al*., 2011), including the volatile compounds responsible for off-flavors in the olive oils (in more detail in section 5.2.3.6.).

UV spectrophotometric analysis

During oxidation of oils and fats, hydroperoxides are formed from unsaturated fatty acids. These molecules have a conjugated dienic system, resulting from stabilization of the radical state by double bond rearrangement, absorbing in the UV range (235 nm) and forming a shoulder on the main absorption peak of non-conjugated double bonds (Laguerre *et al*., 2007). Since these molecules are very unstable, as mentioned in the previous section, secondary products of oxidation are formed with triene conjugated double bounds that absorb in the 270 nm region. Therefore, UV spectrophotometric analysis, namely the specific extinction coefficients at 232 and 270 nm (K_{232} and K_{270}), is an important tool to monitor the oxidative status and its extent in vegetable oils.

The oviposition of *B. oleae* in olive fruits influence significantly the coefficients of extinction, in a way that in some cases olive oils cannot be classified as EVOO (Table 4.3). The combination of *B. oleae* infestation and olives maturation influences K_{232} and K_{270} values. At 15% infestation in ripe olives from cv. Chemlali, maximum legal values for K_{232} (2.50) are exceeded, with 2.68 (Mraicha *et al*., 2010). At 100% infestation K_{232} raised to 3.06 and K_{270} values also exceeded the legal maximum values (0.22). Such data proves that the actions of *B. oleae* not only induces primary oxidative reactions, but promote indirectly (by air exposition due to exit holes created in the fruits) the occurrence of secondary oxidative reactions (Koprivnjak *et al*., 2010).

Sensorial analysis

Sensorial analysis is determinant for the classification of olive oils into the different legal categories. The descriptors fruity, bitter and pungent, most appreciated by consumers, depend on many factors, like the olive cultivar, maturation, and composition, the extraction technological process conditions, but also on *B. oleae* infestation. The attack of olive fly leads to the reduction of positive sensorial attributes in the olive oils (Angerosa *et al*., 1992; Koprivnjak *et al*., 2010; Mraicha *et al*., 2010; Tamendjari *et al*., 2009a; Tamendjari *et al*., 2009b). At the same time, sensorial defects arise with the infestation of *B. oleae*. The most common defects detected by panelists are fusty, musty, winey, grubby, and in some cases rancid. In fact, olive oils extracted from olives infected by *B. oleae* are similar to standard defects of winey and musty (Bendini *et al*., 2008). Therefore, with an increasing infestation level, olive oils are less scored for organoleptic evaluation by trained panelists. For instance, cv. Coratina olive oils with 0 and 100% infestation were scored with 7.0 and 6.2 respectively, and cv. Nebbio with 6.9 and 5.7 respectively (Angerosa *et al*., 1992). Again, olives maturation and *B. oleae* infestation are a deleterious combination for sensorial characteristics as well. Olive oils from mature olives infested with *B. oleae* have poor sensorial attributes, with a drastic decrease in positive attributes and negative attributes start to be perceived (Tamendjari *et al*., 2009a; Tamendjari *et al*., 2009b). Fusty is the defect detected with more intensity in olive oils prevenient from infested olives, for example in Croatian olive oils extracted from olives infected by *B. oleae* (Koprivnjak *et al*., 2010). In this case, Koprivnjak *et al*. (2010) also observed the influence of cultivar, reporting a low intensity of the defect fusty in cv. Buža, while cv. Istarska bjelica was connoted with fusty, musty and rancid defects. Olive oils

from unripe infested fruits highlight musty as the main defect, while in olive oils from mature olive fruits fusty is the main defect perceived (Mraicha *et al*., 2010).

The influence of *B. oleae* on sensorial component of olive oils is a serious question as it might declassify olive oils (Tamendjari *et al*., 2009a; Tamendjari *et al*., 2009b). Besides a noticed sensorial quality decrease, this fact leads to a considerable economic loss to both producers and industrials, since olive oil cannot be marketed as extra-virgin olive oil.

Bactrocera oleae and olive oil composition

Bactrocera oleae infestation induces quality degradation in olive oils. Such degradation is related with the changes inflicted in the composition of olive oils, mainly in their minor components. These minor components are responsible in major part for the organoleptic (phenolic compounds and volatiles), visual (chlorophylls and carotenoids), nutritional (fatty acids, sterols, tocopherols), and health promoting properties of olive oils.

Fatty acids profile

Fatty acids are among the most influencing molecules that modulates oxidative reactions in lipids. According to Frankel (2005), “The oxidation of unsaturated fatty acids is one of the most popular reactions in lipid chemistry”. The unsaturation degree of fatty acids influences lipids resistance to oxidation, being unsaturated fatty acids prone to oxidative reactions (Kamal-Eldin, 2006). The degradation of unsaturated fatty acids leads to nutritional and quality losses in vegetable oils, developing off-flavors and hazard compounds while reducing essential fatty acids (Laguerre *et al*., 2007). As previously discussed, *B.oleae* cause quality degradation, namely by hydrolysis and oxidative reactions, being important to observe the changes registered in the fatty acids profile of olive oil, its major component.

Some authors studied the fatty acids profile of olive oils extracted from olives with different levels of infestation, reporting no important changes in such profile, as it was the case of the following olive cultivars: Chemlali from Tunisia (Mraicha *et al*., 2010); Cobrançosa, Madural and Verdeal Transmontana from Portugal (Pereira *et al*., 2004); and Chemlal from Algeria (Tamendjari *et al*., 2004). However, some other studies report important changes in the fatty acids profile. For instance the same Chemlal olive cultivar

from Algeria, in other study made by Tamendjari *et al*. (2009a) report a positive correlation between the saturated fatty acids and the infestation level, while negative correlations were established for oleic ($C_{18:1}$) and linoleic acids ($C_{18:2}$) content. The same observation was checked for two others Algerian olive cultivars (cvs. Azzeradj and Bouchouk) but in lower extent. The ratio between unsaturated fatty acids and saturated fatty acids decrease with the increment of infestation level (Tamendjari *et al*., 2009a). This means that the oxidation of unsaturated fatty acids is positively correlated with the infestation level, leading to a higher degradation of the oil, a fact that will compromise oils stability.

Sterols

The role of sterols in human health is still a controversial aspect. Some studies reveal that high levels of plasma sterol are associated with lower cardiovascular problems, namely coronary heart disease (Sudhop *et al*., 2002), while others defend the opposite hypothesis (Wilund *et al*., 2004).

Few studies about the influence of infestation of *B. oleae* on olive oil sterols amounts and composition are available. The main affected sterols in olive oils seem to be β-sitosterol and cholesterol. In cvs. Canino, Coratina, Leccino and Frantoio, sterols loss was correlated with the olives infestation level (Parlati *et al*., 1990). With 100 % of infestation β-sitosterol losses between 1.87 and 2.67% and cholesterol losses between 2.05 and 2.80% were reported by these authors. In the case of uvaol, it was totally loss for fruits infestation above 20%. Being expressed on a percentage levels, an opposite trend is expected in the unaffected sterol, with campesterol and stigmasterol increasing their percentage in the olive oils, the first from 0.38 to 0.55% and the last one from 1.20 to 1.40% with 100% infestation levels (Parlati *et al*., 1990).

Pigments

Pigments influences consumers' preference regarding olive oil. Infestation level of *B. oleae* in olive fruits is negatively related with pigments content in olive oils (Mraicha *et al*., 2010; Tamendjari *et al*., 2004). A direct consequence of infestation is that olive oils extracted from infested olives are lighter in color, being more golden-yellow than green. This loss of pigmentation is naturally incremented by maturation, with higher losses of chlorophylls and carotenoids reported in riper infested olives (Mraicha *et al*., 2010;

Tamendjari *et al*., 2004). Chlorophylls reduce their content from 16% (5% infestation) to 74% (100% infestation), while losses in carotenoids are ranged from 14 to 39% under the same infestation levels (Mraicha *et al*., 2010). Losses in chlorophylls and carotenoids will also affect the oxidative stability of olives oils, as discussed ahead, since these pigments contributes for the total stability of olive oils (Aparicio *et al*., 1999).

Tocopherols

Studies regarding tocopherols composition of olive oils extracted from olives infested by *B. oleae* are scarce. However these minor components of olive oils are essential since they play a dual role, being important antioxidant components (Baldioli *et al*., 1996) and nutritionally are well recognized for their vitamin action as vitamin E (Sen *et al*., 2006).

With increasing levels of infestation, tocopherols content in olive oils from different cultivars respond differently to the aggression. Infestation of *B. oleae* has no repercussions in β- and γ-tocopherols contents while α-tocopherol was reduced with increasing levels of infestation (Pereira *et al*., 2004). Losses vary according to the olive cultivar assessed: 6% in cv. Cobrançosa; 22% in cv. Madural; and 38% in cv. Verdeal Transmontana (100% infestation for all cultivars) (Pereira *et al*., 2004). Some authors state that olives infestation by *B. oleae* is more critical for tocopherols than olives storage prior olive oil extraction (Frega *et al*., 2000).

Phenolics

Phenolic compounds in olive oils display a diversified array of functions and properties. Beginning from its sensorial aspects, phenols are responsible for the bitter and pungent positive attributes of olive oils (Servili *et al*., 2004). Regarding their pharmacological potential, phenols possess antioxidant, anti-inflammatory, cardiovascular, immunomodulatory, gastrointestinal, endocrine, respiratory, and autonomic effects. Furthermore they intervene at the central nervous system, and display antimicrobial, chemotherapeutic, anticancer and chemopreventive properties (Obied *et al*., 2012).

In olive oils the content of phenolic compounds is negatively correlated with the infestation level of the olives (Gucci *et al*., 2012; Koprivnjak *et al*., 2010; Mraicha *et al*.,

2010; Tamendjari *et al*., 2009a). In Figure 4.4 is observed that losses of total phenols content are dependent on the olive cultivar and it can range from 21% in Croatian olive cultivar Istarska bjelica (Koprivnjak *et al*., 2010) to 83% in the Tunisian Chemlali olive cultivar (Mraicha *et al*., 2010).

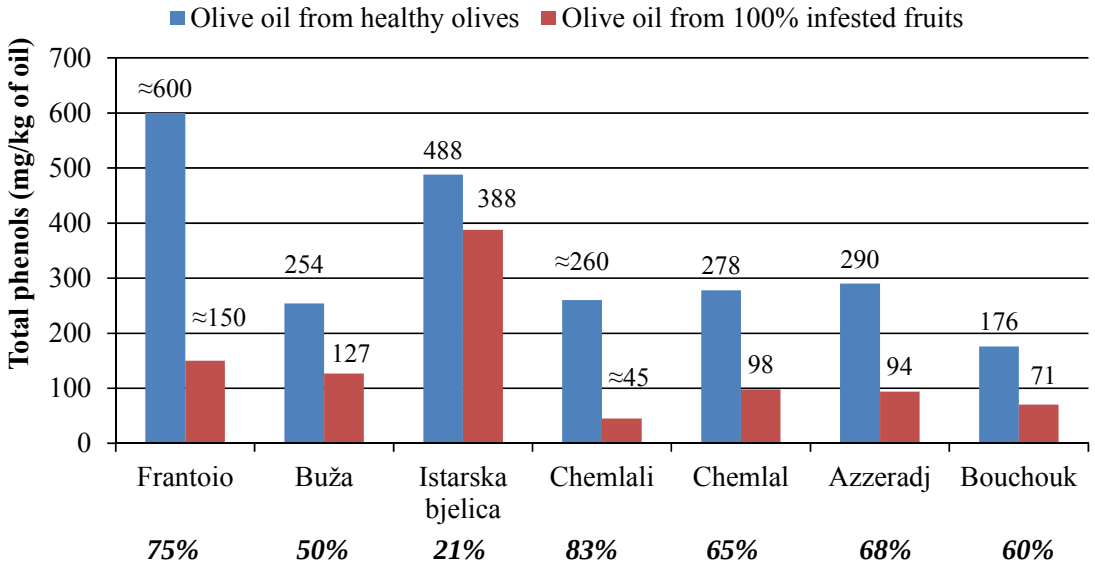

Figure 4.4. Total phenols content (mg/kg of oil) of olive oils extracted from healthy olives and from olives 100% infested by *B. oleae*. Above each identification flag, values represented in bold and italic express the percentage of total phenols lost from healthy to 100% infested olives. Data updated from: Gucci *et al*. (2012); Koprivnjak *et al*. (2010); Mraicha *et al*. (2010); Tamendjari *et al*. (2009a).

Such results are due to the specific phenolic profile of olives and olive oils which is dependent on the cultivar (Vinha *et al*., 2005). The main phenolic compounds influenced by *B. oleae* infestation in cv. Frantoio olive oils were the secoiridods (specially an isomer of oleuropein aglycone) (Gucci *et al*., 2012). Some phenolic alcohols like hydroxytyrosol and tyrosol also decrease drastically their contents with the infestation levels. Other compounds like (+)-pinoresinol and (+)-1-acetoxypinoresinol weren't affected by the attack of *B. oleae*. In several Italian olive cultivars, Gómez-Caravaca *et al*. (2008) reported significant losses in simple phenols, lignans, secoiridoids and elenolic acid in olive oils with different infestation levels. The same authors reported that above 30% of infestation level, olive oils total phenols content were always below 80 mg/kg (Gomez-Caravaca *et al*., 2008), which is considerably low for a virgin olive oil.

Volatiles

The effect of *B. oleae* infestation in the volatile composition of olive oils is an important topic of study since these compounds are responsible for the sensorial characters scented by panelists and consumers, and can affect considerably consumers' choices and loyalty.

Olive oils obtained from *B. oleae* infested olives report lower amount of volatile compounds comparatively to olive oils extracted from healthy olives. The loss in volatiles can reach 15.6% in olives extracted from green fruits and 8% when olive oils are extracted from mature fruits (Tamendjari *et al*., 2004). This happens due to the combination of maturation process and *B. oleae* infestation. Olive oils extracted from mature olives are characterized by lower green notes, and one of the compounds responsible for that attribute is (*E*)-2-hexenal. This compound is one of the most abundant volatile compounds in olive oils (Kalua *et al*., 2007) and one of the green leaf volatiles (GLV's), responsible for the green and cut grass odors found in olive oils (Angerosa *et al*., 2000; Angerosa *et al*., 2004), and playing a critical role in olive oils sensorial quality and characterization. (*E*)-2-hexenal decreases in olive oils volatile fraction during olives maturation, but *B. oleae* infestation induces greater losses (Tamendjari *et al*., 2004). In fact, generally, (*E*)-2-hexenal content in negatively correlated with *B. oleae* infestation level of the olives that originate them (Bendini *et al*., 2008). In an opposite trend, alcohols arise in olive oils extracted from infested olives. Methanol, ethanol, and iso-amylic alcohol are the main alcohols that arise in olive oils, being their increase also related with olives maturation (Bendini *et al*., 2008; Tamendjari *et al*., 2004). Acetic acid register also an increase in olive oils extracted from infected olives (Tamendjari *et al*., 2004). This compound, together with the alcohols mentioned earlier, is responsible for the winey defect, and their content is generally higher in olive oils extracted from olives with high infestation levels (Bendini *et al*., 2008), probably associated with some degree of fermentation. Nonanal and 6-methyl-5-hepten-2-one are two volatile compounds positively related with *B. oleae* infestation level (Bendini *et al*., 2008). In fact, Tamendjari *et al*. (2004) recorded that the ratio hexenal/alcohols decrease considerably in olive oils from infested olives. This situation is directly related with the loss of sensorial attributes (green, fruity, cut grass) due to decrease in GLV's and the arise of alcohols and volatile acids that are related with sensorial defects (mainly winey). Such fact can declassify an olive oil to lower grade categories, leading to economic losses by producers.

Bactrocera oleae and olive oil antioxidant activity and stability

The changes inflicted in the composition of the minor components of olive oils due to the infestation of *B. oleae*, cause inherent consequences at the bioactivity level, namely antioxidant potential of the olive products and also display important changes in stability and therefore in the shelf-life of olive oils.

With an increase in infestation level, the capacity of olive oil to scavenge the free radicals of DPPH (2,2-diphenyl-1-picrylhydrazyl) is reduced, being necessary a higher quantity of olive oil extract to scavenge 50% of the free radicals of this molecule (Mraicha *et al*., 2010). Along the maturation process the results are even more pronounced with the infestation level, since maturation naturally leads to a series of metabolic reactions that reduce the quantity of antioxidants (phenols, sterols, pigments and tocopherols) in olive fruits and consequently in olive oils (Jemai *et al*., 2009; Morelló *et al*., 2004). The same authors established that phenolic composition and antioxidant activity are positively correlated (Mraicha *et al*., 2010). In Figure 4.5A is detailed the antioxidant potential, expressed as µg quercetin equivalents/mL of phenolic extract, of Italian olive oils from different cultivars, different extraction systems, and different infestation levels (from 2 to 85%).

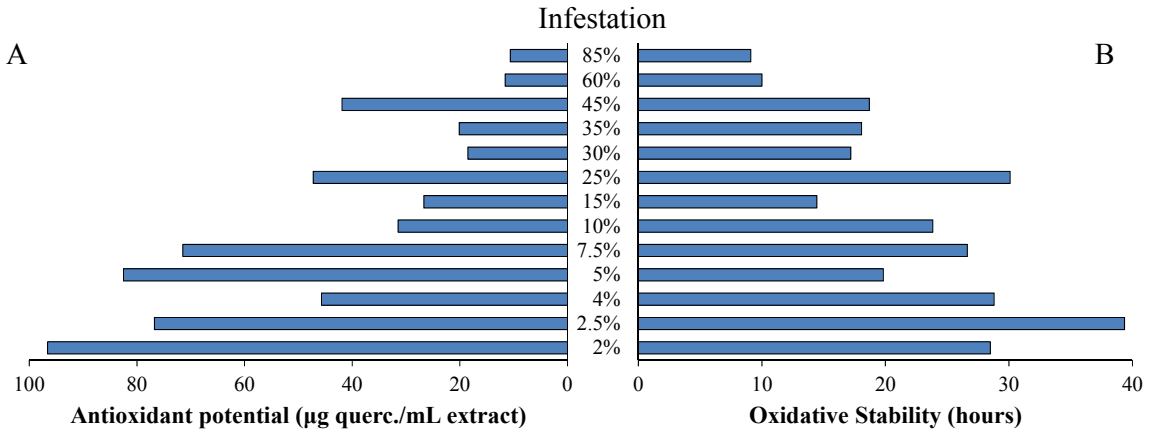

Figure 4.5. Antioxidant potential (Fig. 4.5A - µg quercetin/mL of phenolic extract) and oxidative stability (Fig. 4.5B - hours) of olive oils extracted from olives with different *B. oleae* infestation levels. Data updated from: Gómez-Caravaca *et al*. (2008).

It is observed that higher infestation levels are ascribed with lower antioxidant activity (Gómez-Caravaca *et al*., 2008). In fact, below 30% of infestation olive oils report 2.5 times more antioxidant activity than those with infestation level higher that 30%.

The reduction in antioxidant activity influences directly the capacity of olive oils to resist against oxidation. In Figure 4.5B the nefarious effect of *B. oleae* infestation in olive oil oxidative stability is noticed. Above 30% infestation level, olive oils lost around 40% of their oxidative stability (Gómez-Caravaca *et al*., 2008). In Portuguese olive cultivars it was verified that olive oils extracted from 100% infested fruits lost around 9, 16 and 53% of oxidative stability, respectively in cvs. Madural, Cobrançosa and Verdeal Transmontana when compared to olive oils extracted from healthy fruits (Pereira *et al*., 2004).

Loss of antioxidant activity and oxidative stability are intrinsically related with the changes inflicted in the minor components of olive oil. As discussed in the previous sections many antioxidant compounds are lost due to *B. oleae* infestation, particularly phenols (Figure 4.4). Among them, the phenolic compounds contribute for about 30% of olive oils stability, while tocopherols contribution is around 9% (Aparicio *et al*., 1999). Fatty acids unsaturation degree also influences vegetable oils stability, with the ratio between oleic and linoleic acid contributing to around 27% for olive oils stability (Aparicio *et al*., 1999). Overall and analyzing the contribution of phenols, tocopherols, pigments, and fatty acids, around 66% of olive oil stability may be compromised with *B. oleae* infestation.

Concluding remarks

Bactrocera oleae is a menace to olive crop worldwide, being spread in the last decades to new areas of the globe where olives are being cultivated. The global impact of *B. oleae* in olive crop, from the field itself until consumers' table, is summarized in Figure 4.6.

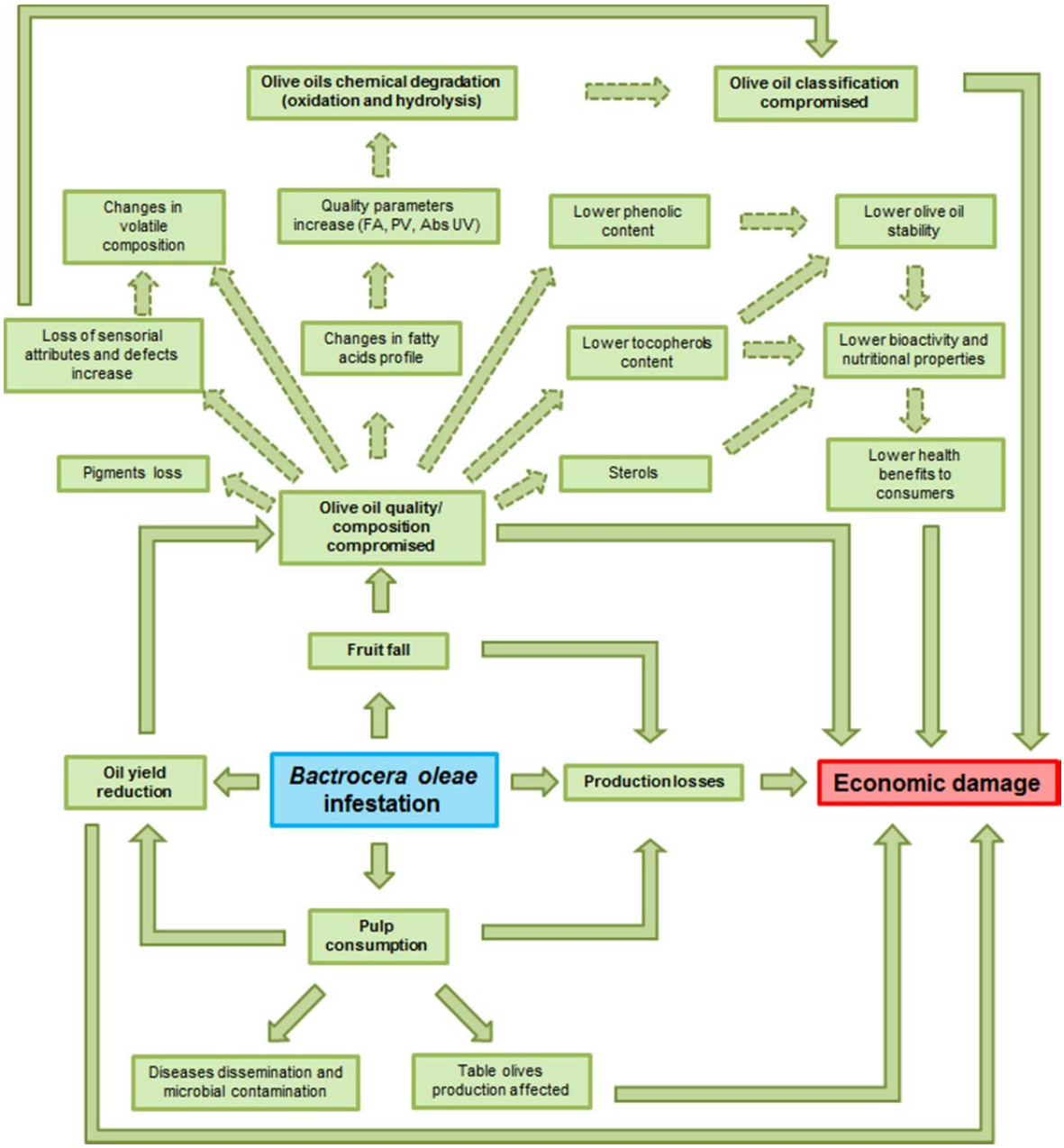

Figure 4.6. *Bactrocera oleae* global impact in olive crop economic losses.

The damages affect the entire olive chain, starting in the olive orchard affecting severely producers, passing through the industrials, and ending at consumers, the final receptors of the olive products.

1. Field and farmers' impact:

i) *Bactrocera oleae* larvae pulp consumption lead to severe fruit drop, affecting olives production and yield at harvest; ii) attacked fruits are prone to fruit contamination and fruit rot; iii) low fruits quality and producers income; iv) necessity of additional investments for *B. oleae* populations control strategies.

2. Industrials:

i) the quality of raw materials is severely affected, influencing table olives production and olive oil quality; ii) oil yield reduction due to pulp consumption by *B. oleae* larvae; iii) olive products classification is compromised, being relegated to lower classification categories, reducing drastically the income from those products; iv) difficulty to drain those products, many times considered as waste.

3. Consumers:

i) Higher crop losses means lower offer and high prices for final consumer; ii) olive products with lower quality; iii) consumers' loyalty and trust in compromised; iv) degraded and oxidized olive oil with formation of possible hazard compounds for health; iv) loss of nutritional, pharmacological and functional properties due to olive oils composition changes; v) lower healthy beneficial inputs by consumers.

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PART II

Experimental section

Chapter 5. Olive leaves volatiles along fruit maturation and their possible role in olive fly oviposition preference

Chapter 6. Olive volatiles from Portuguese cultivars Cobrançosa, Madural and Verdeal Transmontana: role in oviposition preference of *Bactrocera oleae* (Rossi) (Diptera: Tephritidae)

Chapter 7. Electrophysiological response of *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) adults to olive leaves essential oils from different cultivars and olive tree volatiles

Chapter 8. Influence of olive cultivar and maturation process on the oviposition preference of *Bactrocera oleae* (Rossi) (Diptera: Tephritidae)

Chapter 9. Physico-chemical characteristics of *Olea europaea* L. olives and leaves and *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) cultivar oviposition preference

CHAPTER 5.

Olive leaves volatiles along fruit maturation and their possible role in olive fly oviposition preference

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Abstract

The olive fly, *Bactrocera oleae* (Rossi), is the key pest of olive crop worldwide, causing severe damages in production and quality of olive products. This pest reveals cultivar preference to oviposit. Volatiles emitted by plants influence insects behavior and may be behind the olive preference of olive fly.

In order to clarify the oviposition preference, olive leaves volatile composition from three cultivars (Cobrançosa, Madural and Verdeal Transmontana) with different susceptibilities to olive fly was assessed by headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC/MS), at six different periods along fruit maturation. Maturation index and olive infestation level were also assessed.

A total of 39 volatiles were identified, mainly esters and alcohols, with a minor percentage of aldehydes, ketones and terpenic compounds, including sesquiterpenes. In the sampling dates with higher infestation degrees, cv. Cobrançosa had, simultaneously, significantly lower infestations and higher volatile amounts than the other two cultivars, with a probable deterrent effect for oviposition. Green leaf volatiles [(*Z*)-3-hexen-1-ol and (*Z*)-3-hexen-1-ol acetate)] were the main compounds identified on all cultivars, together with toluene. The abundance of the formers decreased significantly along maturation, without significant differences among cultivars, while toluene showed a general increase and positive correlations with olive fly infestation levels. This is the first report establishing

relations between olive fly cultivar preference and leaf volatiles composition from olive cultivars with different susceptibility degrees. The results obtained could be an open window to understand the role of olive volatiles type and amounts in the environment, especially in host selection and cultivar preference of olive fly, and a basis to find new alternative and sustainable ways to control this pest.

Keywords: *Olea europaea* L.; olive leaves; green leaf volatiles; *Bactrocera oleae* (Rossi); cultivar susceptibility; oviposition preference.

Introduction

Plant volatiles are ascribed with important roles and functions, representing the way that plants use to communicate and interact with the surrounding environment. Volatiles intervene at plant reproduction, in tritrophic interactions, in belowground defense systems and in the abiotic stress of plants (Dudareva *et al*., 2006). Regarding defense mechanisms, plants release volatile compounds with deterring and repelling purposes to maintain herbivores and pests away or, when attacked, to attract specific pest predators, parasitoids and other natural enemies and alert neighboring plants (Tamiru *et al*., 2011; Wu and Baldwin, 2010), globally recognized as semiochemicals (Paré and Tummlinson, 1999).

Several pests and diseases attack olive tree each year, causing serious production losses. *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), the olive fly, is considered the key-pest in several regions in the world, particularly in the Mediterranean area (Daane and Jonhson, 2010), causing important economical and quality losses (Figure 5.1).

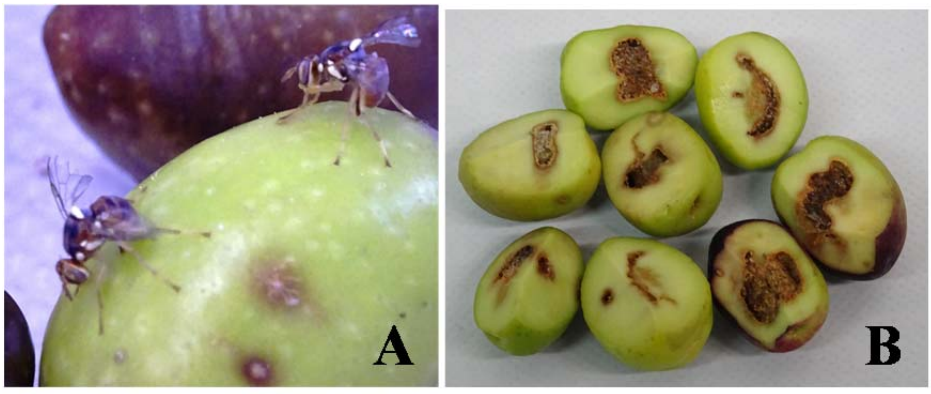

Figure 5.1. Olive fly females laying eggs in olive (A); damages caused by olive fly larvae in olive pulp (B).

Olive fly females lay their eggs inside the olive, from where larvae hatch and start to feed on the pulp. The larvae passes through three instars, creating galleries inside the fruit, once ready to pupate, the larvae exit the olive and leave behind the destruction observed in Figure 5.1B.

The female of this dipteran shows cultivar preference, by attacking specific olive cultivars (Burrack and Zalom, 2008; Iannotta *et al*., 2007; Navrozidis *et al*., 2007). Host selection for oviposition is believed to be based in chemical (Corrado *et al*., 2012; Imperato *et al*., 2012; Spadafora *et al*., 2008), physical (Rizzo *et al*., 2012), and molecular aspects.

Regarding chemical factors, olive tree volatiles (fruits and leaves) may play an important role in olive fly cultivar selection (Aluja and Mangan, 2008). However, most studies on cultivar preference are mainly focused in olive fruits, rather than olive leaves or the olive tree as a whole. Therefore, studies on olive leaves volatile characterization are scarce, and most are based on hydro-distillate volatile fractions, different from the natural emission (Brahmi *et al*., 2012; Campeol *et al*., 2003). Nevertheless, some studies do report the potential effect of olive leaves volatiles in olive fly behavior and, according to Scarpati *et al*. (1993) two abundant olive leaves volatiles, toluene and α-pinene, were the most attractive and repellent cues in olive fly oviposition bioassays, respectively. However, Liscia *et al*. (2013) by studying the electrophysiological response of females (mated and unmated) to volatiles from olive leaves and fruits, did not found significant differences. These apparently contradictory responses suggest that the host-pest interaction, *Olea europaea* - *Bactrocera oleae*, is complex and difficult to study, and therefore, yet scarcely known. A detailed knowledge of the volatile patterns produced by olive leaves from cultivars with different degrees of susceptibility to olive fly attack would be very useful to clarify this interaction.

In this sense, in the present work the volatile compounds emitted by olive leaves from cvs. Cobrançosa, Madural and Verdeal Transmontana, were assessed at different harvest times during fruit maturation. These olive cultivars were selected due to their different susceptibilities to olive fly in Portugal, where Verdeal Transmontana and Madural cultivars are highly susceptible, while cv. Cobrançosa is less attacked (Gonçalves *et al*., 2012). Possible relations between volatile type, amounts, and cultivar susceptibility to *B. oleae* attack are discussed as well. To the authors knowledge this is the first investigation relating olive fly oviposition preference with the volatiles from olive leaves from different cultivars.

Material and methods

Sampling

For the present study olive leaves from three Portuguese olive cultivars, the most representative from Trás-os-Montes region (Northeast of Portugal) were assessed: *cvs.* Cobrançosa, Madural and Verdeal Transmontana. The work occurred in 2011, and samples were collected in an organic olive grove located in Paradela (Mirandela - 41º32'35.72''N; 7º07'27.17''W). Five trees were marked per olive cultivar and olive branches with leaves were collected at six different dates: 18th July; 18th August; 20th September; 4th October; 21st October; and 9th November. After collection, branches were transported at refrigeration temperatures and volatile analysis was performed in the first 24 to 48 hours.

Simultaneously, fruits were collected per tree for calculation of the maturation index, as described by Hermoso *et al*. (2001). Briefly, samples of 100 olive fruits (20 fruits per tree) were separated in 8 levels based on epidermis and pulp color (0 to 7). Therefore, the fruit is classified as "0" if the epidermis is green; "1" for yellowish green; "2" if the epidermis shows red spots in less than half fruit; "3" if the epidermis is red or purple in more than half fruit; "4" for black epidermis and white pulp; "5" if the epidermis is black and less than half pulp is purple; "6" if the epidermis is black and more than half pulp purple (without reaching the stone); "7" if the epidermis is black and total pulp purple (reaching the stone). The maturation index was calculated as follows: MI = (a × 0 + b × 1 + c × 2 + d × 3 + e × 4 + f × 5 + g × 6 + h × 7) / 100, where the letters are the number of fruits in each level of classification considered.

To assess infestation level, from 4th August to 23rd November 20 random handpicked fruits from each olive tree (5 trees per cultivar; 100 fruits) were collected fortnightly and inspected in a binocular stereomicroscope for signs of infestation (oviposition sites or exit holes). Infestation level was expressed as the percentage of infested olive fruits.

Volatile characterization

SPME fibers

For the headspace solid-phase microextraction (HS-SPME) a fiber coated with divinylbenzene/carbonex/polydimethylsiloxane (DVB/CAR/PDMS), 50/30 μm was selected based on a preliminary assay conducted with further two fibers (CAR/PDMS 75 μm and PDMS 100 μm), all from Supelco (Bellefonte, USA). Selection of the fiber was based on the highest qualitative (number of volatiles extracted) and quantitative data (peak areas) of a sample of olive leaves from *cv.* Cobrançosa.

HS-SPME

The HS-SPME was carried out according to the methodology applied by our research group in other matrices (Malheiro *et al*., 2013), with some modifications. Five healthy leaves (approximately 1 g) were placed in 50 ml vials, deuterated chloroform (99.96%, Aldrich) was added as internal standard (250 ppm in methanol; 10 μl) and immediately sealed with a polypropylene cap with silicon septum. The volatiles were released at 40 ºC during 30 min, in an ultrasonic bath. After that, the DVB/CAR/PDMS fiber was exposed during 30 min at 40 ºC for volatiles adsorption, and then inserted into the injection port of the GC system for thermal desorption and reconditioning (10 min at 280 ºC). For each harvest moment and olive cultivar the HS-SPME analysis was performed in quintuplicate.

Gas chromatography mass spectrometry (GC-MS) conditions

Chromatographic analysis was performed using an Agilent 6890 series GC (Agilent, Avondale, PA, USA), with split less injection, coupled to a MS detector (Agilent 5973). Volatiles were separated using a bounded phase fused-silica capillary column (SPB-5, 60 m × 0.32 mm × 1 μm, Supelco, Bellefonte, USA), operating at constant flow with helium at 1 ml min^{-1}. The oven temperature program was isothermal for 5 min at 40 ºC, raised to 220 ºC at a rate of 3 ºC min^{-1} and maintained at 220 ºC for 2 min, with a total run of 67 min. The transfer line to the mass spectrometer was maintained at 250 ºC. Mass spectra were obtained by electronic impact at 70 eV, with a multiplier voltage of 2056 V,

collecting data at a rate of 1 scan s^{-1} over the range 30 – 500 *m/z*. The constituents were identified by comparing the experimental spectra with spectra from NIST 98 data bank (NIST/EPA/NISH Mass Spectral Library, version 1.6, U.S.A.), and also by comparison of their GC Kovats index (Adams, 2007). For quantification purposes, the chromatographic peak areas were determined using for each compound the correspondent base ion (m/z 100% intensity), using the internal standard area as reference.

Statistical analysis

Analysis of variance

An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov-Smirnov with Lilliefors correction (if n>50) or the Shapiro-Wilk`s test (if n<50), and the Levene´s tests, respectively. All dependent variables were analysed using a one-way ANOVA with or without Welch correction, depending if the requirement of the homogeneity of variances was fulfilled or not. The main factor studied was the effect of harvest moment in the volatile composition of the three studied olive cultivars. If a statistical significant effect was found, means were compared using Tukey´s honestly significant difference multiple comparison test or Dunnett T3 test also depending if equal variances could be assumed or not. All statistical tests were performed at a 5% significance level.

Principal component analysis

Principal components analysis (PCA) was applied for reducing the number of variables in the three olive leaves cultivars to a smaller number of new derived variables (principal component or factors) that adequately summarize the original information, i.e., the effect of collection time and cultivar on the volatile composition of olive leaves and their relation to olive fly cultivar preference. Variables corresponding to 11 of the most abundant volatile compounds identified, olive fly infestation levels, and total volatile amounts (mg/kg) at the last three sampling dates (4^{th} Oct; 21^{st} Oct, and 9^{th} Nov) were

combined. PCA was performed by using SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.).

Results

Infestation level and maturation index

Fruit infestation levels in cvs. Cobrançosa, Madural and Verdeal Transmontana were assessed fortnightly in order to observe the preferences of olive fly towards the three olive cultivars during fruits maturation. The results obtained are reported in Figure 5.2A. During the assessed period (from 4th Aug. to 23rd Nov.) higher infestation levels are clearly observed in cv. Verdeal Transmontana, followed by cv. Madural, and finally, cv. Cobrançosa the less attacked olive cultivar,. During August, low infestation levels were observed, with 16% in cv. Verdeal Transmontana 16%, while cvs. Madural and Cobrançosa reported 5 and 2%, respectively. In this period olives maturation index (MI) was 0 for all cultivars (Figure 5.2A).

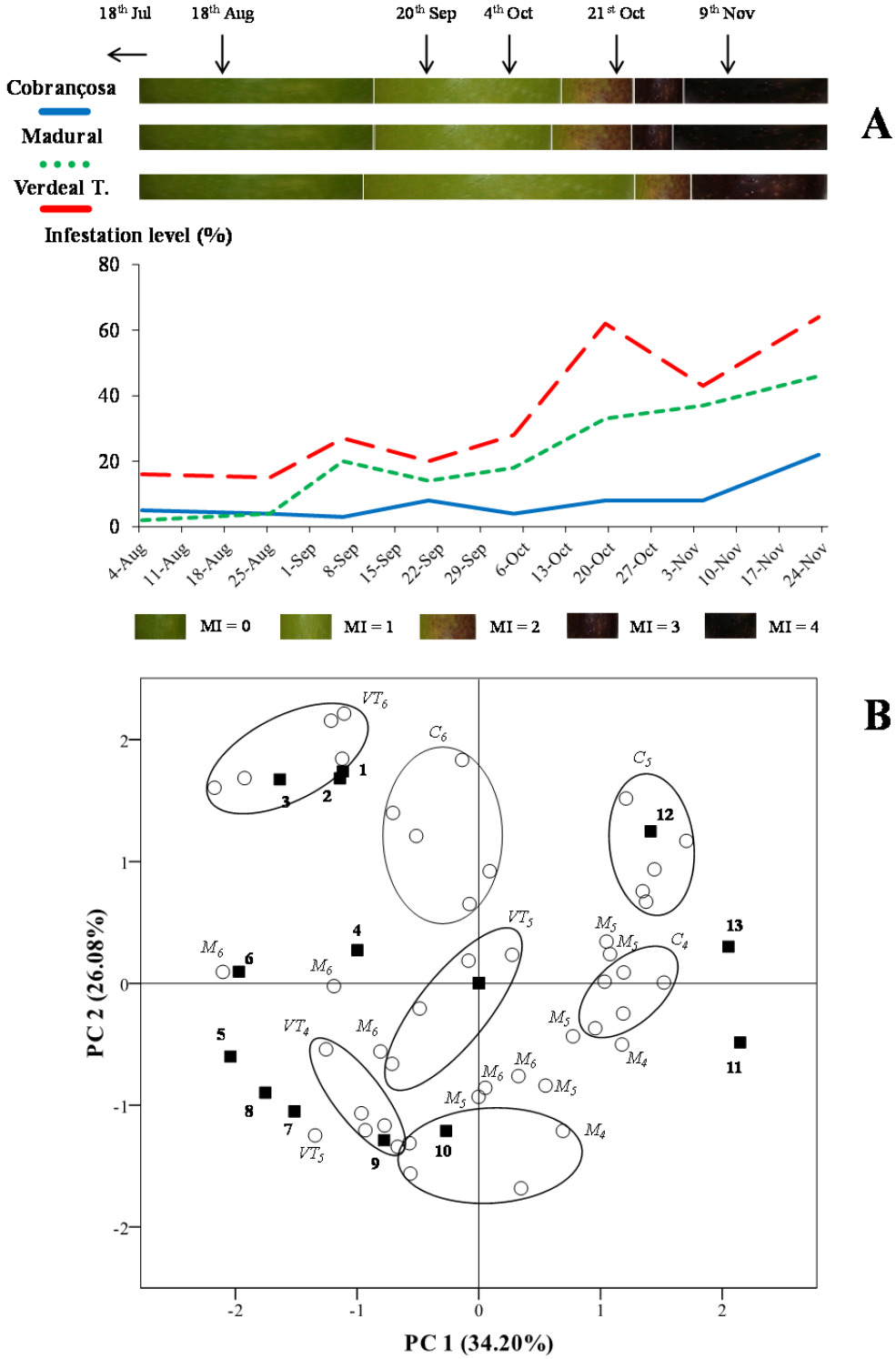

Figure 5.2. *Bactrocera oleae* infestation levels (%) and olives maturation from the cultivars Cobrançosa, Madural and Verdeal Transmontana (Fig. 5.2A). Olive cultivar Verdeal Transmontana is the main "target" of olive fly with higher infestation levels, followed by cv. Madural, and by last, the less susceptible olive cultivar, Cobrançosa. Comparatively to cvs. Madural and Cobrançosa, cv. Verdeal Transmontana presents a slower maturation process.Principal component analysis (Fig. 5.2B) obtained from the

main volatile compounds of olive leaves from cvs. Cobrançosa, Madural, and Verdeal Transmontana at different harvesting times along fruit maturation (4th Oct (C_4, M_4 and VT_4); 21st Oct (C_5, M_5 and VT_5); 9th Nov (C_6, M_6 and VT_6)). 1 – Butanoic acid, 3-methyl-methyl ester; 2 – hexanoic acid methyl ester; 3 – limonene; 4 – infestation level; 5 – toluene; 6 – β-caryophyllene; 7 - butanoic acid, 2-methyl- methyl ester; 8 – benzoic acid methyl ester; 9 – ρ-xylene; 10 – butanoic acid methyl ester; 11 – (*Z*)-3-hexen-1-ol acetate; 12 - (*Z*)-3-hexen-1-ol; 13 – total volatiles (µg of volatiles/100 g of olive leaves). The principal components (PC) explain 60.28% of the total variance. According to the most abundant volatiles found, it was possible to differentiate olive cultivars and harvest dates, mainly cvs. Cobrançosa and Verdeal Transmontana.

In September infestation levels increased in cvs. Madural and Verdeal Transmontana while cv. Cobrançosa remained with a low infestation rate. In this period all cultivars reported MI between 0 and 1 (Figure 5.2A). In October a considerable infestation increase was observed in cvs. Verdeal Transmontana and Madural olive fruits, achieving 62 and 33% respectively at the 19th Oct, while cv. Cobrançosa remained with only 8% of infested fruits. At the end of October, olive fruits from cvs. Cobrançosa and Madural reported a MI around 3, while cv. Verdeal Transmontana reported a MI between 1 and 2 (Figure 5.2A). Near to olives harvest moment (23rd Nov) the infestation levels in cvs. Verdeal Transmontana and Madural were 64 and 46%, respectively, while Cobrançosa had about 22%. At that date olives from Cobrançosa and Madural were already at advanced maturation (MI between 4 and 5) while cv. Verdeal Transmontana showed a slower maturation process, since olives from this cultivar were at a MI of 3 (Figure 5.2A), being a natural characteristic of this cultivar.

Volatile amounts and composition

The volatile fraction of olive leaves from cvs. Cobrançosa, Madural, and Verdeal Transmontana were assessed at six different harvest periods during olive fruits maturation. Overall 39 volatile compounds were identified: 5 alcohols, 3 aldehydes, 12 esters, 3 ketones, 9 sesquiterpenes, 4 terpenes, and 3 aromatic hydrocarbons (Tables 5.1-3).

Table 5.1. Volatile composition (%; mean ± standard error) and total volatile amounts (µg.100 g^{-1} of olive leaves) of cv. Cobrançosa olive leaves at different harvest times (in the same line, mean values with different letters differ significantly ($P < 0.05$); (1) $P < 0.05$, by means of Levene test. *P* values are those from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since equal variances could not be assumed; (2) $P > 0.05$, be means of Levene test. *P* values are those from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed).

Chemical class	Compound	18th Jul	18th Aug	20th Sep	4th Oct	21st Oct	9th Nov	*P*-value
	Total volatiles (µg 100 g^{-1})	519 ± 81 a	1334 ± 164 b	2194 ± 175 b,c	5143 ± 617 c,d	6830 ± 654 d	608 ± 31 a	< 0.001(1)
Alcohols								
	3-Methyl-1-butanol	0.5 ± 0.2 a	0.3 ± 0.0 a	0.4 ± 0.2 a	tr.	0.2 ± 0.0 a	-	0.172(1)
	3-Hexanol	-	1.3 ± 0.5 a	0.1 ± 0.0 a	tr.	-	-	0.094(1)
	(*Z*)-3-hexen-1-ol	4.0 ± 1.7 a,b	1.8 ± 0.5 a	4.3 ± 1.1 a,b	12.6 ± 1.3 c	27.4 ± 2.1 d	9.8 ± 2.7 b,c	< 0.001(2)
	Hexanol	-	-	-	-	1.4 ± 0.3	-	-
	Octanol	-	-	-	-	tr.	-	-
	Σ of alcohols	4.4 ± 1.6 a,b	3.4 ± 0.5 a	4.9 ± 1.0 a	12.7 ± 1.2 b	29.0 ± 2.3 c	9.8 ± 2.7 a,b	< 0.001(1)
Aldehydes								
	Hexanal	0.8 ± 0.3 b	-	0.1 ± 0.0 a	-	tr.	-	0.030(2)
	Nonanal	-	0.5 ± 0.2 b	0.1 ± 0.0 a	tr.	tr.	-	0.044(2)
	Decanal	-	0.6 ± 0.1	-	tr.	-	-	-
	Σ of aldehydes	0.8 ± 0.3 a,b	1.0 ± 0.3 b	0.2 ± 0.0 a	0.1 ± 0.0 a	0.1 ± 0.0 a	-	0.023(1)
Esters								
	Butanoic acid methyl ester	8.2 ± 2.6 b	8.4 ± 1.6 b	3.7 ± 1.5 a	8.9 ± 2.8 b	2.4 ± 0.6 a	2.0 ± 0.3 a	0.031(2)
	Butanoic acid, 3-methyl-, methyl ester	6.1 ± 2.8 a	1.3 ± 0.5 a	5.6 ± 1.9 a	0.5 ± 0.1 a	2.2 ± 0.4 a	39.3 ± 6.3 b	0.003(1)
	Butanoic acid, 2-methyl-, methyl ester	14.9 ± 2.1 c	10.3 ± 3.4 b,c	1.4 ± 0.7 a	3.3 ± 0.7 a,b	0.6 ± 0.2 a	-	0.002(1)
	Hexanoic acid methyl ester	0.7 ± 0.1 a,b	1.0 ± 0.3 a,b	0.4 ± 0.0 a	1.0 ± 0.1 a,b	1.7 ± 0.5 b	1.2 ± 0.2 a,b	0.006(1)
	(*Z*)-3-hexenoic acid methyl ester	4.3 ± 1.9 a	-	2.7 ± 0.9 a	4.5 ± 0.8 a	11.8 ± 1.5 b	-	0.001(2)
	(*E*)-2-hexenoic acid methyl ester	-	-	-	-	1.0 ± 0.2	-	-
	(*Z*)-3-hexen-1-ol acetate	49.8 ± 6.6 a-c	58.9 ± 8.7 b,c	68.7 ± 2.0 c	57.6 ± 4.2 b,c	42.6 ± 4.1 a,b	32.4 ± 4.6 a	0.001(1)
	Hexyl acetate	1.0 ± 0.3 a	1.4 ± 0.3 a	0.7 ± 0.1 a	0.5 ± 0.1 a	1.1 ± 0.2 a	-	0.065(2)
	Benzoic acid methyl ester	2.2 ± 1.1 b	2.4 ± 0.6 b	0.4 ± 0.1 a	2.6 ± 0.7 b	0.5 ± 0.1 a	-	0.043(1)
	(*Z*)-3-hexenyl isobutyrate	-	-	-	0.2 ± 0.0	-	-	-
	(*Z*)-3-hexenyl butyrate	-	-	1.1 ± 0.2 a	3.3 ± 0.3 b	-	-	< 0.001(2)
	(*E*)-3-hexenyl ester	2.4 ± 1.0	-	-	-	-	-	-
	Σ of esters	89.5 ± 1.9 a	83.7 ± 4.0 a	84.9 ± 1.4 a	82.5 ± 1.3 a	63.8 ± 1.6 b	74.8 ± 2.2 a,b	< 0.001(2)
Sesquiterpenes								
	α-Cubebene	-	tr.	-	-	-	-	-
	α-Copaene	0.1 ± 0.0 a	0.3 ± 0.1 a	0.1 ± 0.0 a	tr.	tr.	0.8 ± 0.1 b	< 0.001(2)
	β-Bourbonene	-	0.2 ± 0.0 b	0.1 ± 0.0 a	-	-	-	0.025(2)
	β-Caryophyllene	0.6 ± 0.3 a	0.5 ± 0.1 a	tr.	0.2 ± 0.1 a	0.1 ± 0.0 a	-	0.160(2)
	α-Caryophyllene	-	0.2 ± 0.0	-	-	-	-	-

	Germacrene D	-	0.1 ± 0.0	-	-	-	-	-
	α-Farnesene	-	-	-	0.3 ± 0.2	-	-	-
	Valencene	-	-	-	tr.	-	-	-
	Δ-Cadinene	-	0.1 ± 0.0	-	-	-	-	-
	Σ of sesquiterpenes	0.7 ± 0.3 a,b	1.4 ± 0.3 b	0.3 ± 0.1 a	0.6 ± 0.2 a,b	0.2 ± 0.0 a	0.8 ± 0.1 b	0.001[(1)]
Terpenes								
	α-Pinene	-	-	0.1 ± 0.0	-	-	-	-
	ρ-Cymene	-	0.5 ± 0.1 b	-	0.1 ± 0.0 a	-	-	0.047[(2)]
	Limonene	0.8 ± 0.2 a,b	2.9 ± 1.0 b	0.6 ± 0.2 a	0.1 ± 0.0 a	0.2 ± 0.0 a	1.8 ± 0.3 a,b	0.004[(1)]
	β-Ocymene	-	0.5 ± 0.1	-	tr.	tr.	-	-
	Σ of terpenes	0.8 ± 0.2 a	3.9 ± 1.2 a	0.7 ± 0.2 a	0.3 ± 0.0 a	0.3 ± 0.0 a	1.8 ± 0.3 a	0.083[(1)]
Aromatic hydrocarbons								
	Toluene	1.8 ± 0.7 a	3.1 ± 1.4 a,b	8.0 ± 1.8 b,c	3.5 ± 0.1 a,b	6.4 ± 0.8 a,b	12.8 ± 1.0 c	< 0.001[(1)]
	ρ-Xylene	2.0 ± 0.7 a,b	2.4 ± 0.6 b	0.9 ± 0.2 a,b	0.4 ± 0.0 a	0.2 ± 0.0 a	-	0.007[(1)]
	σ-Xylene	-	1.5 ± 0.4 b	0.5 ± 0.1 a,b	0.2 ± 0.0 a	tr.	-	0.015[(1)]
	Σ of aromatic hydrocarbons	3.7 ± 1.4 a	6.9 ± 2.4 a	9.4 ± 2.1 a,b	3.8 ± 0.2 a	6.7 ± 0.8 a	12.8 ± 1.0 b	0.047[(1)]

Table 5.2. Volatile composition (%; mean ± standard error) and total volatile amounts (μg 100 g^{-1} of olive leaves) of cv. Madural olive leaves at different harvest times (in the same line, mean values with different letters differ significantly ($P < 0.05$); [(1)] $P < 0.05$, by means of Levene test. *P* values are those from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since equal variances could not be assumed; [(2)] $P > 0.05$, be means of Levene test. *P* values are those from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed).

Chemical class	**Compound**	**18th Jul**	**18th Aug**	**20th Sep**	**4th Oct**	**21st Oct**	**9th Nov**	***P*-value**
	Total volatiles (μg 100 g^{-1})	379 ± 52 a	1360 ± 128 b	307 ± 128 a	2298 ± 236 b	2616 ± 290 b	475 ± 65 a	< 0.001[(1)]
Alcohols								
	3-Methyl-1-butanol	0.4 ± 0.1 a	0.4 ± 0.1 a	0.3 ± 0.0 a	-	-	-	0.605[(2)]
	3-Hexanol	-	2.6 ± 0.9 b	1.0 ± 0.3 a,b	0.3 ± 0.2 a	-	-	0.050[(2)]
	(Z)-3-hexen-1-ol	1.3 ± 0.4 a	1.9 ± 0.7 a	2.2 ± 0.5 a	4.7 ± 1.4 a	12.5 ± 6.6 a	6.1 ± 1.5 a	0.116[(1)]
	Σ of alcohols	1.7 ± 0.4 a	4.9 ± 0.8 a	3.6 ± 0.6 a	5.0 ± 1.2 a	12.5 ± 6.6 a	6.1 ± 1.5 a	0.186[(1)]
Aldehydes								
	Hexanal	-	-	0.5 ± 0.1	-	-	-	-
	Nonanal	-	0.6 ± 0.2 a	0.3 ± 0.0 a	0.2 ± 0.1 a	-	-	0.136[(1)]
	Decanal	-	0.3 ± 0.0 a	-	0.2 ± 0.1 a	-	-	0.128[(2)]
	Σ of aldehydes	-	1.0 ± 0.3 a	0.8 ± 0.0 a	0.4 ± 0.1 a	-	-	0.088[(2)]
Esters								
	Butanoic acid methyl ester	5.9 ± 1.4 a	5.7 ± 0.5 a	4.8 ± 1.4 a	21.2 ± 10.6 b	4.1 ± 1.0 a	7.2 ± 0.3 a	0.030[(2)]
	Butanoic acid, 3-methyl-, methyl	6.5 ± 1.7 a	1.3 ± 0.5 a	1.5 ± 0.5 a	-	1.2 ± 0.6 a	9.0 ± 5.1 a	0.166[(1)]

	ester							
	Butanoic acid, 2-methyl-, methyl ester	26.5 ± 2.9 c	22.2 ± 6.9 b,c	15.5 ± 5.0 a-c	4.0 ± 0.8 a	5.2 ± 1.2 a,b	11.8 ± 2.4 a-c	0.002(1)
	Hexanoic acid methyl ester	-	0.5 ± 0.1 a	-	0.5 ± 0.1 a	0.5 ± 0.1 a	1.4 ± 0.3 b	0.013(2)
	(*Z*)-3-hexenoic acid methyl ester	-	-	-	0.7 ± 0.3	-	-	-
	(Z)-3-hexen-1-ol acetate	49.7 ± 6.6 a	45.9 ± 10.5 a	32.8 ± 6.8 a	40.2 ± 11.8 a	52.3 ± 5.7 a	38.6 ± 12.3 a	0.685(2)
	Hexyl acetate	4.1 ± 1.0 b	1.3 ± 0.4 a	2.3 ± 0.5 a,b	0.7 ± 0.3 a	1.2 ± 0.3 a	-	0.005(2)
	Benzoic acid methyl ester	3.0 ± 1.6 a	4.4 ± 1.3 a	3.2 ± 1.2 a	4.5 ± 1.3 a	1.3 ± 0.4 a	-	0.378(2)
	(Z)-3-hexenyl isobutyrate	-	-	-	0.4 ± 0.2	-	-	-
	(Z)-3-hexenyl butyrate	-	-	-	3.2 ± 1.3	-	-	-
	Σ of esters	95.6 ± 0.8 b	81.4 ± 3.9 a,b	60.1 ± 6.1 a	75.4 ± 7.9 a,b	65.8 ± 6.5 a	68.0 ± 7.0 a	
Ketones								
	6-Methyl-5-hepten-2-one	-	0.7 ± 0.2	-	-	-	-	-
	Σ of ketones	-	0.7 ± 0.2	-	-	-	-	-
Sesquiterpenes								
	α-Copaene	0.2 ± 0.0 a	0.1 ± 0.0 a	-	0.1 ± 0.0 a	0.1 ± 0.1 a	-	0.855(2)
	β-Caryophyllene	0.4 ± 0.1 a	0.2 ± 0.0 a	0.2 ± 0.0 a	0.2 ± 0.1 a	0.1 ± 0.0 a	0.3 ± 0.1 a	0.242(2)
	α-Caryophyllene	-	0.1 ± 0.0	-	-	-	-	-
	α-Farnesene	-	0.5 ± 0.2	-	-	-	-	-
	Σ of sesquiterpenes	0.5 ± 0.1 a	1.0 ± 0.3 a	0.2 ± 0.0 a	0.3 ± 0.1 a	0.3 ± 0.1 a	0.3 ± 0.1 a	0.237(1)
Terpenes								
	α-Pinene	-	-	0.2 ± 0.0	-	-	-	-
	ρ-Cymene	-	0.5 ± 0.1 a	-	0.5 ± 0.2 a	-	-	0.912(2)
	Limonene	0.4 ± 0.1 a	3.0 ± 1.0 b	0.8 ± 0.2 a	0.4 ± 0.2 a	0.3 ± 0.1 a	1.9 ± 0.5 a,b	0.004(2)
	β-Ocymene	-	1.8 ± 1.1 a	-	0.4 ± 0.2 a	-	-	0.265(2)
	Σ of terpenes	0.4 ± 0.1 a	5.3 ± 1.3 b	1.1 ± 0.2 a	1.4 ± 0.6 a	0.3 ± 0.1 a	1.9 ± 0.5 a,b	0.019(1)
Aromatic hydrocarbons								
	Toluene	0.8 ± 0.2 a	2.1 ± 0.7 a	27.4 ± 4.5 c	14.2 ± 7.3 b	20.7 ± 7.4 b	23.7 ± 7.9 b	0.014(2)
	ρ-Xylene	0.9 ± 0.2 a	2.2 ± 0.6 a,b	4.3 ± 1.0 b	2.0 ± 0.9 a,b	0.4 ± 0.1 a	-	0.007(2)
	σ-Xylene	-	1.5 ± 0.4 a	2.4 ± 0.5 a	1.4 ± 0.7 a	-	-	0.372(2)
	Σ of aromatic hydrocarbons	1.6 ± 0.4 a	5.8 ± 1.7 a	34.1 ± 6.0 b	17.6 ± 8.2 a,b	21.1 ± 7.5 a,b	23.7 ± 7.9 a,b	0.009(1)

Table 5.3. Volatile composition (%; mean ± standard error) and total volatile amounts (µg 100 g^{-1} of olive leaves) of cv. Verdeal Transmontana olive leaves at different harvest times (In the same line, mean values with different letters differ significantly ($P < 0.05$); (1) $P < 0.05$, by means of Levene test. *P* values are those from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since equal variances could not be assumed; (2) $P > 0.05$, be means of Levene test. *P* values are those from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed).

Chemical class	Compound	18th Jul	18th Aug	20th Sep	4th Oct	21st Oct	9th Nov	*P*-value
	Total volatiles (µg 100 g^{-1})	503 ± 72 a,b	1598 ± 36 d	330 ± 8 a	834 ± 71 b,c	1009 ± 149 c	332 ± 37 a	< 0.001(2)
Alcohols								
	3-Methyl-1-butanol	-	15.1 ± 8.1 b	0.9 ± 0.2 a	-	-	-	0.030(2)
	3-Hexanol	-	2.0 ± 1.5 a	0.8 ± 0.3 a	-	-	-	0.498(1)
	(Z)-3-hexen-1-ol	5.6 ± 0.7 a	19.0 ± 14.2 b	4.3 ± 1.4 a	7.0 ± 0.7 a	20.6 ± 8.7 b	12.6 ± 4.2 a,b	< 0.001(2)
	Hexanol	-	1.2 ± 0.8	-	-	-	-	-
	Octanol	-	0.1 ± 0.0	-	-	-	-	-
	Σ of alcohols	5.6 ± 0.7 a	37.4 ± 15.3 b	6.0 ± 1.3 a	7.0 ± 0.7 a	20.6 ± 8.7 a,b	12.6 ± 4.2 a,b	0.023(2)
Aldehydes								
	Nonanal	-	0.5 ± 0.1a	0.2 ± 0.0 a	-	-	-	0.095(2)
	Decanal	-	0.3 ± 0.1	-	-	-	-	-
	Σ of aldehydes	-	0.8 ± 0.3	0.2 ± 0.0	-	-	-	0.051(2)
Esters								
	Butanoic acid methyl ester	7.5 ± 2.0 a	2.8 ± 1.1 a	7.9 ± 4.8 a	8.0 ± 0.1 a	8.9 ± 1.3 a	5.1 ± 2.0 a	0.051(1)
	Butanoic acid, 3-methyl-, methyl ester	5.4 ± 1.6 b	0.9 ± 0.4 a	4.8 ± 2.2 b	1.1 ± 0.0 a	1.7 ± 0.4 a	33.6 ± 5.5 c	0.007(1)
	Butanoic acid, 2-methyl-, methyl ester	13.6 ± 2.3 b	2.5 ± 0.9 a	7.9 ± 1.6 a,b	7.2 ± 1.9 a,b	6.3 ± 2.8 a,b	3.4 ± 0.9 a	0.005(2)
	Hexanoic acid methyl ester	-	0.5 ± 0.1 a	1.0 ± 0.2 a	1.2 ± 0.2 a,b	-	2.1 ± 0.4 b	0.004(2)
	(*Z*)-3-hexenoic acid methyl ester	-	-	-	0.6 ± 0.1	-	-	-
	(Z)-3-hexen-1-ol acetate	50.4 ± 8.3 b	40.2 ± 16.9 a,b	27.4 ± 1.7 a,b	21.1 ± 4.2 a,b	22.9 ± 4.2 a,b	10.4 ± 3.0 a	0.013(1)
	Hexyl acetate	1.3 ± 0.5 a	0.9 ± 0.4 a	-	-	-	-	0.889(2)
	Benzoic acid methyl ester	5.9 ± 2.1 b	1.4 ± 0.8 a	2.2 ± 0.2 a	13.6 ± 0.4 c	9.4 ± 3.5 b,c	-	< 0.001(2)
	(Z)-3-hexenyl butyrate	-	-	-	1.8 ± 0.1	-	-	-
	Σ of esters	83.9 ± 3.1 b	49.3 ± 17.0 a	51.1 ± 6.4 a,b	54.8 ± 2.8 a,b	50.6 ± 1.6 a	54.7 ± 5.5 a,b	< 0.001(2)
Ketones								
	3-Pentanone	3.9 ± 1.1 a	4.2 ± 2.1 a	-	-	-	-	0.890(2)
	3-Hexanone	-	1.1 ± 0.8	-	-	-	-	-
	6-Methyl-5-hepten-2-one	-	1.2 ± 0.6	-	-	-	-	-
	Σ of ketones	3.9 ± 1.1	6.5 ± 3.4	-	-	-	-	0.446(2)
Sesquiterpenes								
	α-Copaene	0.4 ± 0.2 a	-	-	-	0.2 ± 0.1 a	-	0.273(2)
	β-Bourbonene	-	0.2 ± 0.1	-	-	-	-	-
	β-Caryophyllene	1.1 ± 0.4 b	0.1 ± 0.0 a	0.2 ± 0.1 a	0.3 ± 0.0 a	0.3 ± 0.1 a	0.4 ± 0.1 a	0.003(1)
	Valencene	-	-	0.1 ± 0.0a	0.6 ± 0.2 b	-	-	0.016(2)
	Δ-Cadinene	-	-	0.2 ± 0.0	-	-	-	-
	Σ of sesquiterpenes	1.5 ± 0.3 b	0.3 ± 0.0 a	0.6 ± 0.1 a	0.9 ± 0.2 a,b	0.4 ± 0.0 a	0.4 ± 0.1 a	0.001(2)

Terpenes								
	α-Pinene	-	-	0.3 ± 0.1	-	-	-	-
	Limonene	1.4 ± 0.5 a	1.5 ± 0.6 a	2.1 ± 0.6 a	0.7 ± 0.1 a	0.3 ± 0.1 a	8.1 ± 1.8 b	< 0.001[(2)]
	β-Ocymene	-	0.3 ± 0.1 a	-	0.3 ± 0.1 a	-	-	0.833[(2)]
	Σ of terpenes	1.4 ± 1.0 a	1.8 ± 0.6 a	2.4 ± 0.7 a	1.0 ± 0.2 a	0.3 ± 0.1 a	8.1 ± 1.8 b	< 0.001[(2)]
Aromatic hydrocarbons								
	Toluene	2.0 ± 1.1 a	1.5 ± 0.5 a	34.2 ± 6.0 c	34.0 ± 3.6 c	27.9 ± 7.1 b,c	24.2 ± 6.8 b	< 0.001[(1)]
	ρ-Xylene	1.7 ± 0.6 a,b	1.3 ± 0.5 a	3.6 ± 0.7 b	1.2 ± 0.2 a	0.2 ± 0.1 a	-	0.003[(2)]
	σ-Xylene	-	0.9 ± 0.4 a	1.9 ± 0.3 a	1.2 ± 0.2 a	-	-	0.143[(2)]
	Σ of aromatic hydrocarbons	3.7 ± 1.6 a	3.8 ± 1.4 a	39.7 ± 7.0 b	36.3 ± 3.6 b	28.1 ± 7.1 b	24.2 ± 6.8 a,b	< 0.001[(2)]

Quantitative and qualitatively changes were observed among olive cultivar and harvest period. Considerable quantitative variations were also observed in the volatile composition of olive leaves according to the collection date assessed and olive cultivar. Higher amounts of volatile emission are observed with cv. Cobrançosa leaves (Table 5.1), followed by cvs. Madural and Verdeal Transmontana (Tables 5.2 and 5.3 respectively). At the 18th Jul (first sampling date) no significant differences (P = 0.325) were observed between the three olive cultivars, varying the total volatile emission between 379 µg/100 g leaves (cv. Madural) and 519 µg/100 g (cv. Cobrançosa). One month later, total volatile emission almost triplicate in the three cultivars, also without significant differences between the studied cultivars (P = 0.280). Afterwards, in the subsequent harvest moments a high volatile emission was consistently verified in cv. Cobrançosa comparatively to the other two olive cultivars: 2194, 5143, and 6830 µg/100 g respectively at 20th Sep, 4th Oct, and 21st Oct (P < 0.001 for the three assessed harvest moments). At the last sampling date volatile emissions from the three olive cultivars dropped considerably to values from 332 µg/100 g (cv. Verdeal Transmontana) and 608 µg/100 g (cv. Cobrançosa), still with significant differences among cultivars (P = 0.006).

Globally, 36 compounds were identified in cv. Cobrançosa (Table 5.1), 28 in cv. Madural (Table 5.2), and 30 in cv. Verdeal Transmontana (Table 5.3).

Independently of olive cultivar, characteristic green leaf volatiles (GLV's) were the predominant volatiles present in olive leaves. Esters were the main chemical class identified in all cultivars analyzed during the different sampling dates assessed. At the first sampling date their content varied between 83.9% in cv. Verdeal Transmontana and 95.6% in cv. Madural. During the entire study esters decrease in relative abundance, with small variations in cv. Verdeal Transmontana between the second (18th Aug) and last sampling dates (9th Nov). In cv. Cobrançosa a considerable increase was observed from the fifth to the sixth and last sampling date. Such trends were dependent on the individual esters identified in the volatile fraction of the olive leaves. For instance, (*Z*)-3-hexen-1-ol acetate was the most abundant compound among all the volatiles identified in the three cultivars. This ester decreased significantly during the surveyed period, especially in cv. Verdeal Transmontana (P = 0.013). In cv. Cobrançosa it increased from the 18th Jul to 20th Sep, and afterwards decrease until the end of the surveyed period (9th Nov), reaching a minimum value of 32.4% (Table 5.1). By contrast, in cv. Madural the same compound decreased from 18th Jul to 20th Sep and, afterwards increased to maximum value of 52.3% at 21st Oct. From this date until the end (*Z*)-3-hexen-1-ol acetate decreased (Table 5.2). For cv. Verdeal Transmontana the reduction was more evident, from 50.4% to 10.4% (first and last dates; Table 5.3). Other ester, the methyl ester of 2-methyl-butanoic acid, was

present in relative high abundance at first sampling date with 14.9, 26.5, and 13.6% in cvs. Cobrançosa, Madural and Verdeal Transmontana, respectively. This compound also decreased significantly (P = 0.002 for cvs. Cobrançosa and Madural; P = 0.005 for cv. Verdeal Transmontana) along fruit maturation. In cv. Madural it decreased until 4th Oct with a minimum value of 4.0%, increasing then until 9th Nov (11.8%; Table 2). In cv. Verdeal Transmontana the maximum values were reported at 18th Jul (13.6%), decreasing significantly until 18th Aug (2.5%), increasing again to 20 Sep (8.3%), and decreasing steadily afterwards until 9th Nov (3.4%). In an opposite trend was the methyl ester of 3-methyl-butanoic acid. This compound increased considerably during fruits maturation. In cv. Cobrançosa it varied between 6.1 and 2.2% from 18th Jul to 21st Oct, with minimum abundance at 4th Oct (0.5%). A significant maximum value was quantified at the 9th Nov with 39.3%. A similar trend was observed in cv. Verdeal Transmontana with significant higher abundance at 9th Nov (33.6%). In this cultivar butanoic acid, 3-methyl-, methyl ester reported values between 5.4 and 1.7% between 18th Jul and 21st Oct, reporting minimum values at 18th Aug (0.9%). In cv. Madural butanoic acid, 3-methyl-, methyl ester was absent from the third sampling (20th Sep), and reported higher values at 9th Nov, 9.0%, significantly lower comparatively to cvs. Cobrançosa and Verdeal Transmontana for the same period assessed. From the remaining esters identified, the significant higher abundance of butanoic acid methyl ester in cv. Madural at 4th Oct with 21.2% should be highlighted (Table 5.2).

Regarding alcohols, five compounds were identified, namely 3-methyl-1-butanol, 3-hexanol, (*Z*)-3-hexen-1-ol, 1-hexanol and 1-octanol (Table 5.1 to 5.3). These five compounds occurred in cvs. Cobrançosa and Verdeal Transmontana, while in cv. Madural only 3-methyl-1-butanol, 1-hexanol, and (*Z*)-3-hexen-1-ol were detected. Nevertheless, only (*Z*)-3-hexen-1-ol was present in the entire surveyed period in all cultivars, being the main alcohol identified. During the surveyed period a similar trend of alcohols production was observed in the three cultivars. (*Z*)-3-hexen-1-ol and total alcohols abundance increased generally from 18th Jul to 21st Oct (Table 5.1 to 5.3) and afterwards decrease until 9th Nov. In cv. Cobrançosa minimum values of (*Z*)-3-hexen-1-ol were detected at 18th Aug (1.8%), in cv. Madural at 18th Jul (1.3%), and in cv. Verdeal Transmontana at 20th Sep (5.0%). Maximum values of (*Z*)-3-hexen-1-ol were reported at 21st Oct in the three cultivars in the following increasing order: cv. Madural (12.53%; Table 5.2) > cv. Verdeal Transmontana (20.6%; Table 5.3) > cv. Cobrançosa (27.4%; Table 5.1). Regarding total alcohols abundance an additional peak production in cv. Verdeal Transmontana must be highlighted, at the 18th Aug, with 37.4%. Such increase is related to the high abundance of

3-methyl-1-butanol (15.1%; Table 5.3), while in the remaining cultivars this alcohol had values always below 1%.

Aldehydes were present in low amounts. The identified aldehydes were: hexanal, nonanal and decanal. These aldehydes were present in cvs. Cobrançosa and Madural, but nonanal was absent in cv. Verdeal Transmontana (Table 5.3). Maximum percentage in aldehydes was reported at the second date (18th Aug) in all cultivars, with a maximum of 1.0% in cv. Cobrançosa. After that, aldehydes decreased until being undetected in the volatile fraction of the olive leaves from the three cultivars.

Regarding ketones, three compounds were identified: 3-pentanone, 3-hexanone, and 6-methyl-5-hepten-2-one. Ketones were not found in cv. Cobrançosa, and in cv. Madural only 6-methyl-5-hepten-2-one was identified at 18th Aug with a low percentage, 0.7% (Table 5.2). In cv. Verdeal Transmontana ketones were only identified in the two first surveyed dates, being responsible for about 6.5% of the volatile fraction in 18th Aug, particularly 3-pentanone, exclusively found in this olive cultivar.

Sesquiterpenes are a minority fraction of volatile composition of olive leaves from the cultivars studied. Relative percentages of sesquiterpenes varied between 0.2 and 1.5%, being β-caryophyllene the most representative one (for detailed sesquiterpene composition consult Table 5.1 to 5.3). β-caryophyllene reported significantly higher content (P = 0.003) at 18th Jul in cv. Verdeal Transmontana (1.1%), dropping abruptly to 0.1% at 18th Aug, and showing slight increases onwards until 0.4% at 9th Nov (Table 5.3).

Terpenes, namely monoterpenic compounds, reported a similar trend in cvs. Cobrançosa and Madural. For these cultivars, higher contents of terpenes were reported at the 18th Aug and 9th Nov (second and last dates respectively). In the case of cv. Verdeal Transmontana higher terpenes abundance was reported at 20th Sep and 9th Nov. Limonene was consistently the most abundant monoterpene, with higher values observed in cvs. Cobrançosa and Madural at the 18th Aug and 9th Nov. while in cv. Verdeal Transmontana it represented 8% of the volatile fraction in 9th Nov, significantly higher than in the remaining cultivars (P = 0.007). α-Pinene was identified in the three cultivars but only at the third date (20th Sept), ranging between 0.1 and 0.3%. ρ-Cymene was not identified in the volatile profile of cv. Verdeal Transmontana, but was present in cvs. Cobrançosa and Madural (Table 1 to 3) in low amounts (≤ 0.5%).

Finally, three aromatic hydrocarbons were identified during the entire study: toluene, σ- and ρ-xylene. Comparatively to toluene, σ- and ρ-xylene were present in lower amounts. ρ-Xylene in cvs. Madural and Verdeal Transmontana increased from 18th Jul until 20th Sep, reducing their content gradually until 21st Oct, being absent in 9th Nov (Table 5.2 and 5.3). In cv. Cobrançosa this aromatic hydrocarbon increased from 18th Jul

to 18th Aug, being observed after that the same trend observed in the other two cultivars, a gradual decrease until 21st Oct and being absent at 9th Nov (Table 5.1). Toluene content represented a low percentage in the first date (at 18th Jul) with 1.8, 0.8, and 2.0% for cvs. Cobrançosa, Madural and Verdeal Transmontana, respectively (Table 5.1 to 5.3). Meanwhile, maximum significant values were reported between 20th Sep and 4th Oct, for cv. Madural (27.4%; $P = 0.014$) and cv. Verdeal Transmontana (34.0%; $P < 0.001$) respectively. For cv. Cobrançosa maximum values were reported later, at the 9th Nov, and 12.8% ($P < 0.001$) only, a significant lower value when compared to the other two cultivars ($P < 0.001$). The volatile composition found in our cultivars is quite different from that reported by Scarpati *et al*. (1993). These authors reported the chromatographic profile of main volatiles found in olive leaves but there is no information about the cultivar studied in their work.

Considering the periods where higher infestation levels were reported (4th and 21st Oct, and 9th Nov), and the most abundant volatiles present in those surveyed periods as well as total volatile emission (µg/100 g of olive leaves), a PCA was applied (Figure 5.2B). From Figure 5.2B it is perceived that samples from cvs. Cobrançosa and Verdeal Transmontana are easily grouped, while samples from cv. Madural are mainly dispersed in the entire region of principal component 1 (PC1). Olive leaves from cv. Cobrançosa are mainly grouped in the positive regions of both principal components. Samples from 4th (C_4) and 21st Oct (C_5) (fourth and fifth sampling dates) were mainly characterized by a higher content in GLV's, like (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol acetate and therefore, also, higher total volatile emissions in this sampling dates. Represented in an extreme opposite position are cv. Verdeal Transmontana olive leaves samples, those who exhaled lower amounts of volatiles. Samples from 9th Nov. of cv. Verdeal Transmontana were characterized by high amounts in esters, like butanoic acid, 3-methyl- methyl ester, and hexanoic acid methyl ester, as well as the monoterpene limonene. An interesting observation is that all the samples from cv. Verdeal Transmonatana are around the variable corresponding to infestation level, fomenting the idea that this cultivar is clearly susceptible to olive fly oviposition. It is also perceptible that toluene is represented in the extreme opposite of samples from cv. Cobrançosa, which means close to cv. Verdeal Transmontana olive leaves samples (Figure 5.2B).

Discussion

Secondary plant metabolites, like volatiles, are metabolized through enzymatic pathways. Their formation is therefore monitored by enzymes and influenced by several factors, being the cultivar (Brahmi *et al*., 2012), time of year (Campeol *et al*., 2003), and region important aspects. Therefore, the quantitative and qualitative changes observed in the present work are mainly related to cultivar specificities and the harvest time assessed, since olive trees surveyed were all cultivated in the same olive grove. Our results highlight for the presence of qualitative and quantitative differences among the volatile composition of the different cultivars assayed as well as the harvest moment assessed (Tables 5.1-5.3).

The main compounds found in the volatile fraction (GLV's) of the three cultivars are formed through the lipoxygenase pathway (LOX). The hydrolysis of polyunsaturated fatty acids (linoleic and α-linolenic acids) present in olive leaves is carried out by endogenous acyl-hydrolases, leading to the formation of free acids, modified by lipoxygenase enzymes at chain C9 and C13 positions and forming 9- and 13-hydroperoxydes. These fatty acids derivatives are then metabolized and excised by hydroperoxide lyases, leading to the formation of short chain C6 aldehydes, like (*Z*)-3-hexenal and hexanal. These C6 aldehydes are unstable and, spontaneously or by enzymatic action (enal-isomerases), can be converted to positional isomers or can be reduced to alcohols by alcohol dehydrogenases action. These C6 alcohols formed can be then esterified by alcohol acyltransferases yielding volatile esters ((*Z*)-3-hexen-1-ol is esterified to (*Z*)-3-hexen-1-ol acetate) (Akacha and Gargouri, 2009; Dudareva *et al*., 2006; Salas *et al*., 2005). The high contents of (*Z*)-3-hexen-1-ol acetate and (*Z*)-3-hexen-1-ol found in olive leaves volatile fraction, rather than hexanol and hexyl acetate is plausible since olive leaves are three times richer in linolenic acid than in linoleic acid (Guerfel *et al*., 2008), with higher activity in the LOX linoleic acid branch reported.

Along the surveyed period, the reduction of C6 esters and GLV's (more specifically (*Z*)-3-hexen-1-ol acetate), may be related to LOX pathway. We hypothesize that lipoxygenase activity in olive leaves was significantly influenced by the climatic conditions. Lipoxygenase is more active in stressed plants and drought conditions (Sofo *et al*., 2004). Therefore, high levels of GLV's in the first dates could be plausible due to a higher activity of lipoxygenase, since plants were in drought conditions for a long period. During collection time, mainly in the end of September and beginning of October, with the increasing rainfall, GLV's and (*Z*)-3-hexen-1-ol acetate reduce drastically their content, since lipoxygenase may reduce 3 times its activity in plants with water availability (Sofo *et*

al., 2004). Furthermore, LOX substrate (α-linoleic free fatty acids) could also be reduced, since olive leaves from irrigated olive trees reduce their linoleic acid content (Guerfel *et al*., 2008), influencing therefore LOX mechanisms.

A contrary trend to GLV's was observed for the aromatic hydrocarbon toluene. Toluene has been already described in olive oil and table olives (Baccouri *et al*., 2008; Iraqui *et al*., 2005). The origin of toluene in olive leaves and olive food products is still unknown. Some authors claim that toluene presence in plants can be from exogenous contamination, as well as from endogenous mechanisms (Biedermann *et al*., 1995), while others claim that toluene emission is enhanced in stressed plants (Heiden *et al*., 1999). However, in our study, the increase in toluene was observed in the period where olive tree was less stressed due to rainfall. In fact, in the present study, rainfall can be related to toluene significant increase in the olive leaves volatile composition. Some authors proved that aromatic hydrocarbons formation is improved and considerably enhanced by epiphytic microorganisms present in the olive leaves surface during the late summer rainfall (Scarpati *et al*., 1996). These authors verified the attraction of olive fly to olive orchards after first summer rainfall. The same research group proved that the aromatic hydrocarbons styrene and toluene are oviposition attractants to olive fly (Scarpati *et al*., 1993; Scarpati *et al*., 1996). In our work we found a positive correlation between toluene abundance in the olive leaves from the three olive cultivars and the infestation levels of the respective olive fruits (R^2 = 0.249; $P < 0.001$; $y = 0.31x+8.04$). Therefore, higher toluene contents could be ascribed with higher infestation levels, possibly related to the oviposition attractant properties of this aromatic hydrocarbon, as demonstrated by Scarpati *et al*. (1993) and Scarpati *et al*. (1996).

Another compound with important attractant activity to olive fly is the monoterpene limonene. This compound was present in higher amounts in cv. Verdeal Transmontana rather than cvs. Cobrançosa and Madural, especially at 9th Nov. The R-enantiomer of limonene, R-(+)-limonene, is highly attractive in wind tunnel assays to both olive fly sexes (De Cristofaro *et al*., 2007).

Tephritids fruit flies are influenced by several factors in host selection where volatiles display an important role (Aluja and Mangan, 2008). Our hypothesis is that volatiles emitted by olive leaves may interfere in olive fly females host selection in conjunction to other factors, like physical (fruit color, shape, volume) (Rizzo *et al*., 2012) and biochemical aspects (olives maturation) (Gonçalves *et al*., 2012). In fact, when host volatiles from olive leaves and olive fruits were tested in electroantenographic studies in males and females of olive fly (mated and unmated) higher EAG signals were obtained in olive leaves volatiles, especially in mated males (Liscia *et al*., 2013). Such result is

indicative that host volatiles are recognized by olive fly and their recognition is higher in olive leaves volatiles rather than olive fruit ones. Since volatiles are highly influenced by olive cultivar, olive leaves volatiles are probable important cues in host selection. A second factor in observation was the volatile amounts, clearly higher in cv. Cobrançosa olive leaves, representing probably a deterrent factor against other less intensive emissions from the other cultivars.

Conclusions

In conclusion, olive fly has cultivar preference towards cv. Verdeal Transmontana and Madural rather than cv. Cobrançosa. Volatiles type and amounts emitted by olive leaves are dependent on the olive cultivar and suffer considerable changes according to the harvest time surveyed. Olive leaves are mainly composed by esters, alcohols and the aromatic hydrocarbon toluene. For the olive cultivars studied correlations between toluene and infestation levels during olives maturation were reported. This compound may play an important role in the attractiveness of olive fly and in cultivar preference of this olive pest. Olive leaves volatiles may act as a short-range cue for olive fly, interacting with factors of other nature (other chemical and physical cues), and intervening in host selection by olive fly.

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CHAPTER 6.

Olive volatiles from Portuguese cultivars Cobrançosa, Madural and Verdeal Transmontana: role in oviposition preference of *Bactrocera oleae* (Rossi) (Diptera: Tephritidae)

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Abstract

The olive fly, *Bactrocera oleae* (Rossi), a serious threat to the olive crop worldwide, displays ovipositon preference for some olive cultivars but the causes are still unclear. In the present work, three Portuguese olive cultivars with different susceptibilities to olive fly (Cobrançosa, Madural, and Verdeal Transmontana) were studied, aiming to determine if the olive volatiles are implicated in this interaction. Olive volatiles were assessed by SPME-GC-MS in the three cultivars during maturation process to observe possible correlations with olive fly infestation levels. Overall, 34 volatiles were identified in the olives, from 7 chemical classes (alcohols, aldehydes, aromatic hydrocarbons, esters, ketones, sesquiterpenes, and terpenes). Generally, total volatile amounts decrease during maturation but toluene, the main compound, increased in all cultivars, particularly in those with higher susceptibility to olive fly. Sesquiterpenes also raised, mainly α-copaene. Toluene and α-copaene, recognized oviposition promoters to olive fly, were correlated with the infestation level of cvs. Madural and Verdeal Trasnmontana (intermediate and highly susceptible cultivars respectively), while no correlations were established with cv. Cobrançosa (less susceptible). No volatiles with inverse correlation were observed.

Volatile composition of olives may be a decisive factor in the olive fly choice to oviposit and this could be the basis for the development of new control strategies for this pest.

Keywords: olive cultivar; olives; volatile composition; olive fly; oviposition preference

Introduction

Olea europaea L. has registered a considerable growth and dissemination worldwide in the last decades, attracting the attention of new producing countries worldwide. Therefore productive records are being registered since the beginning of this decade, exceeding for the first time the 20 million tons barrier (20.4 million tons of olives in 2011; 20.3 million tons in 2013; FAOSTAT 2015). Escorting such dissemination, the dispersion of *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), the olive fly, is also verified, being a key pest of olives worldwide, with special importance in the Mediterranean region (Daane and Johnson, 2010). This dipteran causes severe olive production losses due to fruit drop (Neuenschwander *et al*., 1980), leads to the production of low quality olive oils (Pereira *et al*., 2004), and olives infected by this pest cannot be used for table olives processing. Olive fly is also a vehicle of phytopathogenic agents (Iannota *et al*., 2007; Latinović *et al*., 2013) leading to the appearance and development of other olives diseases. Altogether, pests and diseases are believed to reduce olives production by 15% on average (Bueno and Jones, 2002), which means that about 3.6 million tons of olives were wasted in 2013, with olive fly being responsible for tremendous share in this loss.

Regarding olive fly infestation, olive cultivars display different susceptibilities to this pest, with some cultivars having systematically low infestation levels, while others, within the same agro-ecosystem, are usually more heavily affected (Burrack and Zalom, 2008; Gonçalves *et al*., 2012; Iannota *et al*., 2007; Navrozidis *et al*., 2007). In this particular olive fly/olive tree interaction several factors, including physical, chemical and molecular aspects influence preference of olive fly towards specific olive cultivars. Concerning chemical cues, volatiles emission may exert a strong influence in olive fly varietal preference. Several works highlight the attractant and/or repellent activity of chemical volatiles in olive fly: pheromones and other semiochemicals (Canale *et al*., 2013; Carpita *et al*., 2012); host volatiles (Scarpati *et al*., 1993; Scarpati *et al*., 1996); and bacterial filtrate volatiles (Liscia *et al*., 2013). More recently, a possible link with sesquiterpenes was also raised (Alfonso *et al*., 2014). However, studies reflecting the susceptibility of

different olive cultivars and their volatile emissions are scarce. These studies, involving cultivars with different vulnerability degrees to olive fly oviposition, could give important information about host selection causes and possible volatiles involved in the binomial *O. europaea – B. oleae*.

In this sense, three Portuguese olive cultivars, Cobrançosa, Madural and Verdeal Transmontana, were selected based on their susceptibility to olive fly. Olives from cvs. Madural and Verdeal Transmontana are highly susceptible to olive fly, while cv. Cobrançosa displays lower susceptibility than the others (Gonçalves *et al*., 2012). The main objective of this work was to characterize the volatile fraction of the olives during ripening by HS-SPME-GC-MS (headspace solid-phase microextraction/gas chromatography with mass spectrometry detector), while trying to establish possible relations between infestation levels observed on each cultivar with the volatiles of the drupes.

The determination of volatile composition of olives from cultivars with different susceptibility degrees to olive fly could give answers to the binomial olive fly-olive tree. By monitoring the volatile fraction and infestation levels of the different cultivars through the maturation process, important information could be retained regarding the possible existence of volatiles responsible for the olive fly attraction and/or repellence. From the results obtained, new hypothesis may be elaborated in order to turn the control of olive fly more eco-friendly and sustainable by the application of volatiles naturally present in olives.

To the authors knowledge, this is the first investigation of this kind with these cultivars, and the first study reporting possible relations between olive fly varietal preference and olive fruit volatile composition.

Material and methods

Sampling

For the present study olives from three Portuguese olive cultivars, the most representative from Trás-os-Montes region (Northeast of Portugal) were assessed: *cvs.* Cobrançosa, Madural and Verdeal Transmontana. The work occurred in 2011, and samples were collected in a private organic olive grove located in Paradela (Mirandela - 41º32'35.72''N; 7º07'27.17''W) (permission to carry out samples collection was kindly granted by the olive grove owner). Five trees were marked per cultivar and branches with olives were collected at six different dates: 18th July; 18th August; 20th September; 4th

October; 21st October; and 9th November. The number of sampling dates was settled until the harvest of olives by olive grove owner, which occurred one week later to the last sampling date, in order to naturally preserve the field conditions to which olive fly is up against. After collection, branches were transported at refrigeration temperatures and volatile analysis was performed in the first 24 to 48 hours.

Simultaneously, fruits were collected per tree for calculation of the maturation index, as described by Hermoso *et al*. (2001). Briefly, samples of 100 olive fruits (20 fruits per tree) were separated in 8 levels based on epidermis and pulp color (0 to 7). Therefore, the fruit is classified as "0" if the epidermis is green; "1" for yellowish green; "2" if the epidermis shows red spots in less than half fruit; "3" if the epidermis is red or purple in more than half fruit; "4" for black epidermis and white pulp; "5" if the epidermis is black and less than half pulp is purple; "6" if the epidermis is black and more than half pulp purple (without reaching the stone); "7" if the epidermis is black and total pulp purple (reaching the stone). The maturation index was calculated as follows: MI = ($a \times 0 + b \times 1 + c \times 2 + d \times 3 + e \times 4 + f \times 5 + g \times 6 + h \times 7$) / 100, where the letters are the number of fruits in each level of classification considered.

To assess infestation level, from 4th August to 23rd November, 20 random handpicked fruits were collected fortnightly from each olive tree (5 trees per cultivar; 100 fruits) and inspected in a binocular stereomicroscope for signs of infestation (oviposition sites or exit holes). Infestation level was expressed as the percentage of infested olive fruits.

Volatile characterization

SPME fibers

For the headspace solid-phase microextraction (HS-SPME) a fiber coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS; 50/30 µm) was selected based on a preliminary assay conducted with further two fibers (CAR/PDMS 75 µm and PDMS 100 µm), all from Supelco (Bellefonte, USA). Selection of the fiber was based on the highest qualitative (number of volatiles extracted) and quantitative data (peak areas) of a sample of olives from *cv.* Cobrançosa.

HS-SPME

The HS-SPME was carried out according to the methodology applied by our research group in other matrices (Malheiro *et al*., 2013), with some modifications. Healthy olives (one per replicate) were placed in 50 ml vials, deuterated chloroform (99.96%, Aldrich) was added as internal standard (250 ppm in methanol; 10 μl) and immediately sealed with a polypropylene cap with silicon septum. The volatiles were released at 40 ºC during 30 min, in an ultrasonic bath. After that, the DVB/CAR/PDMS fiber was exposed during 1 hour at 40 ºC for volatiles adsorption, and then inserted into the injection port of the GC system for thermal desorption and reconditioning (10 min at 280 ºC). For each harvest moment and olive cultivar the HS-SPME analysis was performed in quintuplicate (five different olives).

Gas chromatography-mass spectrometry (GC-MS) conditions

Chromatographic analysis was performed on an Agilent 6890 series GC (Agilent, Avondale, PA, USA), with splitless injection, coupled to a MS detector (Agilent 5973). Volatiles were separated using a bonded phase fused-silica capillary column (SPB-5, 60 m × 0.32 mm × 1 μm, Supelco, Bellefonte, USA), operating at constant flow with helium at 1 ml min^{-1}. The oven temperature program was isothermal for 5 min at 40 ºC, raised to 220 ºC at a rate of 3 ºC min^{-1} and maintained at 220 ºC for 2 min, with a total run of 67 min. The transfer line to the mass spectrometer was maintained at 250 ºC. Mass spectra were obtained in electronic impact mode at 70 eV, with a multiplier voltage of 2056 V, collecting data at a rate of 1 scan s^{-1} over the range 30 – 500 *m/z*. The constituents were identified by comparing the experimental spectra with spectra from NIST 98 data bank (NIST/EPA/NISH Mass Spectral Library, version 1.6, U.S.A.), and also by comparison of their GC retention index (Adams, 2007). Retention indices were obtained using a commercial n-alkanes series C_7-C_{30} (Sigma-Aldrich, St. Louis, U.S.A.) by direct splitless liquid injection (1 μL) while all further conditions of GC and MS as settled for the volatile analysis. Retention indices were calculated according to van Den Dool and Kratz (1963). The compounds on Tables 6.1 to 6.3 are expressed on the basis of the relative areas achieved for the individual base ions (m/z 100% intensity). For semi-quantification purposes (Fig. 6.2), total volatile amounts were calculated by the ratio of each individual base ion peak area to the area of the internal standard and converted to mass equivalents on the basis on the internal mass added.

Statistical analysis

Analysis of variance

An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov-Smirnov with Lilliefors correction (if n>50) or the Shapiro-Wilk`s test (if n<50), and the Levene´s tests, respectively. All dependent variables were analysed using a one-way ANOVA with or without Welch correction, depending if the requirement of the homogeneity of variances was fulfilled or not. The main factor studied were the changes in volatile composition of olives from three different olive cultivars during crop maturation. If a statistical significant effect was found, means were compared using Tukey´s honestly significant difference multiple comparison test or Dunnett T3 test also depending if equal variances could be assumed or not. All statistical tests were performed at a 5% significance level.

Principal component analysis

Principal components analysis (PCA) was applied for reducing the number of variables in olives from cvs Cobrançosa, Madural and Verdeal Transmontana (variables corresponding to the amount of total volatiles, most abundant volatile compounds identified, and olives infestation levels during the olives maturation; overall 24 variables), to a smaller number of new derived variables (principal components or factors) that adequately summarize the original information, i.e., the effect of harvest period and olive cultivar on the volatile composition of olives from different olive cultivars with different susceptibility degrees to olive fly. Moreover, it allowed recognizing patterns in the data by plotting them in a multidimensional space, using the new derived variables as dimensions (factor scores). PCA was performed by using SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.).

Results

Infestation level

Infestation of olives in the three cultivars was monitored from early August until harvest, end of November). The results obtained clearly showed preference of olive fly females to lay their eggs in olives from cv. Verdeal Transmontana, followed by cv. Madural, while Cobrançosa was the less susceptible olive cultivar during the entire assessed period (Fig. 6.1). Verdeal Transmontana displayed higher infestation levels during the entire study, reporting 16% of infestation at 24th Aug, increasing continuously until mid-October, reporting 62% of olives infested. At the end of monitoring Verdeal Transmontana olives reported the highest infestation level, 64% (Fig. 6.1). Olives from cv. Madural reported initially a low infestation of 2%, increasing steadily until 46% of infested olives at the end of monitoring. Olives from cv. Cobrançosa reported infestation levels below 10% until the 9th Nov (Fig. 6.1), while the highest infestation level was verified at the end of the assessed period, with 22% infestation levels, three times less than cv. Verdeal Transmontana, and half of infestation verified at cv. Madural.

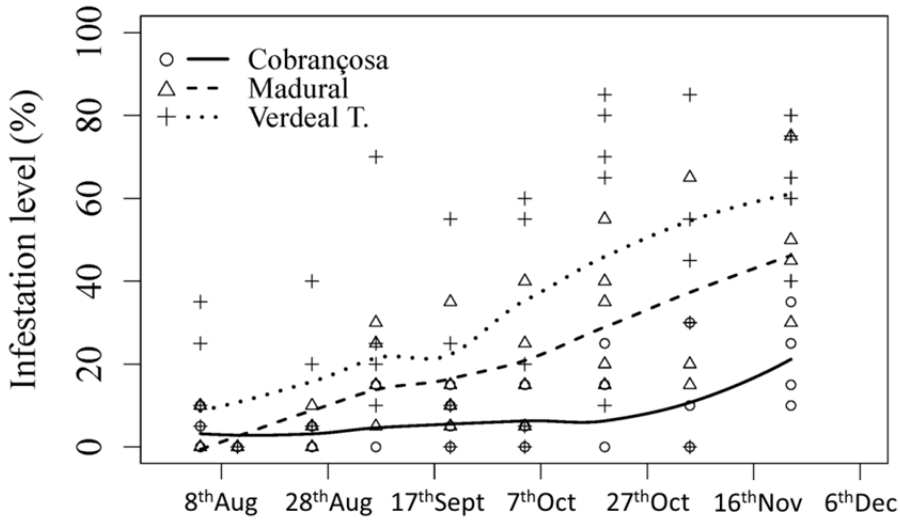

Figure 6.1. Olive fly infestation level (%) in olives from cvs. Cobrançosa, Madural and Verdeal Transmontana during crop maturation.

Volatile content and characterization

Volatile composition of olives from cvs. Cobrançosa, Madural and Verdeal Transmontana were assessed by HS-SPME-GC/MS during crop maturation at six sampling dates. The detailed relative volatile composition is reported in Tables 6.1-6.3 for cv. Cobrançosa, cv. Madural, and cv. Verdeal Transmontana, respectively. A total of 34 volatile compounds were identified in the three olive cultivars, distributed by 7 chemical classes: alcohols (5); aldehydes (6); aromatic hydrocarbons (3); esters (5); ketone (1); sesquiterpenes (7); and terpenes, more specifically monoterpenes (7).

Table 6.1. Volatile composition (relative %; mean ± standard error) of cv. Cobrançosa olives at different harvest times.

		18th Jul	18th Aug	20th Sep	4th Oct	21st Oct	9th Nov	
Maturation index		0	0	1	1	2	4	
Chemical class	**Compound**							***P*-value**
Alcohols								
	3-methyl-1-butanol	-	-	23.0 ± 1.3	-	-	-	-
	2-methyl-1-butanol	-	-	12.6 ± 0.7	-	-	-	-
	(*Z*)-3-hexen-1-ol	5.8 ± 1.2 a	-	9.1 ± 2.1 a	25.7 ± 5.4 b	6.2 ± 0.3 a	5.0 ± 0.8 a	< 0.001(1)
	Octanol	-	-	-	-	-	0.9 ± 0.1	-
Aldehydes								
	Hexanal	5.9 ± 1.1 a	11.0 ± 1.0 b	6.5 ± 0.7 a	-	4.3 ± 0.2 a	4.4 ± 0.6 a	< 0.001(1)
	Heptanal	-	-	-	-	2.26 ± 0.1 a	3.12 ± 0.3 b	0.036(1)
	Benzaldehyde	0.7 ± 0.1 a	-	-	3.5 ± 0.4 b	-	4.6 ± 0.6 b	< 0.001(2)
	Octanal	-	-	-	2.7 ± 0.2 a	5.0 ± 0.3 b	5.4 ± 0.8 b	0.001(2)
	Nonanal	-	8.6 ± 0.4 b,c	5.8 ± 0.6 a	10.3 ± 0.8 c	9.5 ± 0.7 c	6.3 ± 0.6 a,b	< 0.001(1)
	Decanal	-	7.5 ± 0.4 c	2.3 ± 0.2 a	4.8 ± 1.0 b	4.5 ± 0.4 a,b	2.4 ± 0.3 a	< 0.001(1)
Esters								
	Butanoic acid methyl ester	12.6 ± 0.8 b	13.7 ± 1.1 b	4.9 ± 0.4 a	-	-	-	< 0.001(1)
	Butanoic acid, 3-methyl-, methyl ester	1.1 ± 0.1	-	-	-	-	-	-
	Butanoic acid, 2-methyl-, methyl ester	3.1 ± 0.3	-	-	-	-	-	-
	(*Z*)-3-hexen-1-ol acetate	8.6 ± 1.7	-	-	-	-	-	-
Ketones								
	6-Methyl-5-hepten-2-one	-	26.0 ± 3.7 b	-	-	-	1.8 ± 0.2 a	< 0.001(1)
Sesquiterpenes								
	α-Copaene	1.7 ± 0.1a	4.2 ± 0.2 b	2.4 ± 0.2 a	3.3 ± 0.3 a,b	3.0 ± 0.2 a,b	6.1 ± 0.8 c	< 0.001(2)
	β-Caryophyllene	0.3 ± 0.0	-	-	-	-	-	-
Terpenes								
	α-Pinene	-	-	-	2.2 ± 0.3 a	1.4 ± 0.0 a	-	0.063(1)
	ρ-Cymene	-	-	-	-	-	1.8 ± 0.2	-
	Limonene	0.4 ± 0.0a	5.7 ± 1.1 b	7.2 ± 1.0 b,c	8.7 ± 0.9 b-d	12.1 ± 1.1 d	10.6 ± 1.5 c,d	< 0.001(1)
	Eucalyptol	-	-	-	-	-	1.4 ± 0.1	-
	(L)-Menthone	-	-	-	-	-	0.9 ± 0.1	-
	Menthol	-	-	-	-	1.8 ± 0.2 a	3.4 ± 0.4 b	0.010(1)
Aromatic hydrocarbons								
	Toluene	46.4 ± 1.7d,e	20.4 ± 2.3 a	26.3 ± 2.7 a,b	38.8 ± 2.9 c,d	49.8 ± 2.4 e	35.3 ± 1.4 b,c	< 0.001(1)
	para-Xylene	8.8 ± 1.5 b	2.9 ± 0.5 a	-	-	-	6.6 ± 0.7 a,b	0.004(1)
	ortho-Xylene	4.6 ± 0.7	-	-	-	-	-	-

In the same line, mean values with different letters differ significantly ($P < 0.05$); (1) $P > 0.05$, be means of Levene test. *P* values are those from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed; (2) $P < 0.05$, by means of Levene test. *P* values are those from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since equal variances could not be assumed

Table 6.2. Volatile composition (relative %; mean ± standard error) of cv. Madural olives at different harvest times.

		18th Jul	18th Aug	20th Sep	4th Oct	21st Oct	9th Nov	
Maturation index		0	0	1	1	2	4	
Chemical class	**Compound**							***P*-value**
Alcohols								
	(*Z*)-3-hexen-1-ol	5.2 ± 0.7 a,b	2.8 ± 0.4 b	14.8 ± 1.7 c	-	7.7 ± 1.2 b	1.5 ± 0.2 a	< 0.001(2)
	Hexanol	-	-	-	-	5.5 ± 1.1 b	1.8 ± 0.2 a	0.010(1)
	Octanol	-	-	-	-	-	0.5 ± 0.0	-
Aldehydes								
	Hexanal	2.9 ± 0.4 a,b	8.2 ± 1.5 c	5.2 ± 0.9 b,c	2.9 ± 0.2 a,b	1.7 ± 0.1 a	1.3 ± 0.2 a	< 0.001(2)
	Benzaldehyde	0.7 ± 0.1 a	1.3 ± 0.2 a	-	-	-	2.2 ± 0.3 b	0.001(1)
	Octanal	-	-	-	2.2 ± 0.2 a	-	1.7 ± 0.2 a	0.125(1)
	Nonanal	-	3.8 ± 0.2 a	3.6 ± 0.4 a	6.5 ± 0.8 b	3.4 ± 0.2 a	4.8 ± 0.4 a,b	0.001(1)
	Decanal	-	3.1 ± 0.1 c	1.5 ± 0.2 a,b	2.6 ± 0.2 c	1.8 ± 0.1 b	1.0 ± 0.1 a	< 0.001(1)
Esters								
	Butanoic acid methyl ester	1.6 ± 0.1 a	6.6 ± 0.8 b	3.4 ± 0.2 a,b	-	-	-	< 0.001(2)
	(*Z*)-3-hexen-1-ol acetate	69.5 ± 2.8 c	19.6 ± 3.4 a,b	28.7 ± 6.6 b	-	11.1 ± 2.2 a	-	< 0.001(1)
Ketones								
	6-Methyl-5-hepten-2-one	-	11.4 ± 0.6 b	-	4.8 ± 0.3 a	4.4 ± 0.1 a	-	< 0.001(2)
Sesquiterpenes								
	α-Cubebene	0.8 ± 0.1 a	1.4 ± 0.3 a	0.8 ± 0.1 a	-	-	-	0.319(2)
	(+)-Cycloisosativene	-	-	0.7 ± 0.2 a	2.6 ±c 0.2	1.3 ± 0.1 a,b	1.8 ± 0.2 b	< 0.001(1)
	α-Copaene	4.5 ± 0.4 a	7.9 ± 1.6 a,b	12.3 ± 2.5 b,c	28.7 ± 1.9 e	17.5 ± 1.5 c,d	22.4 ± 1.3 d,e	< 0.001(1)
	α-Muurolene	-	-	-	-	1.1 ± 0.1	-	-
	β-Caryophyllene	0.4 ± 0.1	-	-	-	-	-	-
	α-Farnesene	-	-	-	-	-	0.5 ± 0.0	-
	Δ-Cadinene	0.5 ± 0.1 a	1.1 ± 0.2 b	0.6 ± 0.1 a	-	-	-	0.009(1)
Terpenes								
	α-Pinene	-	-	-	2.6 ± 0.3 b	-	1.4 ± 0.1 a	0.010(1)
	ρ-Cymene	-	-	-	-	-	1.3 ± 0.1	-
	Limonene	0.6 ± 0.1 a	16.2 ± 2.1 c	6.2 ± 1.1 b	4.7 ± 0.3 a,b	1.0 ± 0.1 a	7.5 ± 0.8 b	< 0.001(2)
	Eucalyptol	-	-	-	-	-	1.0 ± 0.1	-
	(*E*)-β-Ocimene	-	-	-	-	-	3.0 ± 0.4	-

	(L)-Menthone	-	-	-	-	-	0.6 ± 0.1	-
	Menthol	-	-	-	-	-	1.9 ± 0.1	-
Aromatic hydrocarbons								
	Toluene	5.2 ± 0.9 a	15.5 ± 3.5 a,b	16.3 ± 2.3 b	42.4 ± 2.7 c	43.4 ± 2.0 c	37.6 ± 2.2 c	< 0.001[(1)]
	para-Xylene	5.5 ± 1.1 b	1.0 ± 0.0 a	5.9 ± 0.6 b	-	-	6.0 ± 0.4 b	< 0.001[(1)]
	ortho-Xylene	2.6 ± 0.4	-	-	-	-	-	-

In the same line, mean values with different letters differ significantly ($P < 0.05$); [(1)] $P > 0.05$, be means of Levene test. P values are those from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed; [(2)] $P < 0.05$, by means of Levene test. P values are those from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since equal variances could not be assumed

Table 6.3. Volatile composition (relative %; mean ± standard error) of cv. Verdeal Transmontana olives at different harvest times.

		18th Jul	18th Aug	20th Sep	4th Oct	21st Oct	9th Nov	
Maturation index		0	0	1	1	1	3	
Chemical class	**Compound**							***P*-value**
Alcohols								
	3-methyl-1-butanol	-	1.2 ± 0.1 a	17.2 ± 0.4 b	-	-	-	< 0.001[(2)]
	2-methyl-1-butanol	-	-	9.5 ± 0.2	-	-	-	-
	(*Z*)-3-hexen-1-ol	3.6 ± 0.5 a	3.9 ± 0.7 a	7.2 ± 1.6 a	17.2 ± 1.1 b	4.4 ± 0.3 a	5.7 ± 0.9 a	< 0.001[(1)]
	Hexanol	-	2.4 ± 0.3 a	-	-	-	4.0 ± 0.1 b	0.002[(1)]
Aldehydes								
	Hexanal	3.8 ± 0.5 a,b	19.9 ± 1.3 c	6.0 ± 1.2 b	-	1.7 ± 0.1 a	4.0 ± 0.5 a,b	< 0.001[(2)]
	Heptanal	-	5.0 ± 0.2	-	-	-	-	-
	Nonanal	-	11.8 ± 0.7 c	5.0 ± 0.6 b	3.3 ± 0.3 b	4.1 ± 0.2 b	1.4 ± 0.1 a	< 0.001[(2)]
	Decanal	1.0 ± 0.1	-	-	-	-	-	-
Esters								
	Butanoic acid methyl ester	17.3 ± 1.3b	3.3 ± 0.1 a	5.1 ± 0.3 a	3.5 ± 0.1 a	-	-	< 0.001[(2)]
	Butanoic acid, 2-methyl-, methyl ester	8.9 ± 1.6	-	-	-	-	-	-
	Hexanoic acid methyl ester	14.7 ± 2.8	-	-	-	-	-	-
	(*Z*)-3-hexen-1-ol acetate	9.0 ± 1.0 a	10.1 ± 0.6 a	-	-	-	-	0.414[(1)]
Ketones								
	6-Methyl-5-hepten-2-one	-	5.5 ± 0.1 b	-	2.6 ± 0.2 a	-	-	< 0.001[(1)]
Sesquiterpenes								
	α-Cubebene	0.8 ± 0.1	-	-	-	-	-	-
	α-Copaene	3.6 ± 0.5 a,b	2.5 ± 0.3 a	3.9 ± 0.3 a,b	3.7 ± 0.4 a,b	5.0 ± 0.3 b	10.2 ± 1.1 c	< 0.001[(2)]
	β-Caryophyllene	2.6 ± 0.3	-	-	-	-	-	-
	α-Farnesene	2.3 ± 0.2	-	-	-	-	-	-
	Δ-Cadinene	0.6 ± 0.1	-	-	-	-	-	-

Terpenes								
	α-Pinene	-	0.9 ± 0.1 a	-	1.0 ± 0.1 a	-	-	0.317[(1)]
	Limonene	1.0 ± 0.1 a	11.2 ± 0.5 d	7.6 ± 0.6 c	2.4 ± 0.1 a,b	2.0 ± 0.1 a,b	2.7 ± 0.2 b	< 0.001[(2)]
	Menthol	-	-	-	-	1.1 ± 0.1	-	-
Aromatic hydrocarbons								
	Toluene	11.1 ± 1.0 a	21.2 ± 1.7 b	38.6 ± 1.0 c	66.3 ± 1.0 d	81.7 ± 0.4 e	66.8 ± 0.5 d	< 0.001[(1)]
	para-Xylene	12.0 ± 1.6 c	1.3 ± 0.4 a	-	-	-	5.2 ± 0.3 b	< 0.001[(2)]
	ortho-Xylene	7.7 ± 0.7	-	-	-	-	-	-

In the same line, mean values with different letters differ significantly ($P < 0.05$); [(1)] $P > 0.05$, be means of Levene test. P values are those from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed; [(2)]$P < 0.05$, by means of Levene test. P values are those from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since equal variances could not be assumed

The semi-quantification of total volatiles revealed always higher emissions in olives from cvs. Verdeal Transmontana and Madural than cv. Cobrançosa, except for the last sampling date, 9th Nov (Fig. 6.2). At the first sampling date, Verdeal Transmontana olives emitted on average a total of approximately 324 µg of volatiles per 100 g of olives, while Madural and Cobrançosa olives reported, 223 and 111 µg per 100 g, respectively. Generally, a sharp decrease was observed in all cultivars at the second sampling date (18th Aug), with a slight increase on the 4th Oct., and again on the last sampling date (9th Nov), similar in the three cultivars. Despite the lower volatile amounts in cv. Cobrançosa olives at all sampling dates, in the last one a higher volatile content was emmited, with 108 µg 100 g^{-1}, while cv. Madural and cv. Verdeal Transmontana had only 91 and 51 µg 100 g^{-1}, respectively (Fig. 6.2). Therefore, and independently from the volatile identities that will be further explored in the next paragraphs, the total volatile amounts seem to have an attractive effect to olive fly oviposition.

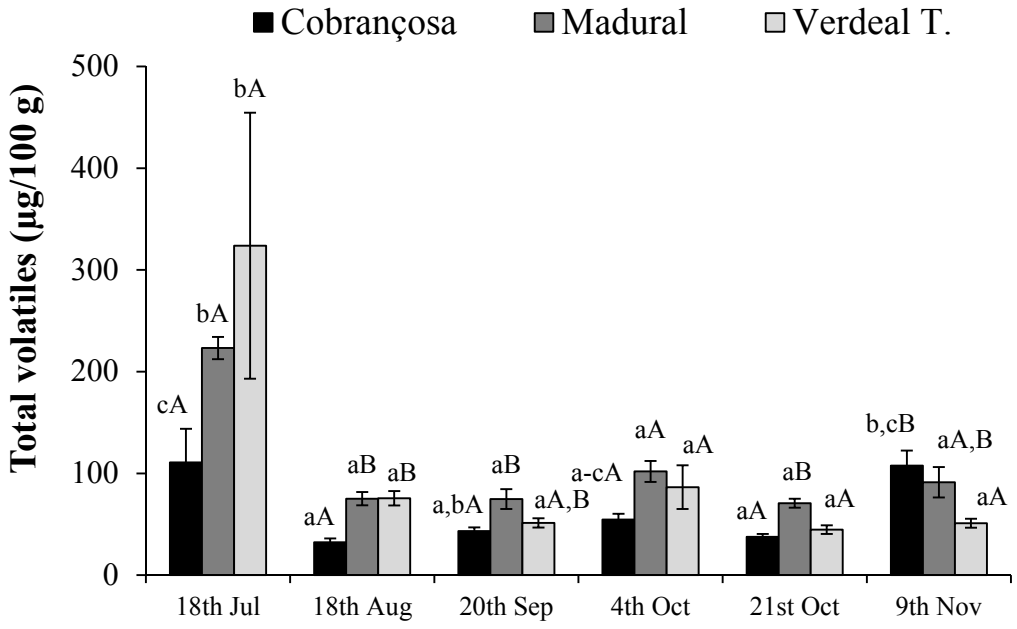

Figure 6.2. Total volatiles emission (µg.100 g^{-1} of olives) of cvs. Cobrançosa, Madural and Verdeal Transmontana olives at different harvesting times (18th Jul; 18th Aug; 20th Sep; 4th Oct; 21st Oct; 9th Nov) during fruit maturation (in each cultivar different minor letters represent significant differences during crop maturation (P < 0.05); in each harvest moment, capital letters represent significant differences between olive cultivars (P < 0.005)).

Regarding volatile composition, aromatic hydrocarbons were present in the three olive cultivars (Fig. 6.3), represented mainly by toluene, followed by *para*- and *ortho*-

xylene. In the case of *ortho*-xylene, it was found only at the first sampling date (18th Jul) in the three olive cultivars (Table 6.2-6.3) while its isomer was only detected at the first two sampling dates (18th Jul and 18th Aug) in cvs. Cobrançosa and Verdeal Transmontana, being persisted to the 20th Sep (third sampling date) in cv. Madural and reappearing in the last sampling date (9th Nov). In opposition, toluene was present during the entire study, with significant variations in the three olive cultivars ($P < 0.001$) (Table 6.1-6.3). The trend observed in the relative area proportion of aromatic hydrocarbons during crop maturation was similar for cvs. Madural and Verdeal Transmontana, increasing until 4th Oct, and then decreasing to the 9th Nov. In the case of cv. Cobrançosa it was already heavily represented in the first sampling date with slight variations over the time course studied (Fig. 6.3).

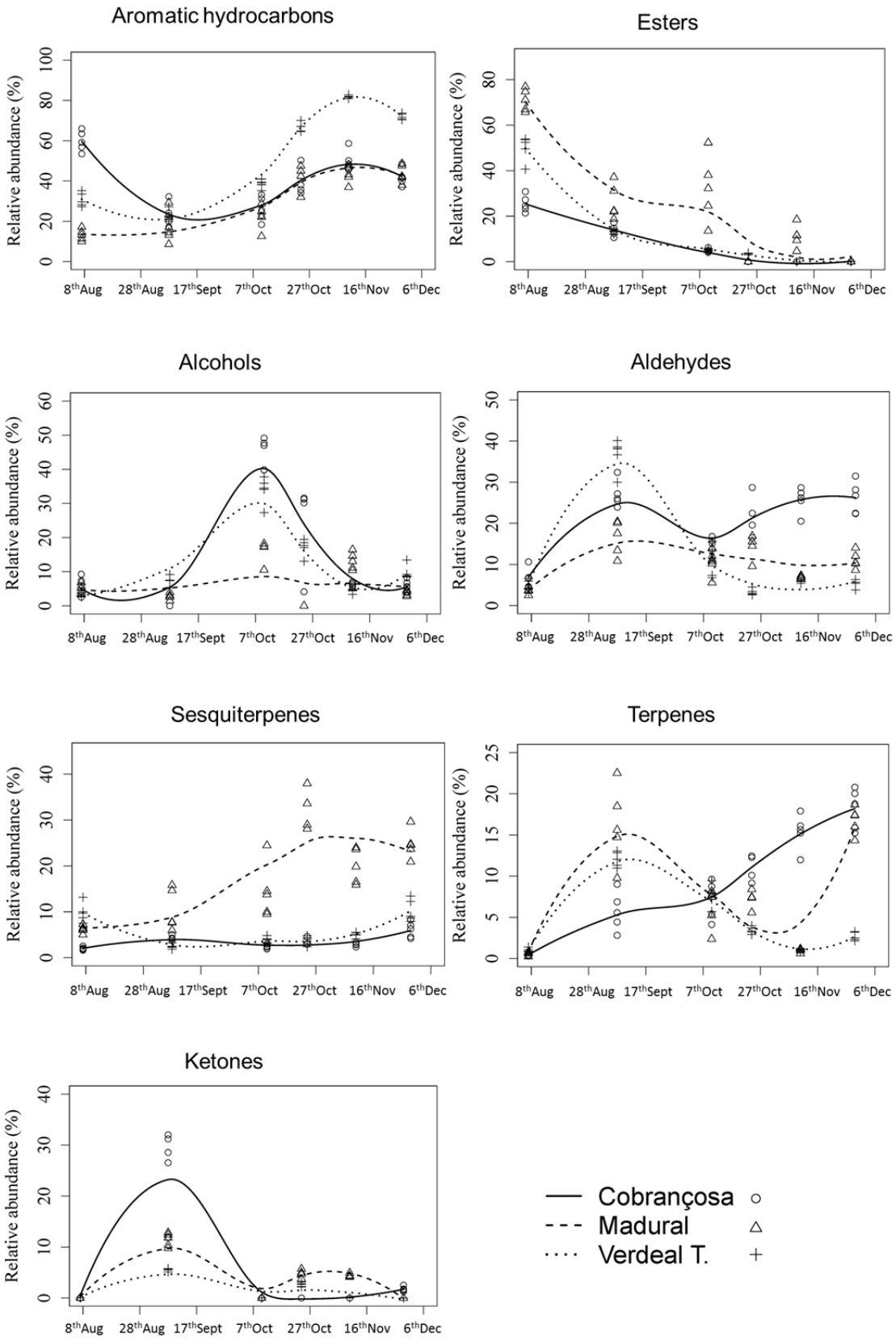

Figure 6.3. Volatile relative changes in the chemical classes identified in olives from cvs. Cobrançosa, Madural and Verdeal Transmontana during crop maturation.

Esters were highly represented in the first sampling dates in cvs. Cobrançosa and Verdeal Transmontana, while in the last sampling dates they were almost absent (Tables 6.1-6.3), following apparently a similar trend in the three olive cultivars with a relative decrease during ripening (Fig. 6.3). A particular note to butanoic acid methyl ester in Cobrançosa and Verdeal transmontana, and to (*Z*)-3-hexen-1-ol acetate, particularly detected in cv. Madural (Table 6.3).

Alcohols trend during olives maturation was also similar in the three cultivars. Alcohols relative abundance increased form 18th Jul until 20th Sep with significant decreases onwards (Fig. 6.3), being present in lower relative abundance in cv. Madural almost during the entire study (except at 21st Oct). The predominant alcohols were (Z)-3-hexen-1-ol and 3-methyl-1-butanol. The first varied significantly during fruits maturation in the three cultivars ($P < 0.001$). In the case of 3-methyl-1-butanol, it was absent in cv. Madural, being present in cv. Cobrançosa only at 20th Sep and in cv. Verdeal Transmontana at 18th Aug and 20th Sep.

Aldehydes were one of the most diversified and consistent classes of volatile compounds found in the olives analyzed. In the three cultivars studied aldehydes reported higher relative abundance at 18th Aug (Fig. 6.3). From 18th Aug until the end of the study, 9th Nov, aldehydes decrease their relative content in cvs. Madural and Verdeal Transmontana, while in cv. Cobrançosa aldehydes remained practically constant. Hexanal, nonanal and decanal were the most representative aldehydes present in the olives.

For sesquiterpenes a cultivar effect was observed. Significant higher values ($P < 0.001$) were reported for cv. Madural in the last three sampling dates (> 20%), with maximum relative proportions at the 4th Oct (Fig. 6.3). The most abundant sesquiterpene present in the volatile fraction of the three olive cultivars was α-copaene (Table 6.1-6.), being present in the three olive cultivars in all sampling periods. The highest diversity of and amounts of sesquiterpenes was detected in cv. Madural. Some of them were only present in the first sampling dates (α-cubebene; β-caryophyllene, and Δ-cadinene), while (+)-cycloisosativene was exclusive from this olive cultivar and was only detected from 20th Sep onwards (Table 6.2). In cv. Verdeal Transmontana four sequiterpenes were identified (α-cubebene; β-caryophyllene; α-farnesene; and Δ-cadinene), however they were present only at the first sampling date (Table 6.3). Olives from cv. Cobrançosa had generally lower diversity and amounts (Table 6.1).

Similar to sesquiterpenes, terpenes presented a marked cultivar dependent trend. In cv. Cobrançosa olives their relative proportion increased continuously during olives maturation (Fig. 6.3). In olives from cvs. Madural and Verdeal Transmontana, the

terpenes increased from 18th Jul to 18th Aug, but decreased considerably after that. However in cv. Madural an increase is observed in the final two harvest periods, reaching similar values to those observed in the beginning of the study, an increase not attended by cv. Verdeal Transmontana olives. The trends observed are partially related to limonene content, the main terpene in the volatile fraction of the three olive cultivars. Limonene was identified during the entire study, reporting higher predominance in the first sampling periods for cvs. Madural and Verdeal Transmontana and in the last ones for cv. Cobrançosa (Tables 6.1-6.3).

Regarding ketones, only one volatile was identified, 6-methyl-5-hepten-2-one (Table 6.1-6.3). A variable pattern was observed between cultivars and through maturation.

In order to summarize all the information obtained, a principal component analysis (PCA) was performed with the relative proportion of the main volatile compounds identified in the olives of the three cultivars, together with the infestation levels and total volatiles emission. From the results obtained (Fig. 6.4), the aromatic hydrocarbon toluene and the sesquiterpene α-copaene, were the two most important volatiles from olives headspace related positively with infestation levels, clearly perceptible in the PCA performed.

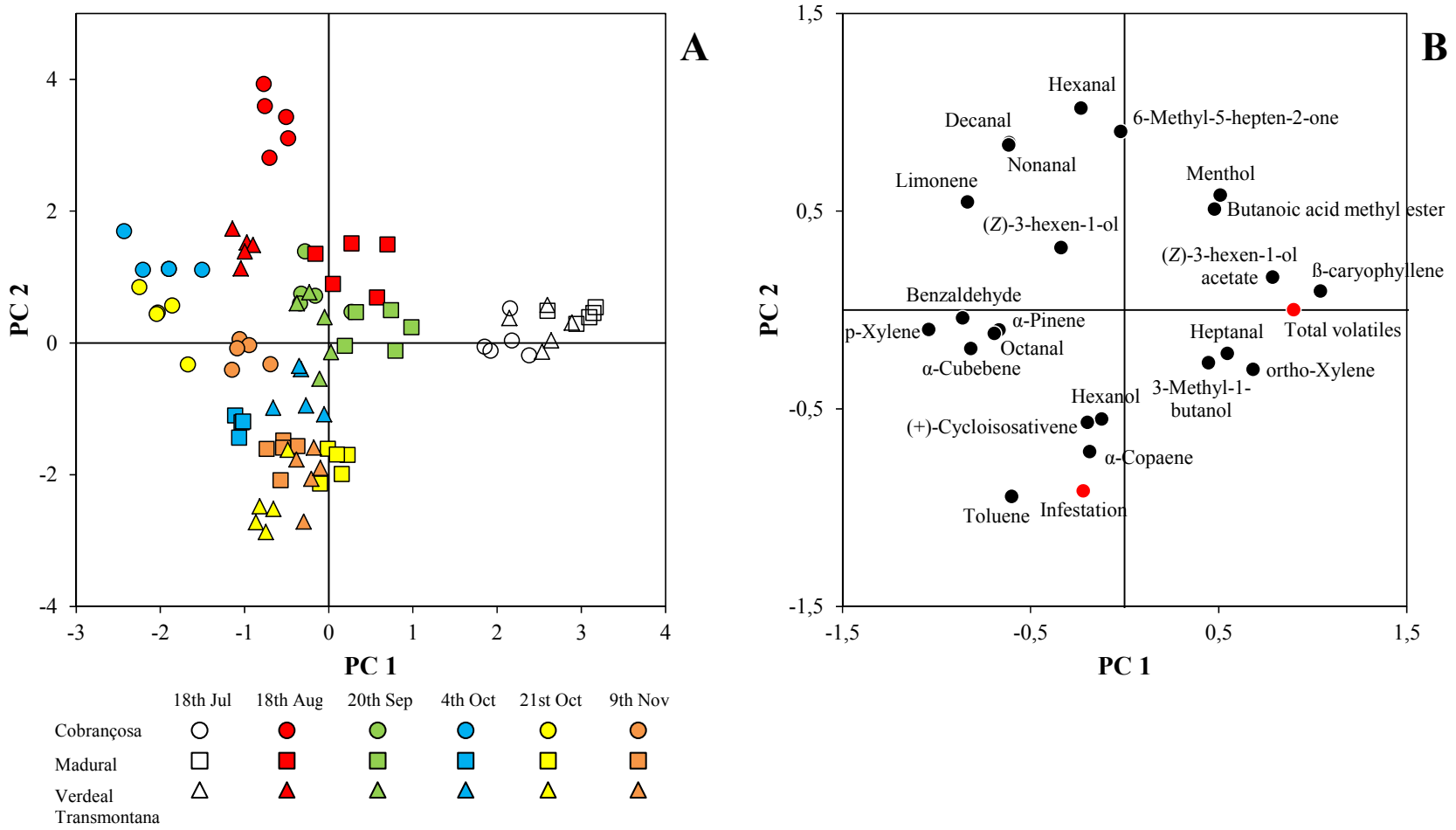

Figure 6.4. Principal component analysis obtained from the volatile composition, total volatiles and infestation levels of olives from cvs. Cobrançosa, Madural, and Verdeal Transmontana (Fig. 6.4A) at different harvesting periods during olives maturation. The variables used in this PCA and their respective loadings are represented in Fig. 6.4B.

These two volatiles were mainly correlated with infestation level being represented in the 21st Oct to 9th Nov region for cvs. Madural and Verdeal Transmontana (negative regions of both principal components). These observations were are also corroborated by the results obtained in the infestation level monitored in the field, with higher infestation levels reported in the same dates and cultivars (Fig. 6.1). If we analyze carefully the PCA obtained, samples from cv. Cobrançosa are represented in the extreme opposite region to toluene, α-copaene and infestation recorded (Fig. 6.4). Even with the use of unsupervised statistical tools is possible to observe the notorious differentiation in the volatile composition of the three olive cultivars during olives maturation and the agglomeration of samples with higher infestation levels around toluene and α-copaene (Fig. 6.4).

In fact these two volatiles were correlated with infestation level in the olive cultivars. In our study, the relative toluene amounts were extremely correlated with infestation level ($P < 0.001$; $R^2 = 0.256$; $y = 0.54x - 1.813$). Only in cv. Cobrançosa toluene contents were not correlated with infestation level ($P = 0.614$; $R^2 = 0.009$; $y = -0.14x + 37.04$). Olive cultivars Madural ($P < 0.001$; $R^2 = 0.368$; $y = 0.50x + 17.62$) and Verdeal Transmontana ($P = 0.003$; $R^2 = 0.274$; $y = 0.49x + 32.55$) infestation levels were extremely correlated with toluene content.

Regarding α-copaene, positive significant correlations were also established between its relative abundance in olives from cvs. Madural and Verdeal Transmontana and the infestation levels caused by olive fly during olives maturation (cv. Madural – $P = 0.02$; $R^2 = 0.168$; $y = 0.20x + 12.04$; cv. Verdeal Transmontana – $P = 0.05$; $R^2 = 0.134$; $y = 0.04x + 3.688$). In cv. Cobrançosa olives no correlation was observed between α-copaene emission and olives infestation level ($P = 0.149$; $R^2 = 0.073$; $y = 0.06x + 3.094$).

In the same region where were represented α-copaene and toluene were also (+)-cycloisosativene and hexanol. The sesquiterpene was only present in cv. Madural and the alcohol was present mainly in the last sampling date in both cvs. Madural and Verdeal Transmontana. Both compounds revealed no direct correlation with infestation levels caused by olive fly.

Discussion

The results observed in infestation levels demonstrate clearly that cv. Verdeal Transmontana is the most susceptible olive cultivar, being cv. Madural an intermediate, and cv. Cobrançosa the less susceptible to olive fly oviposition. Such differences in the

susceptibility of olive cultivars could be ascribed to the volatile content and composition of the olives in conjunction with factors of other nature (physical and molecular).

Total amounts of volatiles reduced during olives maturation (Fig. 6.2). The main contributors are GLV's (green leaf volatiles), mainly alcohols ((*Z*)-3-hexen-1-ol), aldehydes (hexanal), and esters ((*Z*)-3-hexen-1-ol acetate). The biosynthesis of these olives components is guided by lipoxygenase pathway, also known as LOX. LOX activity starts when olive tissues are disrupted and enzymes released, taking contact with fatty acids, mainly polyunsaturated fatty acids (linoleic and linolenic). The polyunsaturated fatty acids are oxidized by LOX and cleaved by hydroperoxide lyase, forming aldehydes. The aldehydes formed are then reduced to alcohols by alcohol dehydrogenase action. Alcohols could be then esterified to yield esters due to alcohol acyltransferase. According to the polyunsaturated fatty acid intervened, different volatiles are formed. For instance hexanal is formed from linoleic acid, while (*Z*)-3-hexen-1-ol and (*Z*)-3-hexen-1-ol acetate are formed from linolenic acid (Kalua *et al*., 2007). In our study intact olives were analyzed, therefore LOX pathway is considerably reduced due to the low/absence of tissues disruption, mainly attributed to olive fly damage (Matsui *et al*., 2006). This explains why in our study volatiles emission from olives is considerably low (from 32 to 324 µg per 100 g of olives). Also, the reduction in volatiles emission during olive maturation is also expected, since many of the enzymes present in LOX pathway reduce their activity during maturation, like lipoxygenase (Salas *et al*., 1999) and alcohol dehydrogenase (Salas and Sanchez, 1998).

Toluene is apparently one of the most abundant volatile in olives from the three cultivars, with special high relative abundance in cv. Verdeal Transmontana. This aromatic hydrocarbon, generally considered an environmental contaminant, is naturally present in the volatile profile of olive leaves and fruits (Flamini *et al*., 2003; Scarpati *et al*., 1993), and in olive food products: olive oil (Kiralan *et al*., 2012), and table olives of different preparation methods (Iraqui *et al*., 2005; Sansone-Land *et al*., 2014). Therefore, toluene is not a strange component in the volatile fraction of olive tree organs or olive food products. Nevertheless, the origin of toluene in olives and derived products is still controversial, since some authors believe that its presence may be due to exogenous contamination, others hypothesize the formation of this aromatic hydrocarbon by endogenous mechanisms (Bierdman *et al*., 1995), and a third part recognize the formation of aromatic hydrocarbons by the microflora naturally present in olive tree (Scarpati *et al*., 1996), with special reference to epiphytic community. Epiphytic community of olive tree is diverse (Ercolani, 1991), and according to Saccheti *et al*. (2008) a strong relationship is observed between olive fly population and epiphytic microorganisms in olives. Therefore, olive

surface epiphytic community may also be a key aspect in the olive cultivar oviposition preference of olive fly, since the survival of adults is also dependent in these communities (Sacchetti *et al*., 2008).

Generally, during maturation, olives exhale higher proportions of toluene, but the amounts released are dependent on olive cultivar, being increased in the olive cultivars with higher susceptibility to olive fly (cvs. Madural and Verdeal Transmontana). For instance, in Italian olive cultivars Cellina di Nardò and Ogliarola Barese toluene amounts also increased from green to mature olives, with higher expression in cv. Ogliarola Barese (Masi *et al*., 2014). The same pattern was verified by Scarpati *et al*. (1993) that found toluene as one of the most abundant volatiles in the headspace of half-ripe olives from cv. Itrana. As we verified earlier, toluene is extremely correlated with infestation level. This observation is very interesting since olives headspace volatiles determined are recognized as being highly attractive to olive fly in attractancy bioassays (Scarpati *et al*., 1993), corroborating that some volatiles present could interfere in the behavior of olive fly. Therefore, toluene could be at least partially responsible for the behavior of olive fly. Indeed, when tested in attractancy bioassays, toluene proved a highly attractive action towards olive fly (Scarpati *et al*., 1993). Nevertheless, toluene could act in a synergic way with others volatiles from olives.

Like toluene, the sesquiterpene α-copaene was also extremely correlated with olive fly infestation. Therefore, α-copaene presence in olives volatile fraction may as well act as an oviposition promoter for olive fly females. In fact, the role of α-copaene in the fruit susceptibility to olive fly is already recognized (Alfonso *et al*., 2014). The amounts of α-copaene released from different cultivars and the infestation levels of olives were also correlated, in accordance to our data. Bioassays revealed that the enantiomer (+)-α-copaene is an oviposition promoter, nearly doubling the number of olives attacked by olive fly females (Alfonso *et al*., 2014). In our study we verified that α-copaene emitted were clearly higher in cvs. Madural, followed Verdeal Transmontana, the more susceptible olive cultivars, while cv. Cobrançosa volatiles reported lower proportion of α-copaene, being this cultivar less susceptible. Therefore, based on our data and on the hypothesis that α-copaene is a potent oviposition promoter (Alfonso *et al*., 2014), this sesquiterpene could be partially responsible for the oviposition preference of olive fly towards different olive cultivars. Nevertheless, this hypothesis needs to be further exploited by field-trials and also electroantennographic studies to prove their impact in olive fly behavior.

Conclusions

In light of the obtained results, it is clear that olive fly has an oviposition preference for cv. Verdeal Transmontana and a lower preference for cv. Cobrançosa. The volatile composition of olives is dependent on the olive cultivar, and is highly influenced by olives maturation. The different susceptibility degrees of olive cultivars could be ascribed to the volatile profile, since positive correlations were established between important components and amounts of olives headspace and the infestation recorded in each cultivar. Despite the inexistence of negative interactions, which could directly open field strategies to repulse olive fly, further studies, namely bioassays, field trials and electroantennographic studies, need to be carried out to elucidate olive fly thresholds for these volatiles and possible mechanisms of action while aiding in the development of new strategies to control olive fly populations in olive groves. Furthermore, this type of studies should be expanded to a higher number of olive cultivars in order to obtain even more reliable and strong data about the influence of volatile composition in the olive fly oviposition preference.

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CHAPTER 7.

Electrophysiological response of *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) adults to olive leaves essential oils from different cultivars and olive tree volatiles

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Abstract

In the present study, the electrophysiological response of olive fly adults, *Bactrocera oleae* (Rossi), to olive leaves essential oils (EO's) from different cultivars (cvs. Cobrançosa, Madural and Verdeal Transmontana) with distinct olive fly susceptibility degrees, as well as host volatiles ([(*E*)-2-hexenal, α-pinene, farnesene, xylene, and nonanal] and semiochemicals (spiroketal, and (*Z*)-9-tricosene) were assessed at different adults ages ([0-5[days old, early young adults; [5-10[days old, corresponding to sexual maturity; and [10-15[days old, when females are gravid).

Results showed clear differences in the EAG response of both sexes to EO's of the different cultivars, with higher signal in cv. Cobrançosa, the less susceptible to olive fly. An inverse proportionality was found between the EAG response to EO's in both sexes and the susceptibility degrees to olive fly. Chemical composition of EO's proved to be highly influenced by olive cultivars, with a clear differentiation between them. A possible deterrent mechanism could be involved in the observed results.

Host volatiles, mainly (*E*)-2-hexenal and nonanal exerted higher EAG responses, (*E*)-2-hexenal mainly in females at sexual maturity and oviposition period, while nonanal elicited higher responses at [5-10[days old in males, corresponding to sexual maturity.

The present results give important results regarding olive fly oviposition preference as well to olive fly – olive tree interactions.

Keywords: olive fly; EAG; oviposition preference; semiochemicals; nonanal, (*E*)-2-hexenal

Introduction

The olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), contrary to many species of the Tephritidae family, is a monophagous specie. Larvae of olive fly develop in species from the genus *Olea*, including *O. verrucosa*, *O. chrysophylla*, and the most important commercial specie, *O. europaea* (Daane and Johnson, 2010). According to the International Olive Council (IOC, 2000), around 250 olive cultivars are considered to have commercial value for table olives and olive oil production, corresponding to only 10% of the cultivars known worldwide. Nevertheless *O. europaea* cultivars are not affected in the same form by olive fly. Consistently, at the same edaphoclimatic conditions, some cultivars are less susceptible to olive fly while others suffer severe attacks by this dipteran (Iannotta *et al*., 2007), causing considerable production losses. The preference of olive fly towards a specific olive cultivar in detriment to others inserted in the same agro-climatic niche is a fact observed worldwide: Croatia (Koprivnjak *et al*., 2010); Greece (Navrozidis *et al*., 2007); Italy (Iannotta *et al*., 2007); Jordan (Al-Zaghal and Mustafa, 1987); Portugal (Gonçalves *et al*., 2012); Syria (Al-SAlti *et al*., 2011; Saour and Makee, 2004); Turkey (Gümusay *et al*., 1990); and U.S.A. (Burrack and Zalom, 2008).

The binomial olive fly – olive tree is mediated by different factors in which chemical cues, more specifically volatiles exhaled from host, exert a preponderant role. Several works were carried out to evaluate the potential of certain stimuli in the electrophysiological response of insects by electroantennographic studies (EAG). In the case of olive fly, EAG studies allowed very recently to discover a male-produced female attractant, (Z)-9-tricosene (Canale *et al*., 2013a; Carpita *et al*., 2012), and to reveal the olive fly males response to diverse components of sex pheromone produced by females (Canale *et al*., *in press*). Liscia *et al*. (2013) verified the electrophysiological response of olive fly to host volatiles (olives, leaves and epiphytic bacteria). Nevertheless, no studies on the electrophysiological response of olive fly related to olive cultivar susceptibility were carried out so far. This type of studies based on olive cultivars with different

susceptibilities to olive fly and their composition, may give important information about possible attractant and/or repellent components.

In this sense, and in order to understand the relation of olive fly with olive tree and cultivar susceptibility, different stimulus were tested in the present work to observe the electrophysiological response of olive fly adults. Essential oils (EO's) extracted from leaves from Portuguese olive cultivars with different susceptibilities to olive fly (Cobrançosa: low susceptibility; Madural: intermediate; and Verdeal Transmontana: high susceptibility) were tested. This approach has never been tested in olive fly, but EO's from diverse species proved to have toxic effects in other Tephritid species, like *Ceratitis capitata* (Benelli *et al*., 2013a) and *Bactrocera tryoni* (Hidayat *et al*., 2013). Some selected host volatiles were also tested [(*E*)-2-hexenal, α-pinene, farnesene, xylene, and nonanal] as well as olive fly recognized semiochemiclas [(*Z*)-9-tricosene; and 1,7-dioxaspiro[5.5] undecane] considered as positive controls. The EAG assays were also conducted attaining olive fly adults sex and age ([0-5[days old, corresponding to early young adults; [5-10[days old, when adults become sexually mature; and [10-15[days old, when olive flies are gravid and need to oviposit). This is the first time that the electrophysiological response of olive fly is recorded against EO´s of olive leaves prevenient from cultivars with different susceptibility degrees.

Material and methods

Reagents and standards

The reagents and standards used in this work were obtained from Sigma-Aldrich (St. Louis, USA): hexane, diethyl ether, magnesium sulphate anhydrous, (*E*)-2-hexenal, α-pinene, farnesene (mixture of isomers), xylene (mixture of isomers), nonanal, (*Z*)-9-tricosene, and 1,7-dioxaspiro[5.5] undecane (spiroketal).

Olive leaves sampling and essential oil extraction

For the present study, olive leaves from three Portuguese olive cultivars, the most representative from Trás-os-Montes region (Northeast of Portugal), were collected: cvs. Cobrançosa, Madural and Verdeal Transmontana. Samples were collected in an organic olive grove located in Paradela (Mirandela - 41º32'35.72''N; 7º07'27.17''W). Per olive

cultivar, olive leaves were collected from three olive trees, with approximately 1 kg per tree (n = 3).

Essential oil (EO) extraction was carried out under the following conditions: 200 g of fresh olive leaves crushed under liquid nitrogen, with 0.5 L of distilled water, during 3 h. EO was collected in diethyl ether a, cleaned and dried with anhydrous magnesium sulphate, and concentrated under a continuous flow of nitrogen at room temperature.

Essential oil characterization by gas chromatography-mass spectrometry (GC-MS)

Olive leaves essential oils from cvs. Cobrançosa, Madural and Verdeal Transmontana were characterized by GC-MS. The GC used was a Thermo FOCUS GC coupled to a Thermo QP-5000 quadropole mass spectrometer. A DB-5 capillary column (30 mm × 0.25 mm i.d.; 0.25 μm film thickness) was used. The oven temperature was programmed at 60 ºC for 5 min with an increase of 5 ºC/min until 280 ºC. The carrier gas was helium, at a constant flow of 1.2 mL/min. The temperature of the ionization source was maintained at 200 ºC, and the ionization energy at 70eV. Compounds were identified based on standard compounds, retention indices and comparing the mass spectrum with those in Wiley 275L library.

***Bactrocera oleae* collection and rearing**

Olive fruits from the three olive cultivars were collected in the same olive grove where olive leaves were previously obtained. Olives with signs of olive fly infestation were selected, spread in trays, and their larvae and pupae were collected daily and transferred to an insectarium for rearing purposes. Once hatched from pupae, adults were separated daily (for age control purposes) from the main insectarium to rearing caches, being both sexes maintained together. Larvae, pupae and adults were maintained at the following conditions: 26 ± 1 ºC, 70 ± 10% of relative humidity, with a photoperiod of 16L:8D. Adults were feed *ad libitum* with a honey solution (10% w/v), artificial diet (sucrose and yeast extract at a ration 4:1), and water, being the diet changed every two days.

Electroantennographic (EAG) assays

For the EAG assays, three groups of olive fly adults were constituted per sex according to their age: [0-5[days old; [5-10[days old; and [10-15[days old. In each olive fly sex and age group, the following stimuli were tested: EO's from olive leaves of cvs. Cobrançosa, Madural, and Verdeal Transmontana; and olive tree host volatiles [(*E*)-2-hexenal, α-pinene, farnesene, xylene, and nonanal]. The results obtained were compared to those of olive fly recognized semiochemicals 1,7-dioxaspiro[5.5] undecane (spiroketal) (Baker *et al*., 1980) and (*Z*)-9-tricosene (Canale *et al*., 2013a; Carpita *et al*., 2012).

Essential oils were tested at two concentrations (1 and 10 $\mu g.mL^{-1}$) and olive tree host volatiles were tested at three concentrations (1, 10, and 100 $\mu g.mL^{-1}$) according to the following described methodology. Electroantennogram bioassays were performed using a Syntech system (Syntech Laboratories, Hilversum, The Netherlands). Freshly dissected head/antennal preparations of olive fly from each sex and age group were mounted between two metal electrodes using conductive gel and placed under a constant stream of humidified air (flow of 500 $mL.min^{-1}$). Test stimulations were carried out by applying puffs of air (200 $mL.min^{-1}$) for 2 s using a stimulus controller CS-01 (Syntech) through a Pasteur pipette containing a small piece of filter paper (1 cm diameter) with 1 µL aliquot of the EO's or olive tree volatiles against hexane (control). Puffs of the tested stimuli were applied at 1 min intervals three times on each antenna dissected, and the order of presentation of the test stimuli was randomized among replicates. Regarding dose-response assays, stimuli were applied in increasing doses. There was no reduction in the response of the reference stimulus throughout the tests in any of the replicates.

The antennal responses to the different stimulus concentrations from the EO's of the three olive cultivars and other chemicals tested were recorded at least in five insects per sex and age (n = 5). The response to hexane was considered a negative control, being the EAG responses reported as relative responses to hexane.

Statistical analysis

Analysis of variance

An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.). The fulfilment of the ANOVA requirements, namely

the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov-Smirnov with Lilliefors correction (if n>50) or the Shapiro-Wilk`s test (if n<50), and the Levene´s tests, respectively. All dependent variables were analysed using a one-way ANOVA with or without Welch correction, depending if the requirement of the homogeneity of variances was fulfilled or not. The main factors studied were: i) the composition of EO's from the different olive cultivars; ii) the effect of EO's and different chemicals tested in the EAG signal according to sex, age and concentration tested. If a statistical significant effect was found, means were compared using Tukey´s honestly significant difference multiple comparison test or Dunnett T3 test also depending if equal variances could be assumed or not. All statistical tests were performed at a 5% significance level.

Principal Component Analysis

Principal components analysis (PCA) was applied for reducing the number of variables in the EO's composition from the olive leaves of the three olive cultivars to a smaller number of new derived variables (principal component or factors) that adequately summarize the original information, i.e., composition of EO's from cv.s Cobrançosa, Madural and Verdeal Transmontana. Overall, 31 variables corresponding to the EO's components of cvs. Cobrançosa, Madural and Verdeal Transmontana were used in PCA. PCA was performed by using SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.).

Results and discussion

EAG responses of olive fly to olive leaves essential oils

Essential oils from olive leaves of cvs. Cobrançosa, Madural and Verdeal Transmontana were extracted in order to verify the EAG response of olive fly adults while trying to establish a possible correlation with olive cultivars susceptibility. Independently of age, sex, and concentration tested, EO's from cv. Cobrançosa elicited always higher EAG response, followed by cv. Madural and by last cv. Verdeal Transmontana (Figure 7.1).

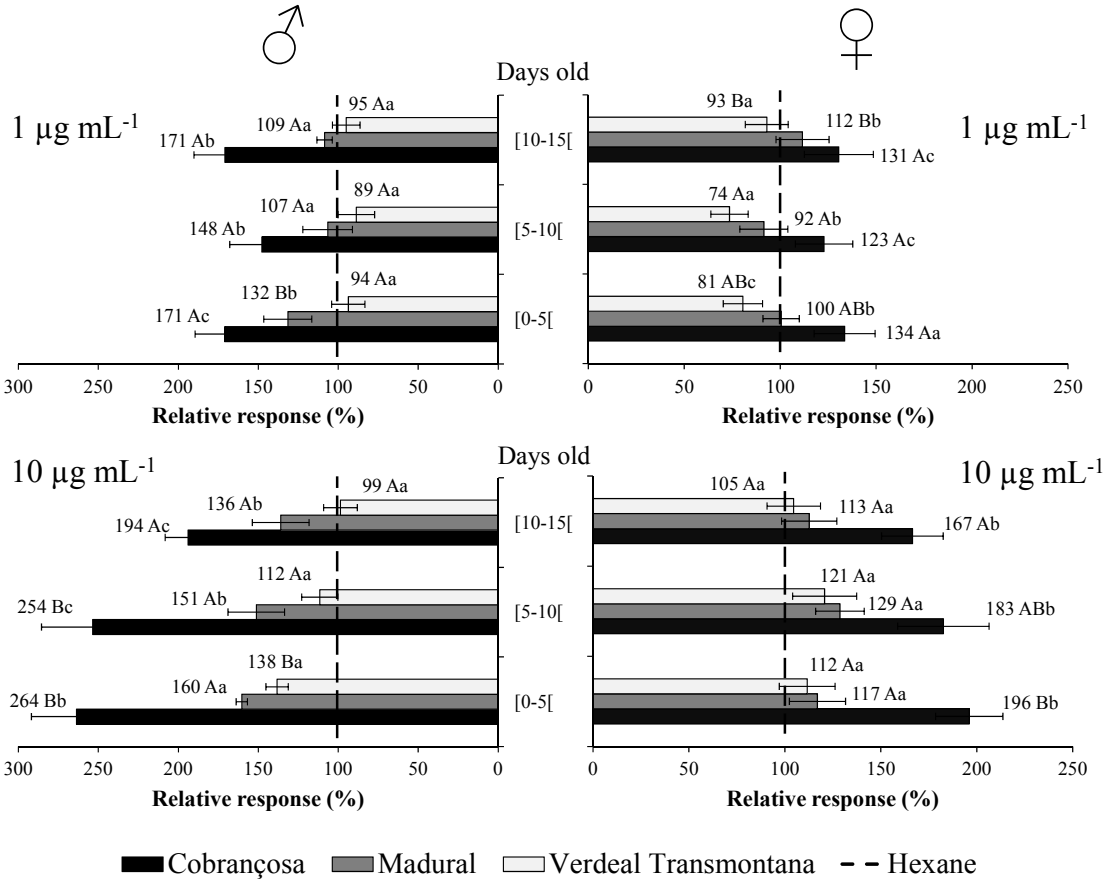

Figure 7.1. EAG responses (% against hexane; mean ± standard deviation) of *Bactrocera oleae* (Rossi) adults to olive leaves essential oils from cvs. Cobrançosa, Madural, and Verdeal Transmontana (In each chart, bars with different capital letters represent significant effect of each olive cultivar according to *B. oleae* adults age ($P < 0.05$); In each chart, bars with different minor letters represent significant differences among olive cultivars for a determined age group).

Within all variables assayed (cultivar; sex; age; and concentration tested) higher EAG signals were generally reported in males rather than females. According to the obtained results males, reported higher sensibility in the antenna sensilla, but no differences were found in the structure and number of sensilla in the antenna of olive fly males and females (Liscia *et al*., 2013). A concentration dependent response was also verified when 1 or 10 µg.mL^{-1} of EO´s were tested. In a general way, and in both sexes, the EAG signals of EO's decrease with olive fly age, with higher signal losses at 10 µg.mL^{-1} (Figure 1). At 1 µg.mL^{-1} cv. Cobrançosa EO's reported EAG signals between 148 and 171%, significantly higher to those presented by females, between 123 and 134%. EO's from cv. Madural elicited higher responses than hexane for males (maximum of 132% at [0-5[days old; minimum values of 107% at [5-10[days old), while in females values

obtained were similar or below 100% (minimum of 92% at [5-10[days old; maximum of 112% at [10-15[days old[). EAG response for cv. Verdeal Transmontana EO's were always below the effect of hexane in both males and females. For this cultivar, at 1 $\mu g.mL^{-1}$ maximum EAG responses of 95% and 93% were reported respectively in males and females at [10-15[days old for both sexes (Figure 1). This data clearly highlight that cvs. Verdeal Transmontana EO at 1 µg has no effect while Madural EO at the same concentration had a reduced effect in the behaviour of olive fly adults. On contrary, cv. Cobrançosa EO appears to affect olive fly electrophysiological response even at a low concentration of 1 $\mu g.mL^{-1}$.

By testing olive fly adults response at 10 $\mu g.mL^{-1}$, a considerable increase in EAG response was verified at all variables assayed. Moreover, at this concentration, EAG signal was completely influenced by adults age. In males, higher EAG responses were always verified at [0-5[days old, while the lowest responses were obtained at [10-15[days old. In females the same trend was only verified for cv. Cobrançosa EO, while in cvs. Madural and Verdeal Transmontana higher EAG signals were reported at [5-10[days old. For males, EO of cv. Cobrançosa elicited always significant higher EAG response comparatively to cvs. Madural and Verdeal Transmontana with 264, 254 and 194% respectively for [0-5[, [5-10[, and [10-15[days old. In females, EAG signals were 196, 183 and 167% respectively for [0-5[, [5-10[, and [10-15[days old (Figure 1),no differences were observed at each age group between cvs. Madural and Verdeal Transmontana at 10 $\mu g.mL^{-1}$, but significant differences were reported for cv. Cobrançosa ($P < 0.001$).

According to these results, it appears that age is an important factor to consider for understanding the sensibility of olive fly adults. In fact, in Tephritids, age is known to reduce antenna sensitivity, as already observed for the fruit fly *Anastrepha suspensa* (Kendra *et al*., 2005), and for olive fly itself (De Cristofaro *et al*., 2007). This observation can point out that effectiveness of control strategies against this pest could be reduced for old flies, like the use of mass-trapping devices together with semiochemicals. Another important fact in olive fly EAG response is the sexual status. Since olive fly adults of both sexes become sexually mature at 7 days old (Benelli *et al*., 2013b), it could explain the results observed in the EAG responses to EO's of the different cultivars. In fact, De Cristofaro *et al*. (2007) verified that mated or unmated adults display different trends. While EAG responses in mated males reduced with age, EAG responses for mated females remained quite stable. In our studies, both males and female become sexually mature at the [5-10[days old group, while at [10-15[days old, females were considered gravid. Therefore, the physiological needs of both sexes are different according to their

age, a possible explanation for the differences observed in the EAG responses to the EO's from the different olive cultivars.

The results obtained in the EAG response of olive fly adults to the EO's from olive cultivars with different susceptibilities to olive fly give a clear idea that they could intervene in the behaviour of olive fly females and interfere at oviposition preference level. According to Gonçalves *et al*. (2012), cv. Cobrançosa olives are less susceptible to olive fly infestation, while cvs. Madural and Verdeal Transmontana are more susceptible. In the last years, cv. Verdeal Transmontana report higher susceptibility, being cv. Madural an intermediate cultivar regarding olive fly infestation (Malheiro *et al*., 2014; Rodrigues *et al*., 2014). When we relate the EAG signals of olive fly females with those of infestation susceptibility, we can observe that higher EAG signals are obtained in the less susceptible olive cultivar, while lower signals are in fact reported in cultivars with higher susceptibility. These results give the idea that olive leaves EO's may act as deterrents towards olive fly. Nevertheless, this hypothesis need to be further exploited with semi-field and field assays to observe the potential repellent action and oviposition deterrent activity towards olive fly females. Such hypothesis has never been raised before, since works with olive fly and EO are scarce. Only one report was carried out by Canale *et al*. (2013b) with EO's from Lamiaceae species, being therefore impossible the establishment of associations between olive fly and olive cultivars susceptibility.

The chemical composition of the EO's from the olive leaves of the three olive cultivars (Table 7.1) could also explain the high differences found in the EAG responses between cv. Cobrançosa EO and the remaining olive cultivars, as discussed in the next section.

Essential oils chemical composition

As discussed earlier, a clear significant higher EAG response (independently of sex, age and concentration tested) was verified with EO's from cv. Cobrançosa, a fact that could be ascribed to EO's chemical composition. The chemical composition of the EO's from olive leaves of cvs. Cobrançosa, Madural and Verdeal Transmontana is reported in Table 7.1.

Table 7.1. Composition (relative abundance %) of the essential oils extracted from olive leaves of cvs. Cobrançosa, Madural and Verdeal Transmontana (mean ± standard deviation; n=3).

Nº	Compound	LRI*	MW[a]	MF[b]	Cobrançosa	Madural	Verdeal Transmontana	*P*-value
1	Benzaldehyde	960	106	C_7H_6O	0.6±0.0	-	-	-
2	3-Ethenylpyridine	968	105	C_7H_7N	1.2±0.2	-	-	-
3	*Unidentified compound*	1003	-	-	2.1±0.2	-	-	-
4	α-Terpinene	1017	136	$C_{10}H_{16}$	-	-	3.2±0.3	-
5	Benzeneacetaldehyde	1045	120	C_8H_8O	1.9±0.1 b	0.2±0.0 a	-	< 0.001(1)
6	*cis*-Linalool oxide	1071	170	$C_{10}H_{18}O_2$	0.1±0.0	-	-	-
7	Nonanal	1100	142	$C_9H_{18}O$	7.1±0.2 c	1.1±0.1 a	3.3±0.3 b	< 0.001(1)
8	Menth-2-en-1-ol	1127	154	$C_{10}H_{18}O$	1.6±0.1 b	-	1.0±0.2 a	0.011(1)
9	α-Terpineol	1189	154	$C_{10}H_{18}O$	2.2±0.3 c	0.8±0.0 a	1.6±0.3 b	0.001(1)
10	Methyl salycilate	1192	152	$C_8H_8O_3$	-	0.7±0.0 a	2.3±0.2 b	0.003(2)
11	α-Fenchyl acetate	1219	196	$C_{12}H_{20}O_2$	3.6±0.3 b	1.1±0.0 a	3.5±0.8 b	0.002(2)
12	(*E*)-2-decenal	1263	154	$C_{10}H_{18}O$	4.0±0.8 b	6.6±0.2 c	2.3±0.3 a	< 0.001(1)
13	Theaspirane	1292	194	$C_{13}H_{22}O$	-	-	4.6±0.7	-
14	4-Vinylguaiacole	1314	150	$C_9H_{10}O_2$	3.6±0.4 a	-	4.5±0.6 a	0.106(1)
15	(*E*)-Isosafrole	1378	162	$C_{10}H_{10}O_2$	1.4±0.4 a	1.7±0.2 a	-	0.237(1)
16	1,1,5,6-Tetramethylindane	1387	174	$C_{13}H_{18}$	2.0±0.1 b	3.9±0.4 c	1.2±0.1 a	< 0.001(1)
17	(*E*)-β-Damascenone	1384	190	$C_{13}H_{18}O$	14.7±0.6 a,b	11.4±0.8 a	17.1±2.7 b	0.017(1)
18	(*Z*)-Jasmone	1394	164	$C_{11}H_{16}O$	3.5±0.2 a	8.9±0.7 b		< 0.001(1)
19	(*Z*)-α-Bergamotene	1414	204	$C_{15}H_{24}$	2.6±0.1 b	1.7±0.1 a	3.6±0.3 c	< 0.001(1)
20	(*Z*)-α-Damascone	1419	192	$C_{13}H_{20}O$	4.7±0.4 b	3.0±0.1 a	2.9±0.6 a	0.003(1)
21	β-Caryophyllene	1420	204	$C_{15}H_{24}$	5.1±0.2 a	-	5.3±0.7 a	0.677(1)
22	α-Ionone	1425	192	$C_{13}H_{20}O$	-	19.1±0.4	-	-
23	α-Humulene	1454	204	$C_{15}H_{24}$	1.5±0.0 b	1.2±0.0 a	1.7±0.2 b	0.002(1)
24	(*E*)-β-Ionone	1485	192	$C_{13}H_{20}O$	0.8±0.0 a	1.2±0.1 b	0.8±0.1 a	0.005(1)
25	(*Z*)-Nerolidol	1543	222	$C_{15}H_{26}O$	3.6±0.1 b	0.7±0.1 a	-	< 0.001(1)
26	Caryophyllene oxide	1580	220	$C_{15}H_{24}O$	-	-	0.8±0.1	-
27	*Unidentified compound*	1609	-	-	0.9±0.0 a	2.8±0.0 c	1.9±0.2 b	< 0.001(1)
28	Myristic acid	1754	228	$C_{14}H_{28}O_2$	-	1.1±0.0	-	-
29	(*E*)-Farnesyl acetate	1842	264	$C_{17}H_{28}O_2$	-	1.2±0.1 a	2.0±0.2 b	0.003(1)
30	Palmitic acid	1987	256	$C_{16}H_{32}O_2$	2.5±0.3 a	3.3±0.2 b	-	0.022(1)
31	Linoleic acid methyl ester	2091	294	$C_{19}H_{34}O_2$	2.3±0.1 b	2.0±0.2 b	1.2±0.1 a	< 0.001(1)

* Linear Retention Index (DB-5 column); [a] Molecular weight; [b] Molecular formula;
In the same line, mean values with different letters differ significantly ($P < 0.05$); (1) $P > 0.05$, be means of Levene test. *P* values are those from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed; (2) $P < 0.05$, by means of Levene test. *P* values are those from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since equal variances could not be assumed.

Important qualitative and quantitative variations were found among EO's composition of the three olive cultivars. Overall 31 compounds were identified in the EO's: 24 in cv. Cobrançosa; 21 in cv. Madural; and 20 in cv. Verdeal Transmontana. One of the main components of olive leaves EO's is (*E*)-β-damascenone (Table 7.1), reporting significant quantitative differences among cultivars ($P = 0.017$). This C13 norisoprenoid was the main component of cvs. Cobrançosa and Verdeal Transmontana EO's, with 14.7 and 17.1% respectively. In cv. Madural, olive leaves were mainly composed by α-ionone, with 19.1%, exclusively found in this cultivar, while β-damascenone appeared in second position (with 11.4%). (*E*)-β-Damascenone content is known to vary considerably in olive cultivars. In Tunisian cultivars, for instance, it ranged from 3.3 to 17.7%, while in Italian cultivars the variation was minor, between 4.7 and 7.9% (Campeol *et al*., 2003). In cv. Cobrançosa, quantitatively following (*E*)-β-damascenone were nonanal (7.1%) and β-

caryophyllene, while in cv. Verdeal Transmontana β-caryophyllene and theaspirane were also found in considerable amounts, with 5.3 and 4.6%, respectively. Theaspirane was exclusively found in cv. Verdeal Transmontana. Theaspiranes 6*S* and 6*R* enantiomers were also found in EO's of Italian cultivars Frantoio, Leccino, and Cipressino (Campeol *et al*., 2001).

Olive cultivar is one of the most important factors that influence olive leaves EO's composition (Campeol *et al*., 2001; Campeol *et al*., 2003). Nevertheless, other aspects like sample conditions (fresh vs. dried sample) (Brahmi *et al*., 2012), and period of the year, also influence EO composition of olive leaves (Flamini *et al*., 2003).

In the present study, the differences between the EO's chemical composition from the three olive cultivars were clearly verified by applying the data in a PCA. In Figure 7.2, the formation of three groups, each one corresponding to an olive cultivar, is observed.

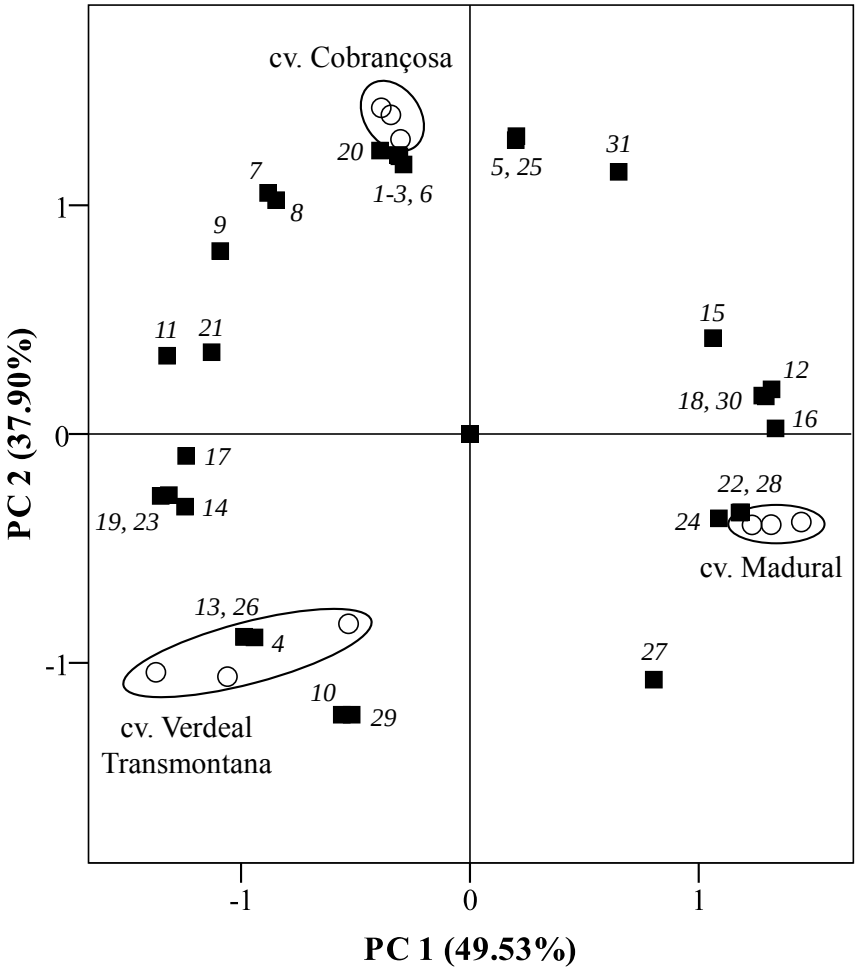

Figure 7.2. Principal component analysis of olive leaves essential oils composition from cvs. Cobrançosa, Madural and Verdeal Transmontana (the numbers represented correspond to the compounds listed in Table 7.1). The principal components (PC) explain 87.43% of the total variance of the data.

In the negative region of first principal component (PC1) and positive region of second principal component (PC2) are represented the EO's from cv. Cobrançosa. A group of 9 components, further sub-divided in two groups (exclusive compounds; and major components), characterized cv. Cobrançosa olive leaves EO's, with four components exclusively found in this olive cultivar: benzaldehyde (1), 3-ethenylpyridine (2), an unidentified compound (3), and *cis*-linalool oxide (6). Some other EO's components present in higher amounts in this cultivar also contributed to characterize cv. Cobrançosa: benzeneacetaldehyde (5), nonanal (7), menth-2-en-1-ol (8), (*Z*)-α-damascone (20), and (*Z*)-nerolidol (25) (Figure 2). Essential oils from cv. Madural were represented in the positive and negative regions of PC1 and PC2, respectively. In cv. Madural, two EO's components were exclusive, α-ionone (22), and myristic acid (28). A

group of five other components were present in higher amounts in cv. Madural EO's: (*E*)-2-decenal (12), 1,1,5,6-tetramethylindane (16), (*Z*)-jasmone (18), and palmitic acid (30) (Figure 2). Verdeal Transmontana EO's were represented in both negative regions of PC, with three EO's components exclusive to this cultivar: α-terpinene (4), theaspirane (13), and caryophyllene oxide (26). Six other components characterized this olive cultivar EO's due to their high amounts: methyl salycilate (10), 4-vinylguaiacole (14), (*E*)-β-damascenone (17), (*Z*)-α-bergamotene (19), α-humulene (23), and (*E*)-farnesyl acetate (29) (Figure 2).

Therefore, the differences found in EAG responses of olive fly adults could be partially related to olive cultivar. However, further studies should be carried out in order to observe seasonal variations as well as possible differences between EO's from dried and fresh leaves. GC-EAD (gas-chromatography with electroantennographic detector) assays could also elucidate if the EO's action, mainly cv. Cobrançosa EO's, could be ascribed to isolated compounds identified in this study or if the action is the result of a synergy between several EO's components.

EAG responses of olive fly to olive tree host volatiles and other semiochemicals

Olive tree volatiles (*E*)-2-hexenal, α-pinene, farnesene, xylene, and nonanal, as well as olive fly sex pheromones spiroketal and (*Z*)-9-tricosene were tested at the same conditions of EO's of olive leaves, at 1, 10 and 100 μg mL^{-1}. The selection of the olive tree volatiles was based on previous knowledge reported in literature, where: (*E*)-2-hexenal, xylene and α-pinene were considered oviposition promoters or deterrents to olive fly (Scarpati et al., 1993); nonanal found to be a component of different lures of olive fly eliciting high EAG responses in both males and females (Seris, 2011); and farnesene that gave EAG signals in a previous screening.

Similar to the observed with the EO's, males generally reported higher EAG signals than females. An increment of the EAG signal was verified according to the concentration tested, and each compound elicited different responses according to adult age. From all the volatiles tested, higher EAG signals were reported with (*E*)-2-hexenal and nonanal in both males and females. The EAG responses of these two aldehydes are represented in Figure 7.3, while the remaining volatiles and semiochemicals tested are reported in Table 7.2.

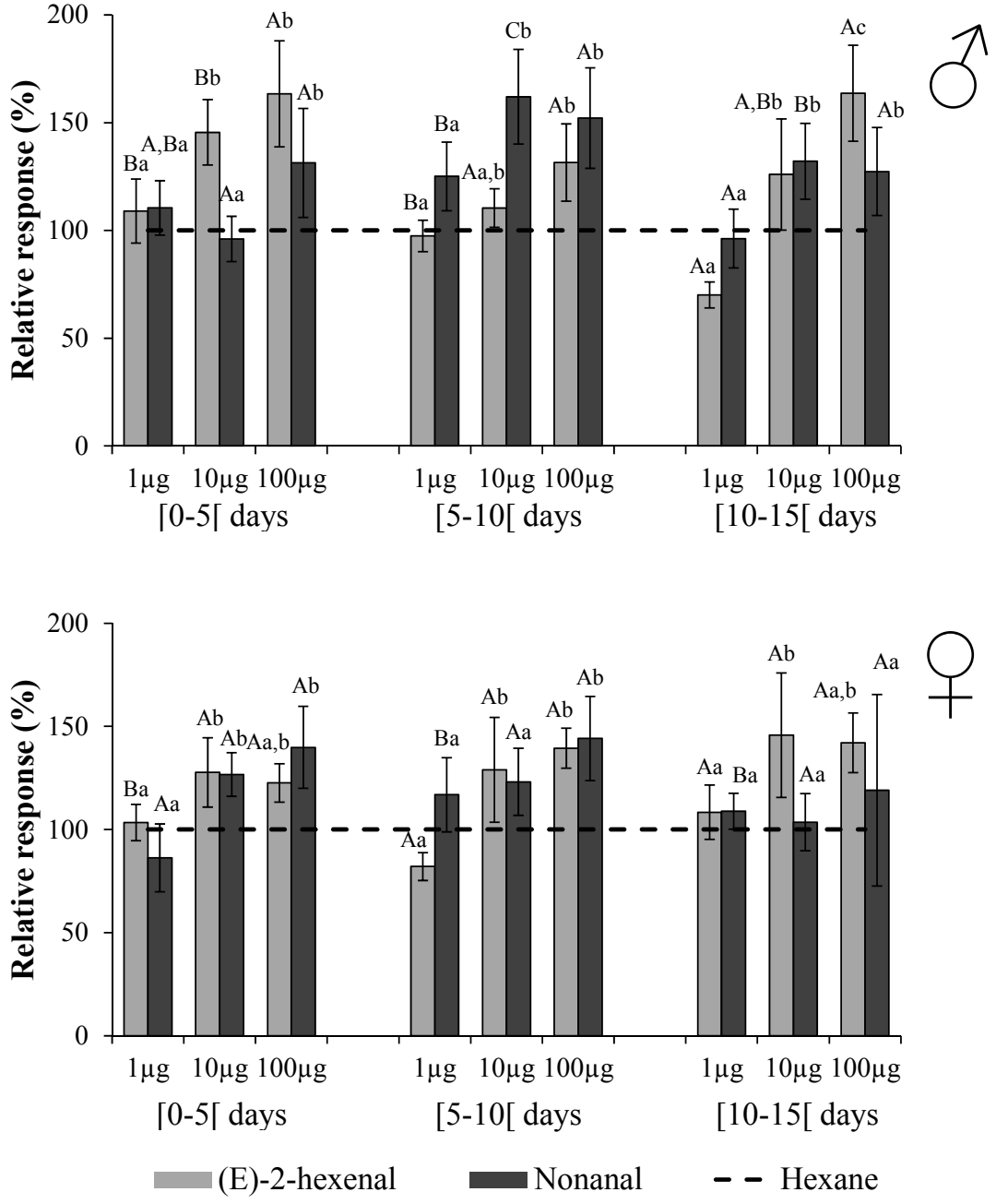

Figure 7.3. EAG responses (% against hexane; mean ± standard deviation) of *Bactrocera oleae* (Rossi) adults to different concentrations of the aldehydes (*E*)-2-hexenal and nonanal. (In each sex, for a determined compound and age group, bars with different minor letter differ significantly, $P < 0.05$; For each sex, for a determined compound and concentration tested, bars with different capital letter differ significantly, $P < 0.05$).

Table 7.2. EAG responses (% against hexane; mean ± standard deviation) of *Bactrocera oleae* (Rossi) males and females at different ages to different concentrations of olive tree volatiles (α-pinene, farnesene, xylene), and semiochemicals [(*Z*)-9-tricosene and spiroketal].

Males	Concentration	α-Pinene	Farnesene	Xylene	(*Z*)-9-Tricosene	Spiroketal
	1 µg mL^{-1}	90 ± 11 Aa	84 ± 12 Aa	85±12 A,Bb	80±10 Aa	77±12 Aa
[0-5[days	**10 µg mL^{-1}**	90 ± 5 Aa	94 ± 13 Aa,b	71±10 Aa	88±10 Aa	79±10 Aa
	100 µg mL^{-1}	97 ± 14 Aa	109 ± 13 Ab	95±15 Ab	103±14 Bb	100±20 Ab
	1 µg mL^{-1}	106 ± 10 Ab	115 ± 13.3 Ba,b	92±12 Ba	89±11 Aa	95±13 Ba
[5-10[days	**10 µg mL^{-1}**	107 ± 2 Bb	132 ± 16 Bb	120±15 Cb	94±11 Aa	113±14 Bb
	100 µg mL^{-1}	89 ± 11 Aa	107±12 Aa	92±12 Aa	86±16 Ba	142±18 Bc
	1 µg mL^{-1}	89 ± 12 Aa	87±9 Aa	75±15 Aa	77±7 Aa,b	79±10 A,Ba
[10-15[days	**10 µg mL^{-1}**	95 ± 14 A,Ba	95±10 Aa	89±13 Ba	87±10 Ab	85±12 Aa
	100 µg mL^{-1}	94 ± 13 Aa	88±13 Aa	91±9 Aa	65±10 Aa	87±11 Aa
Females						
	1 µg mL^{-1}	90 ± 6 Aa	78±10 Aa	67±9 Aa	68±10 Aa	62±16 Aa
[0-5[days	**10 µg mL^{-1}**	90 ± 7 Aa	79±13 Aa	80±7 Aa,b	78±9 Aa	82±9 Aa
	100 µg mL^{-1}	90 ± 10 A,Ba	84±11 Aa	85±11 Ab	81±22 Aa	107±14 Ab
	1 µg mL^{-1}	96 ± 6 A,Bb	94±7 Bb	92±12 Ba	91±14 Ba	91±9 Ba
[5-10[days	**10 µg mL^{-1}**	95 ± 0 Ab	118±20 Bc	104±8 Aa	91±13 Aa	98±14 Aa
	100 µg mL^{-1}	73 ± 3 Aa	67±4 Aa	105±17 Aa	92±14 Aa	123±21 Ab
	1 µg mL^{-1}	104 ± 14 Ba	102±6 Ba	89±9 Ba	87±12 Ba	83±10 Ba
[10-15[days	**10 µg mL^{-1}**	122 ± 35 Aa	100±9 A,Ba	113±53 Aa	104±41 Aa	81±0 Aa
	100 µg mL^{-1}	103 ± 7 Ba	85±22 Aa	108±12 Aa	97±22 Aa	88±42 Aa

Mean values in the same column for each compound and age group with different minor letters differ significantly ($P < 0.05$); Mean values within the same compound and different age groups, at the same concentration tested, with different capital letters, differ significantly ($P < 0.05$).

In males, (*E*)-2-hexenal reported always higher EAG signal than hexane (except at 1 µg.mL^{-1} at [10-15[days old). A general lower EAG signal was observed at the age group of [5-10[days old (minimum of 97% at 1 µg.mL^{-1} and maximum of 132% at 100 µg.mL^{-1}), which could be due to males sexual maturity, having this aldehyde lower activity in the antenna sensilla, due to the higher sensitivity to other compounds in this specific age and sexual status. This could be the example of nonanal. In fact, in Figure 7.3 is observed higher EAG signal to nonanal correspond to [5-10[days old, the period where (*E*)-2-hexenal reports lower EAG response. Furthermore, nonanal is a minor component of sexual pheromone of olive fly (Botsi *et al*., 1995). Interestingly, also in males, higher EAG signals were reported at [5-10[days old for the sexual pheromone spiroketal, mainly at 100 µg.mL^{-1} (142%; Table 7.2). Therefore, the high EAG signal for males regarding nonanal in the specific age of [5-10[days old could be ascribed to sexual maturity and recognition of semiochemicals involved in the reproductive process. The loss of response to sexual pheromone and nonanal by males during age is plausible, since from the third to the sixth day of age males respond greatly to the pheromone, but thereafter the response decreases considerably, with a low response observed at 35 days old (Haniotakis and Pittara, 1994). Regarding (*Z*)-9-tricosene, a male produced attractant to female (Canale *et al*., 2012; Carpita *et al*., 2012), it revealed lower EAG signals in olive fly males, being the highest response obtained at [0-5[days old at 100 µg.mL^{-1} (103%). Farnesene, α-pinene, and xylene reported, in general, EAG responses below 100% at [0-5[and [10-15[days old

at the three concentrations tested. However at [5-10[days old, the period correspondent to sexual maturity, these three compounds elicited greater responses, mainly farnesene (132% at 10 μg.mL^{-1}) and xylene (120% at 10 μg.mL^{-1}).

In females, the EAG signal of (*E*)-2-hexenal increased with age. Lower EAG signals were reported at [0-5[days old while a higher response was reported at [10-15[days old, with 146% (Figure 3). It appears that the EAG response to (*E*)-2-hexenal increases with sexual maturity and when olive fly females are gravid and searching for fruits to oviposit their eggs. This information could be very important for olive cultivar oviposition preference, since this aldehyde is a repellent of olive fly females (Scarpati *et al*., 1993). When olives are intact and in normal conditions, (*E*)-2-hexenal is absent of the volatile profile of fruits. Meanwhile this compound formation is greatly enhanced when olives suffer any kind of tissue disruption. (*E*)-2-Hexenal is formed by the lipoxygenase pathway (LOX) in olives by the release of enzymes that enter in contact with polyunsaturated fatty acids, namely linolenic acid. By the action of hydroperoxyde lyases and isomerases (*E*)-2-hexenal is yielded. Therefore, this aldehyde is the main volatile component found in olive oils (Angerosa *et al*., 1999). When olive fly females select an olive to oviposite their eggs, many times they introduce the ovipositor inside the fruit, but no egg is left inside the pulp, being the tentative fruitless and considered an abortive sting. This reality could be related to the release of (*E*)-2-hexenal when olive tissues are disrupted by the olive fly females ovipositor. In fruit flies the ovipositor is also a sensitive organ, used for the selection of host plant, and for oviposition purposes. Ovipositor is composed by olfactory receptor neurons inside chemosensilla (Zhang *et al*., 2012), like in insect antenna, tarso and palps (Liscia *et al*., 2013). When olive fly female introduce its ovipositor in olives, the release of (*E*)-2-hexenal from the olive pulp could exert a repulsive action, being the amounts of (*E*)-2-hexenal dependent on olive cultivar and olive maturation. The facts mentioned above could explain the high EAG response of olive fly females to (*E*)-2-hexenal, since higher responses are observed when olive flies become sexually mature and ready to oviposite.

Concerning the EAG response of nonanal in olive fly females, a contradictory trend comparatively to (*E*)-2-hexenal was revealed. Nonanal reported similar results at 10 and 100 μg mL^{-1} at both [0-5[and [5-10[days old groups (Figure 3). At [10-15[days old lower EAG signals were obtained, of 103% (10 μg.mL^{-1}) and 119% (100 μg mL^{-1}). It's also clear that nonanal has a more pronounced effect in males than females of olive fly. The same observation was verified by Seris (2011) where nonanal, found as a volatile component of different lures, revealed higher EAG signals in males than females between 14-24 days old.

The remaining olive tree volatiles α-pinene, farnesene and xylene reported interesting EAG signals at distinct age groups. In the case of farnesene, this sesquiterpene elicited higher responses at [5-10[days old in females, similar to the result observed in males. In some Tephritid species, like the West Indian fruit fly *Anastrepha obliqua* (Macquart), several farnesene isomers are emitted by the calling males to attract females, reporting these isomers a high EAG response in females (López-Guillén *et al*., 2011). In the case of olive fly there are no reports on the effect of farnesene in their sexual behaviour or attractant activity. However, and based in the current results obtained, it appears that farnesene and its isomers may influence the behaviour of both males and females of olive fly. In the case of α-pinene and xylene, higher EAG responses were obtained at [10-15[days old at 10 μg mL^{-1}. In the case of α-pinene, higher EAG responses were reported in females comparatively to males (Table 7.2). Nevertheless, both sexes are affected by α-pinene. By one hand α-pinene is an oviposition promoter of olive fly females (Scarpati *et al*., 1993), and by other hand, this terpene is beneficial to olive flies from both sexes enhancing their mating success (Gerofotis *et al*., 2013). Therefore, the EAG response of both olive fly sexes to α-pinene could be related to sexual attraction and also to females oviposition. In the case of xylenes, p-xylene and o-xylene appeared to be weak oviposition promoters according to Scarpati *et al*. (1993). Nevertheless xylenes EAG responses were clearly higher in females after achieving sexual maturity, at [10-15[days old.

Regarding the EAG signal of the sex pheromones in olive fly females, (*Z*)-9-tricosene, showed very low EAG response (Table 7.2), but with increasing signal with olive fly female age. The highest response was verified at [10-15[days old at 10 $\mu g.mL^{-1}$, with 104%. In the case of spiroketal, a pheromone produced by females to attract males, EAG responses were greater than hexane only in two situations, [0-5[(107%) and [5-10[days old (123%), both at a concentration of 100 $\mu g.mL^{-1}$.

Conclusions

In the present work it is possible to conclude that essential oils from olive leaves of cultivars with different susceptibilities to olive fly could influence oviposition preference. An inverse proportionality was verified among the degree of susceptibility and the EAG signal obtained in the antenna sensilla of olive fly males and females. The chemical composition of the essential oils of olive leaves is qualitatively and quantitatively different between cultivars, a fact that could be responsible for the differences found in the EAG assays.

Olive fly males responded, in general, more intensely to the stimuli tested, but a higher decrease in the EAG response is observed during age, while in females the same is not generally observed. Olive fly sex and age influenced the EAG response on individual host volatiles. (*E*)-2-Hexenal and nonanal proved to influence olive fly behaviour, with the first one exerting higher influence in females during oviposition period.

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CHAPTER 8.

Influence of olive cultivar and maturation process on the oviposition preference of *Bactrocera oleae* (Rossi) (Diptera: Tephritidae)

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Abstract

Olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is a key-pest in the main olives producing areas worldwide, and displays preference to different olive cultivars. The present work intended to study oviposition preference to three Portuguese olive cultivars (Cobrançosa, Madural and Verdeal Transmontana) at different maturation indexes. Two types of oviposition bioassays (three-choice and one choice) were conducted during 10 days to assess cultivar preference. One-choice tests were conducted to assess the influence of different maturation indexes (MI 2; MI 3 and MI 4). The longevity of olive fly adults according to the cultivar in which its larvae developed was also evaluated by survival assays.

Cultivar and maturation are crucial aspects in olive fly preference. Field and laboratory assays revealed preference to cv. Verdeal Transmontana olives and a lower susceptibility of cv. Cobrançosa olives. A higher preference is observed for olives at MI 2 and MI 3 in detriment of MI 4. The slower maturation process in cv. Verdeal Transmontana, still green while the other olive cultivars are already at reddish or black stage, seems to have an attractive effect to olive fly females, increasing its infestation levels.. Olive fly adults from both sexes live longer if emerged from pupae developed under cv. Verdeal Transmontana fruits and live less if emerged from cv. Cobrançosa fruits. Therefore, olive cultivar and maturation process are crucial aspects in olive fly

preference, also influencing the longevity of adults, and therefore the potential to expand contamination.

Keywords: olive fly; cultivar preference; olive ripening; oviposition; adult longevity

Introduction

Olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) is one of the most important pest of olive crop worldwide and a key-pest in the Mediterranean region. The impact of olive fly is considerably high, causing olives production losses by pulp consumption, fruit drop, and contamination of olives with microorganisms (Iannota *et al*., 2012), compromising the commercial classification of olive products, reducing olive oil quality, composition and functional properties (Pereira *et al*., 2004), besides enabling table olives production (Kailis and Harris, 2007), with an overall unprecedented economic impact. Nevertheless, this impact varies considerably among cultivars. Some olive cultivars are considerably less susceptible to olive fly attacks, while in others production can be totally lost in years of high infestation levels (Navrozidis *et al*., 2007; Burrack and Zalom, 2008).

The interaction between olive tree and olive fly regarding oviposition preference has been driven in order to understand the parameters involved in such phenomena and while seeking for new alternative paths to reduce this pest populations by natural and sustainable ways. The studies conducted were mainly based in physical (Rizzo *et al*., 2012), chemical (Kombargi *et al*., 1998), and molecular factors (Corrado *et al*., 2012; Imperato *et al*., 2012). In the case of physical parameters, studies were conducted in order to establish correlations between olive size and hardness with olive fly oviposition preference (Gonçalves *et al*., 2012), as well as colour (Katsoyannos and Kouloussis, 2001). However, olive colour is intrinsically correlated with fruits maturation, during which olives undergo modifications induced by metabolic process, but each olive cultivar maturation process advances differently. Some cultivars report a fast ripening process, rapidly changing from green to black fruits and passing through the so-called 'cherry-phase', while others remain green for quite long periods. In this way, the colour of olives in the different maturation indexes may influence olive fly choice towards a specific olive cultivar in detriment of others. Some authors verified a higher attraction of olive fly females to red colours (Katsoyannos and Kouloussis, 2001), while others report the preference of olive fly to green olives (Vlahov, 1992).

In Portuguese cultivars from Trás-os-Montes region, olive fly oviposition preference is also observed (Gonçalves *et al*., 2012), particularly for cvs. Verdeal Transmontana and Madural, while cv. Cobrançosa reports consistent low levels of infestation each year.

In this sense, the main objective in the present work is to study the influence of olive cultivar and maturation index in oviposition preference, under laboratory conditions, of olive fly in Portuguese cultivars with different susceptibility degrees (cvs. Cobrançosa, Madural and Verdeal Transmontana). The effect of the cultivar in which the larvae were developed on olive fly adults survival was also evaluated. To the authors' knowledge, this is the first investigation of this kind with these cultivars, highlighting the effect of cultivar and maturation in the oviposition preference of olive fly in bioassays. Furthermore, this is also the first report available about the survival of olive fly adults according to the olive cultivar in which larvae feed and developed.

Material and methods

In the present work, all olive samples and insects used were collected from an organic olive grove located in Paradela (Mirandela - 41º32'35.72''N; 7º07'27.17''W) Trás-os-Montes region (Northeast of Portugal) in the years of 2013 and 2014. The study focused in three of the main cultivars of Trás-os-Montes region, Cobrançosa, Madural and Verdeal Transmontana.

Infestation level and maturation index determination

From each olive cultivar, five trees were selected and marked to determine infestation level and maturation index. Both parameters were assessed fortnightly from 27th Aug to 6th Nov 2013 (last possible date to be assessed prior to olives harvest).

To assess infestation level, 40 random handpicked fruits were collected from each olive tree (5 trees per cultivar; 200 fruits) in the mentioned periods and inspected in a binocular stereomicroscope for signs of infestation (oviposition sites or exit holes). Infestation level was expressed as the percentage of infested olive fruits.

Simultaneously, fruits were collected per tree for calculation of the maturation index, as described by Hermoso *et al*. (2001). Briefly, samples of 100 olive fruits (20 fruits per tree) were separated in 8 maturation indexes (MI) based on epidermis and pulp color

(0 to 7). Therefore, the fruit is classified as “MI 0” if the epidermis is green; “MI 1” for yellowish green; “MI 2” if the epidermis shows red spots in less than half fruit; “MI 3” if the epidermis is red or purple in more than half fruit; “MI 4” for black epidermis and white pulp; “MI 5” if the epidermis is black and less than half pulp is purple; “MI 6” if the epidermis is black and more than half pulp purple (without reaching the stone); “MI 7” if the epidermis is black and total pulp purple (reaching the stone). The maturation index was calculated as follows: MI = (a × 0 + b × 1 + c × 2 + d × 3 + e × 4 + f × 5 + g × 6 + h × 7) / 100, where the letters are the number of fruits in each MI of classification considered.

Bactrocera oleae collection and rearing

Olives with signs of olive fly infestation were separated and spread in trays, being larvae and pupae collected daily and transferred to an insectarium for rearing purposes. Once hatched from pupae, adults were separated daily (for age control purposes) from the main insectarium to rearing caches, being both sexes maintained together. Larvae, pupae and adults were maintained at the following conditions: 26 ± 1 ºC, 70 ± 10% of relative humidity, with a photoperiod of 16L:8D. Adults were feed *ad libitum* with a honey solution (10% w/v), artificial diet (sucrose and yeast extract at a ratio 4:1), and water, being the diet changed every two days.

Oviposition bioassays

For all the oviposition assays developed, olive fly adults (both males and females) were used with at least 15 days old in order to ensure that all females were gravid and available to oviposit. Olives used in all oviposition bioassays were collected from marked trees of each cultivar. Once in laboratory, all olives were inspected one by one to select only perfectly healthy olives. Olives with signs of diseases were discarded and olives with signs of olive fly infestation were used for survival assays (in more detail in section “Survival bioassays”).

The conditions in which all oviposition bioassays were conducted and the diet given to the flies are the same described in section “*Bactrocera oleae* collection and rearing”.

For all bioassays conducted each set of olives was observed at a binocular stereomicroscope to count the number of oviposition sites on each olive. After that, olives

were maintained during one month at the same conditions described in section "*Bactrocera oleae* collection and rearing". to collect and register the number of pupae and/or adults emerged. The following parameters were considered: number of ovipositions; number of olives recovered without ovipositions; number of olives recovered with ovipositions; number of ovipositions per assayed olive; number of ovipositions per infested assayed olive; number of pupae/adults collected; percentage of recovered pupae/adults to the total amount of ovipositions.

Oviposition bioassays based on olive cultivar

In order to verify the cultivar oviposition preference of olive fly, two types of bioassays were developed:

i) *Three-choice oviposition assays*: males and females of olive fly (10 elements per sex) were placed in cages (0.05 m^3) and maintained without the presence of olives during 24 h. After this period 60 olives (20 from cv. Cobrançosa, 20 from cv. Madural, and 20 from cv. Verdeal Transmontana) were given to the flies during 24 h for oviposition, being removed after this period and immediately replaced by a new set of 60 olives. This operation was repeated during 10 consecutive days in five independent cages (n = 5), totalizing 1000 olives assessed per cultivar.

ii) *One-choice oviposition assays*: in one-choice oviposition bioassays is replicated the same procedure and conditions applied in three-choice oviposition bioassays, however, in this case 60 olives of a single cultivar are given to the flies during 24 hours during 10 consecutive days. Five independent bioassays per cultivar were developed, totalizing 3,000 olives assessed per cultivar.

In both three-choice and one-choice oviposition bioassays olives from cvs. Cobrançosa and Madural were at a MI (maturation index) 3-4, and olives from cv. Verdeal Transmontana were at a MI 2-3.

Oviposition bioassays based on maturation index

In order to verify the impact of MI in oviposition preference of olive fly, an oviposition bioassay similar to the one-choice oviposition bioassay was developed. In this

case, three MI were tested per olive cultivar: MI 2, MI 3, and MI 4. Olives used were separated according to the scale of Hermoso *et al*. (2001) (see section "Infestation level and maturation index determination". for further details). The replicates were limited to the number of available healthy fruits in each MI, so five replicates were conducted at MI 2 and MI 3 for cvs. Cobrançosa and Madural (3,000 olives per cultivar and MI); four replicates were conducted at MI 2 and MI 3 for cv. Verdeal Transmontana (2,400 olives assessed per MI); three and two replicates were conducted at MI 4 for cvs. Cobrançosa and Madural respectively (1800 and 1200 olives assessed, respectively). Due to the slower maturation process of cv. Verdeal Transmontana, prior to harvest date, it was impossible to collect enough amounts of healthy olives to implement oviposition bioassays at MI 4 for this cultivar.

Survival bioassays

Olives destined for oviposition bioassays, but with signs of infestation of olive fly were separated by olive cultivar. These olives were spread in trays, being larvae and pupae collected daily and transferred to three rearing cages, one per cultivar. Rearing cages were daily inspected (at least each 8 h) for signs of adults. Once emerged, olive fly adults were separated by sex and maintained in groups of a maximum of 10 individuals in smaller cages only with water (no diet supplied), being inspected at least each 8 h for signs of dead individuals. For each olive cultivar and sex, 100 individuals were monitored. The most approximate date and hour of emergence and death were recorded to calculate the most proximate survival period in days. Temperatures, relative humidity and photoperiod applied were the same as described in section "*Bactrocera oleae* collection and rearing".

Statistical analysis

Analysis of variance

An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov-Smirnov with Lilliefors correction (if n>50) or the Shapiro-

Wilk`s test (if n<50), and the Levene´s tests, respectively. All dependent variables were analysed using a one-way ANOVA with or without Welch correction, depending if the requirement of the homogeneity of variances was fulfilled or not. The main factors studied were: longevity of olive fly males and females which larvae developed in different olive cultivars. If a statistical significant effect was found, means were compared using Tukey´s honestly significant difference multiple comparison test or Dunnett T3 test also depending if equal variances could be assumed or not. All statistical tests were performed at a 5% significance level.

Results and discussion

Infestation levels and maturation

Olives from cvs. Cobrançosa, Madural and Verdeal Transmontana were monitored from 27th August to 6th November to assess their infestation levels and maturation index. The infestation levels remained low (< 5%), in the three olive cultivars, until 10th October (Figure 8.1).

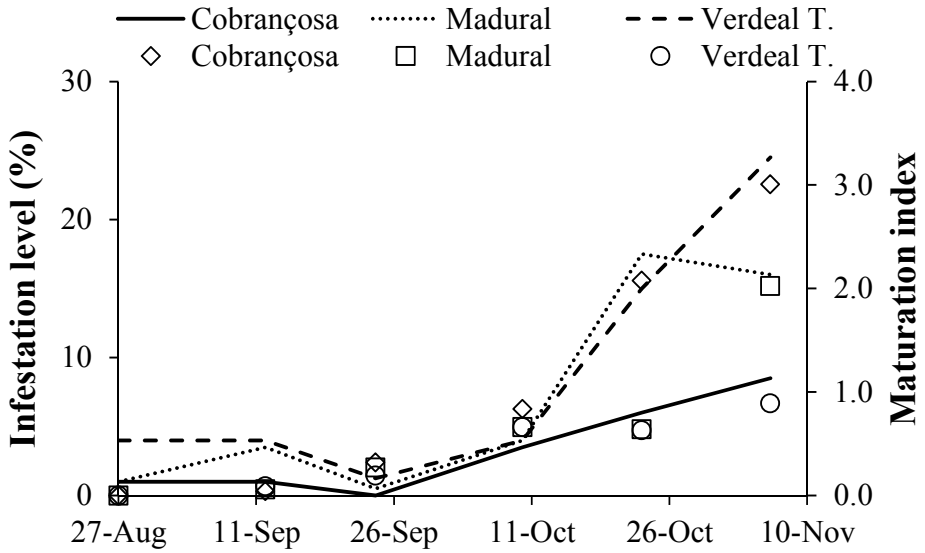

Figure 8.1. Infestation levels (%) (lines) and maturation index (markers) of olives from cvs. Cobrançosa, Madural and Verdeal Transmontana.

After the 10th October, infestation levels began to rise in all cultivars, with higher increment in cvs. Madural (17.5%) and Verdeal Transmontana (15.0%) on that date. In the last sampling date (6th November), infestation level of 24.5% for cv. Verdeal Transmontana, followed by cv. Madural (16.0%), and by last cv. Cobrançosa (8.5%) were

observed. This tendency is reported in other years, being cvs. Madural and Verdeal Transmontana more susceptible, while cv. Cobrançosa display lower susceptibility against olive fly (Gonçalves *et al*., 2012; Malheiro *et al*., 2014). It was interesting to observe that infestation levels start rising at the exact moment when maturation begins (Figure 8.1). This period also coincides with the decrease of the high temperatures observed during July and August, that are not favourable to a fast development of olive fly populations and also causes high mortality of the oviposit eggs (Gonçalves *et al*., 2012). Olives from cv. Verdeal Transmontana reported a very slow maturation process, being completely green at the beginning of the study (MI = 0) and still being yellow-green (MI = 0.89) at the end, coinciding with olives pickup. For cv. Madural, at the end of the study olives were already in the cherry-stage, becoming reddish (MI = 2.03). Regarding olives from cv. Cobrançosa, they reported a faster maturation rate, reporting a MI = 2.08 at the 23rd October and a MI = 3.01 at the last assessed date, being completely reddish. Therefore, olive fly can find simultaneously in the field olives with green coloration, yellow green and reddish, showing a clear preference to green or yellow-green olives as verified by our data (Figure 8.1). Other authors also claim the preference of olive fly females to oviposit in greener olives (Vlahov, 1992). This could be one explanation for the high levels of infestation in olives from cv. Verdeal Transmontana comparatively to the other two cultivars.

Effect of cultivar in olive fly oviposition

In order to verify the olive fly oviposition preference at laboratory conditions, two types of bioassays were conducted: three-choice and one-choice assays. In three-choice assays, olives from the three olive cultivars were simultaneously available at the same time for oviposition to olive fly, while in one-choice assays, each cultivar was assessed independently.

Three-choice oviposition bioassays

For the three olive cultivars it was noted that the number of ovipositions progressively increased as the days assessed passed, increasing from 1 oviposition in day 1 in the three cultivars, to 44, 41 and 54 (day 10) for cvs. Cobrançosa, Madural, and Verdeal Transmontana, respectively (Table 8.1).

Table 8.1. Parameters evaluated in three-choice oviposition bioassays during 10 consecutive days, with olives from cvs. Cobrançosa, Madural and Verdeal Transmontana (mean values; n = 5).

	Days assayed										
Parameter	1	2	3	4	5	6	7	8	9	10	Σ
Ovipositions (n)											
Cobrançosa	1	1	2	6	19	26	27	21	37	44	184
Madural	1	2	2	4	17	28	22	24	29	41	170
Verdeal Transmontana	1	3	2	5	14	24	23	29	52	54	207
Olives not attacked (n)											
Cobrançosa	19	19	18	15	9	5	6	8	4	3	106
Madural	19	19	18	16	9	5	4	6	6	3	105
Verdeal Transmontana	19	18	18	16	10	7	5	4	4	2	103
Olives attacked (n)											
Cobrançosa	1	1	2	5	11	15	14	12	16	17	94
Madural	1	1	2	4	11	15	16	14	14	17	95
Verdeal Transmontana	1	2	2	4	10	13	15	16	16	18	97
Ovipositions per olive											
Cobrançosa	0.04	0.04	0.11	0.31	0.95	1.28	1.36	1.04	1.86	2.22	0.92
Madural	0.04	0.08	0.10	0.20	0.85	1.38	1.10	1.18	1.43	2.04	0.85
Verdeal Transmontana	0.06	0.13	0.10	0.24	0.70	1.21	1.17	1.45	2.60	2.68	1.04
Ovipositions per infested olive											
Cobrançosa	0.40	0.40	0.80	1.33	1.70	1.72	1.90	1.71	2.29	2.61	1.96
Madural	0.40	0.40	0.80	0.85	1.59	1.86	1.35	1.65	1.94	2.36	1.79
Verdeal Transmontana	0.60	0.64	0.65	1.08	1.26	1.73	1.56	1.77	3.07	3.00	2.13
Collected pupae/adults											
Cobrançosa	0	0	2.	4	10	16	13	14	25	31	115
Madural	0	2	1	1	12	16	15	13	19	29	108
Verdeal Transmontana	0	1	1	3	10	17	19	17	33	33	134
Ratio pupae/stings (%)											
Cobrançosa	0	0	100	66.7	52.6	61.5	48.1	66.7	67.6	70.4	62.5
Madural	0	100	50.0	25.0	70.6	57.1	68.1	54.2	65.5	70.7	63.5
Verdeal Transmontana	0	33.3	50.0	60.0	71.4	70.8	82.6	58.6	63.5	61.1	64.7

This result could be related to a gradual adaptation of olive fly females to the presence of olives. During the 10 days assessed, olives from cv. Madural reported the lower oviposition average, 170 (in 200 fruits overall), followed by cv. Cobrançosa with 184, and cv. Verdeal Transmontana reported the highest number of ovipositions, with 207 (Table 8.1). Regarding the number of attacked and non-attacked olives, the three cultivars revealed similar values, with the number of attacked olives varying between 103 and 106. Olives from cv. Verdeal Transmontana reported a general higher number of ovipositions per fruit (1.04), and when we report the number of ovipositions only to the attacked olives it increase to 2.13, against 1.96 and 1.79 of cvs. Cobrançosa and Madural, respectively. From the 207 ovipositions made in cv. Verdeal Transmontana olives, 134 pupae/adults were collected, accounting for 64.7% of ovipositions effectiveness (Table 8.1) while for cvs. Cobrançosa and Madural, 115 and 108 pupae/adults were recovered, accounting for 62.5% and 63.5% respectively.

Therefore, in the results obtained in three-choice bioassays, small differences were found in the three olive cultivars for all parameters assessed and calculated. We

believe that by being presented at the same time it may confuse olive fly females in their choice, diluting the option of preference. Also, once the fruits of three tree cultivars were given simultaneously and after being picked from the tree, the possible stimulus observed in field, like green leaf volatiles were diluted and might confuse fly females. On the other side, considering that all the available fruits had similar maturation index and were freely mixed, it was difficult for the females to select different varieties. Therefore, we implemented a new bioassay with only one option, the one-choice oviposition bioassay.

One-choice oviposition bioassays

In one-choice oviposition bioassays, a single olive cultivar was presented to olive fly females at a time, therefore a real choice is not in question. The results obtained in this type of bioassay were concise and clear: olives from cv. Verdeal Transmontana are highly preferred by females, followed by cv. Madural and by last cv. Cobrançosa olives (Table 8.2).

Table 8.2. Parameters evaluated in one-choice oviposition bioassays during 10 consecutive days, with olives from cvs. Cobrançosa, Madural and Verdeal Transmontana (mean values; n = 5).

	Days assayed										
Parameter	1	2	3	4	5	6	7	8	9	10	Σ
Ovipositions (n)											
Cobrançosa	16	60	26	45	17	29	36	82	55	84	450
Madural	62	60	90	39	51	79	34	89	18	55	577
Verdeal Transmontana	42	148	96	111	140	107	135	85	125	84	1073
Healthy olives (n)											
Cobrançosa	47	26	41	28	44	40	32	21	24	26	329
Madural	11	29	12	33	31	16	35	14	44	22	247
Verdeal Transmontana	28	4	25	9	10	14	12	15	5	11	133
Attacked olives (n)											
Cobrançosa	13	34	19	32	16	20	28	39	36	34	271
Madural	49	31	48	27	29	44	25	46	16	38	353
Verdeal Transmontana	32	56	35	51	50	46	48	45	55	49	467
Ovipositions per olive											
Cobrançosa	0.27	1.00	0.43	0.75	0.28	0.48	0.60	1.37	0.92	1.40	0.75
Madural	1.03	1.00	1.50	0.65	0.85	1.32	0.57	1.48	0.30	0.92	0.96
Verdeal Transmontana	0.70	2.47	1.60	1.85	2.33	1.78	2.25	1.42	2.08	1.40	1.79
Ovipositions per infested olive											
Cobrançosa	1.23	1.76	1.37	1.41	1.06	1.45	1.29	2.10	1.53	2.47	1.66
Madural	1.27	1.94	1.88	1.44	1.76	1.80	1.36	1.93	1.13	1.45	1.63
Verdeal Transmontana	1.31	2.64	2.74	2.18	2.80	2.33	2.81	1.89	2.27	1.71	2.30
Collected pupae/adults											
Cobrançosa	8	41	12	29	5	5	17	50	33	30	230
Madural	104	36	78	24	23	17	22	70	11	34	361
Verdeal Transmontana	35	99	81	80	77	48	71	45	42	79	657
Ratio pupae/stings											
Cobrançosa	50.0	68.3	46.2	64.4	29.4	17.2	47.2	61.0	60.0	35.7	51.1
Madural	74.2	60.0	86.7	61.5	45.1	21.5	64.7	78.7	61.1	61.8	62.6
Verdeal Transmontana	83.3	66.9	84.4	72.1	55.0	44.9	52.6	52.9	33.6	94.0	61.2

Median values of ovipositions were tremendously higher in cv. Verdeal Transmontana, with 1073 ovipositions (in 600 olives overall). Only 133 olives weren´t infested, reporting an overall number of ovipositions per olive of 1.79 (Table 8.2). By dividing the number of ovipositions by the number of infested olives, each infested olive was attacked 2.3 times in cv. Verdeal Transmontana. Regarding cv. Madural 577 ovipositions were recorded, nearly half of those reported by cv. Verdeal Transmontana. An average of 0.96 ovipositions per olive was observed, while per infested olive it raised to 1.63. Olives from cv. Cobrançosa were less attacked by olive fly, with 450 ovipositions overall, less than 1 oviposition per olive assayed (0.75) and an average of 1.66 ovipositions per infested fruit (Table 8.2).

Concerning the number of pupae/adults recovered, 657, 361, and 230 were collected from cvs. Verdeal Transmontana, Madural and Cobrançosa, respectively. This means a percentage of recovery from the total number of oviposition of 61.2%, 62.6% and 51.1%. Besides being the less preferred by olive fly females to oviposit, olives from cv. Cobrançosa reported a lower ratio between pupae/adults recovered and the number of ovipositions. In this case it means that 39.8%, 37.4% and 48.9% of the ovipositions made were unable to lead to adults' formation in cvs. Verdeal Transmontana, Madural and Cobrançosa, respectively. Three aspects could be related to these observations: i) a high number of sterile ovipositions, in which olive fly females perforate the olive epidermis but no egg is laid inside; ii) the egg is laid but it is unable to develop correctly and no larvae hatch; iii) the larvae is capable to hatch but dies inside olive pulp. In the three scenarios presented olive volatiles and pulp composition, namely in phenolic compounds, may have a crucial role. In the case of sterile ovipositions, when olive fly introduce the ovipositor and the olive tissues are disrupted, it cause a series of enzymatic mechanisms (lipoxygenase pathway (LOX) and β-glucosidase activity). These enzymes lead to the formation of highly toxic and deterrent aldehydes like the case of (*E*)-2-hexenal, a linolenic acid degradation product (Salas *et al*., 1999), and a recognized olive fly deterrent (Scarpati *et al*., 1993). The formation of these deterrents is highly dependent on olive cultivar and maturation stage, and it may be formed in higher amounts in cv. Cobrançosa, increasing the number of sterile punctures.

In the second and third hypothesis raised, phenolic compounds may have a direct part in the low rate of pupae/adults collected in cv. Cobrançosa. Indeed, once an olive is attacked by olive fly, a complex internal defence mechanism is trigged. With tissue disruption, oleuropein, the main secoiridoid found in olives, is hydrolysed by β-glucosidase, leading to the formation of highly reactive toxic molecules (Spadafora *et al*., 2008), as glutaraldehyde-like structures with strong protein-denaturing properties

(Koudounas *et al*., 2015). This enzymatic mechanism is more pronounced in less susceptible olive cultivars and reports a low expression in highly susceptible cultivars as observed in cvs. Carolea (highly susceptible) and Cassanese (low susceptibility) (Spadafora *et al*., 2008). In our cultivars, cv. Cobrançosa report high contents of oleuropein at early maturation indexes (33 $g.kg^{-1}$ of olive pulp; Sousa *et al*., 2014) while cv. Verdeal Transmontana contains 13 $g.kg^{-1}$ of olive pulp (Sousa *et al*., 2015). The formation of toxic molecules from oleuropein may abort eggs in olive pulp, since a higher accumulation of this structures are found around the oviposition site (Spadafora *et al*., 2008). Nevertheless, even if the egg is viable and a larvae hatch, the amounts of these compounds during larvae development may compromise as well its normal development leading therefore to death due to toxicity.

Maturation process and cultivar in olive fly oviposition preference

Based on the results obtained in one-choice oviposition bioassays for the determination of the effect of cultivar in preference of olive fly, the same type of procedure was adopted to study the effect of maturation. In these oviposition bioassays, olives from the three olive cultivars at MI 2, MI 3 and MI 4 (except cv. Verdeal Transmontana at IM 4) were tested.

From the results obtained it was observed that, besides olive cultivar, the maturation stage is a preponderant factor influencing olive fly oviposition. At the same maturation index, MI 2, olives from cv. Verdeal Transmontana were the most infested, reporting an average of 778 ovipositions against 614 and 594 from cvs. Cobrançosa and Madural, respectively (Figure 8.2).

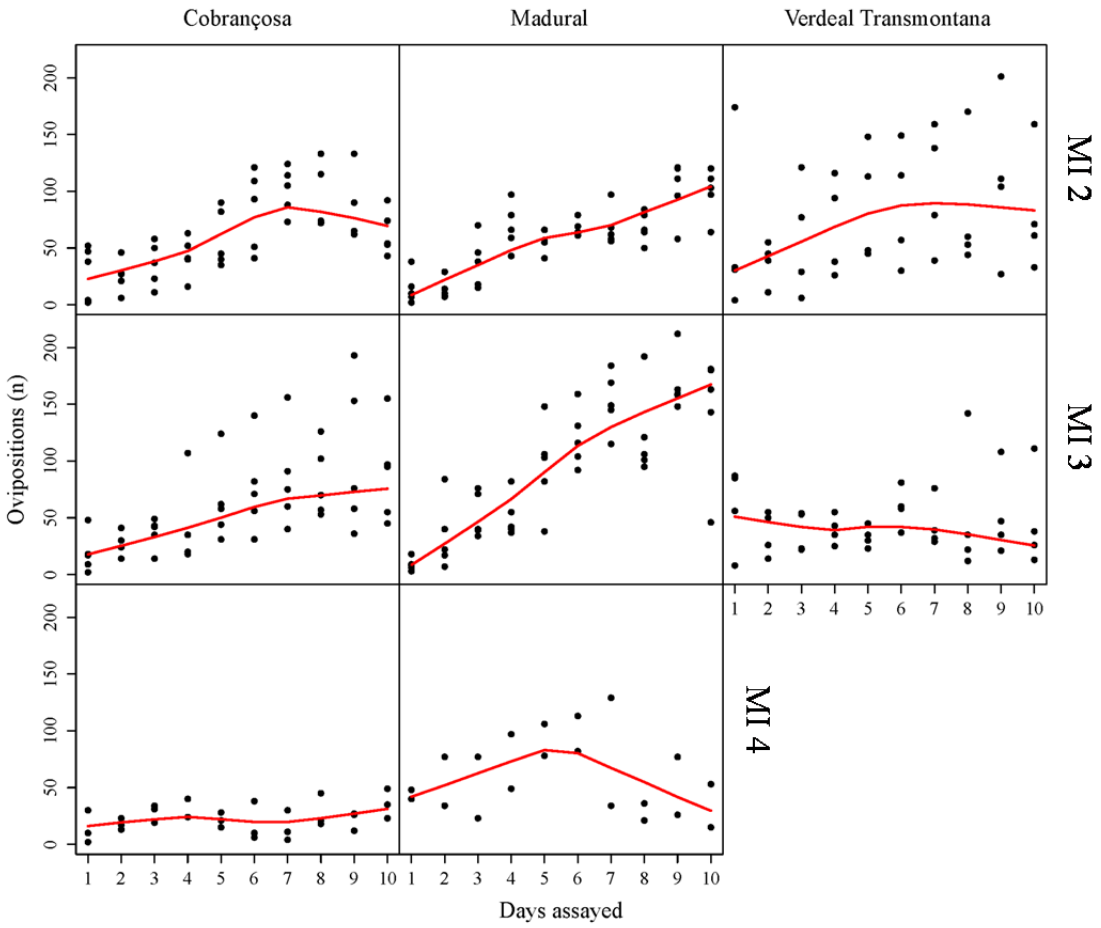

Figure 8.2. Number of ovipositions (mean values; number of replicates available in Table 3) made by olive fly females in olives from cvs. Cobrançosa, Madural and Verdeal Transmontana at different maturation index (MI = 2; MI = 3; and MI =4).

The number of ovipositions decreased slightly from MI 2 to MI 3, mainly in cv. Verdeal Transmontana, while in cv. Madural a considerable increase was observed (Figure 2), to 948. In Figure 8.2 is possible to observe that both olive cultivars tested at MI 4 reduced considerably the number of ovipositions: cv. Cobrançosa decreased from 621 in MI 3 to 229 in MI 4, while cv. Madural decreased from 948 in MI 3 to 608 in MI 4. From these results is clear that olive fly prefer green or reddish olives rather than black olives. Some authors claim that the dark coloration of olive drupes with advanced ripening stages may confuse olives recognition by olive fly females, reducing the risk of infestation (Iannota and Scalercio, 2012). Also, the infestation odds of black olives are clearly low comparatively to red or green olives (Rizzo *et al*., 2012). Our results could also be related to the observations made by Katsoyannos and Kouloussis (2001), in which olive fly females were specially attracted by red color sphere traps.

Another interesting aspect observed in the maturation ovipositions bioassays was the percentage of collected pupae/adults in respect to the mean number of ovipositions. At MI 4 only 7% of cv. Cobrançosa pupae/adults were recovered, which means that about 93% of ovipositions made were sterile punctures or eggs and larvae that died inside the pulp of cv. Cobrançosa olives. At MI 2 and 3 the percentage of recovery was respectively 52.1% and 59.7% (Table 8.3).

Table 8.3. Parameters evaluated in one-choice oviposition bioassays during 10 consecutive days at different maturation stages, with olives from cvs. Cobrançosa, Madural and Verdeal Transmontana (mean values; number of replicates displayed in each row with maturation index).

		Days assayed										
Parameter		1	2	3	4	5	6	7	8	9	10	Σ
Healthy olives (n)	MI*											
Cobrançosa	2 (n = 5)	37	39	33	28	20	13	9	13	13	17	222
	3 (n = 5)	45	40	35	33	21	18	15	11	10	12	240
	4 (n = 3)	49	47	40	41	43	47	50	41	45	35	438
Madural	2 (n = 5)	47	49	34	16	20	12	11	9	11	14	223
	3 (n = 5)	52	38	25	24	12	7	4	7	7	10	186
	4 (n = 2)	36	27	35	25	17	18	19	40	28	37	282
Verdeal Transmontana	2 (n = 4)	33	37	32	29	22	22	22	30	15	24	266
	3 (n = 4)	25	34	34	32	38	25	30	31	27	34	310
Attacked olives (n)												
Cobrançosa	2 (n = 5)	23	21	27	32	40	47	51	47	47	43	378
	3 (n = 5)	15	20	25	27	39	42	45	49	50	48	360
	4 (n = 3)	11	13	20	19	17	13	10	19	15	25	162
Madural	2 (n = 5)	13	11	26	44	40	48	49	51	49	46	377
	3 (n = 5)	8	22	35	36	48	53	56	53	53	50	414
	4 (n = 2)	24	33	25	35	43	42	41	20	32	23	318
Verdeal Transmontana	2 (n = 4)	27	23	28	31	38	38	38	30	45	36	334
	3 (n = 4)	35	26	26	28	22	35	30	29	33	26	290
Ovipositions per olive												
Cobrançosa	2 (n = 5)	0.48	0.43	0.60	0.71	0.97	1.38	1.68	1.56	1.38	1.05	1.02
	3 (n = 5)	0.31	0.41	0.61	0.72	1.06	1.27	1.41	1.36	1.72	1.49	1.04
	4 (n = 3)	0.23	0.29	0.47	0.49	0.36	0.30	0.25	0.47	0.36	0.59	0.38
Madural	2 (n = 5)	0.24	0.23	0.62	1.15	0.92	1.12	1.14	1.14	1.69	1.65	0.99
	3 (n = 5)	0.15	0.57	0.87	0.85	1.59	2.01	2.54	2.05	2.80	2.38	1.58
	4 (n = 2)	0.73	0.93	0.83	1.22	1.53	1.63	1.36	0.48	0.86	0.57	1.01
Verdeal Transmontana	2 (n = 4)	1.01	0.63	0.97	1.14	1.48	1.46	1.73	1.36	1.85	1.35	1.30
	3 (n = 4)	0.98	0.60	0.63	0.66	0.55	0.98	0.73	0.88	0.88	0.78	0.77
Ovipositions per infested olive												
Cobrançosa	2 (n = 5)	1.23	1.20	1.31	1.31	1.42	1.72	1.99	1.95	1.76	1.47	1.62
	3 (n = 5)	1.15	1.23	1.42	1.47	1.53	1.72	1.81	1.62	1.95	1.80	1.72
	4 (n = 3)	1.17	1.38	1.41	1.55	1.28	1.19	1.38	1.43	1.42	1.38	1.41
Madural	2 (n = 5)	1.09	1.21	1.35	1.53	1.36	1.42	1.41	1.35	2.04	2.12	1.58
	3 (n = 5)	1.13	1.44	1.47	1.42	1.93	2.25	2.71	2.32	3.22	2.67	2.29
	4 (n = 2)	1.80	1.60	1.84	2.03	2.10	2.31	1.79	1.38	1.50	1.40	1.91
Verdeal Transmontana	2 (n = 4)	1.70	1.56	1.78	2.02	2.22	2.20	2.67	2.75	2.27	2.16	2.33
	3 (n = 4)	1.58	1.33	1.39	1.40	1.56	1.69	1.43	1.54	1.45	1.62	1.59

Collected pupae/adults												
Cobrançosa	2 (n = 5)	11	13	21	28	33	44	51	47	44	28	320
	3 (n = 5)	13	14	20	27	46	45	51	53	57	45	371
	4 (n = 3)	3	1	3	2	1	1	1	1	0	3	16
Madural	2 (n = 5)	6	8	19	39	20	26	33	30	30	31	242
	3 (n = 5)	5	15	24	29	41	52	66	54	73	34	393
	4 (n = 2)	8	16	11	26	34	24	13	1	6	2	141
Verdeal Transmontana	2 (n = 4)	32	23	31	23	34	32	43	41	40	30	329
	3 (n = 4)	32	15	13	22	11	33	25	32	29	19	231
Ratio pupae/stings												
Cobrançosa	2 (n = 5)	32.2	46.1	55.6	63.9	54.1	53.3	50.6	52.6	54.6	41.0	52.1
	3 (n = 5)	75.4	60.4	52.3	66.0	72.9	57.6	58.2	63.8	55.3	50.6	59.7
	4 (n = 3)	24.4	6.8	14.1	4.7	2.4	8.2	2.2	7.1	1.3	11.3	7.0
Madural	2 (n = 5)	28.9	48.1	49.4	57.7	38.4	40.0	49.4	43.0	30.5	32.5	40.7
	3 (n = 5)	47.2	38.7	44.6	56.2	44.3	44.1	42.6	43.0	42.2	22.7	41.4
	4 (n = 2)	18.5	27.5	25.0	33.9	36.5	26.1	9.7	1.4	11.6	3.8	23.2
Verdeal Transmontana	2 (n = 4)	60.3	62.0	54.9	40.2	42.8	33.5	41.6	46.5	40.7	34.0	42.3
	3 (n = 4)	57.5	45.8	34.3	54.1	33.3	55.3	54.1	51.6	50.7	39.5	50.0

* Maturation index based in Hermoso *et al*. (2001) and according to Figure 8.3.

The same observation was reported in olives of cv. Madural at MI 4. The percentage of recovery of pupae/adults was 40.7% and 41.4% at MI 2 and MI 3, but it decreases to 23.2% at MI 4. These observations clearly state that besides the lower attraction of olive flies to black olives, the ovipositions carried out are highly unsuccessful to maintain this pest populations. These results could be ascribed to the accumulation of toxic molecules of glutaraldehyde-type structures that may remain in olive pulp during maturation, hypothesis in accordance to the decrease of oleuropein in olives of the three olive cultivars (Sousa *et al*., 2014; Sousa *et al*., 2015).

In this case, our results point out that maturation reduces the infestation levels of olive fly, but can also naturally control this insect population by aborting the development of eggs and larvae, by the action of endogenous defence mechanisms mediated by molecular aspects (Koudounas *et al*., 2015).

Survival of olive fly adults

The survival of olive fly adults (males and females) was tested based on the olive cultivar in which the pupae developed. Only water was supplied for the adults and their survival was only due to their body reserves obtained during the larval stages in the fruits. Olive cultivar influences significantly the longevity of both males and females (Figure 8.3), with no significant differences found between males and females (P = 0.423; P = 0.374; and P = 0.868; respectively for cvs. Cobrançosa, Madural and Verdeal Transmontana).

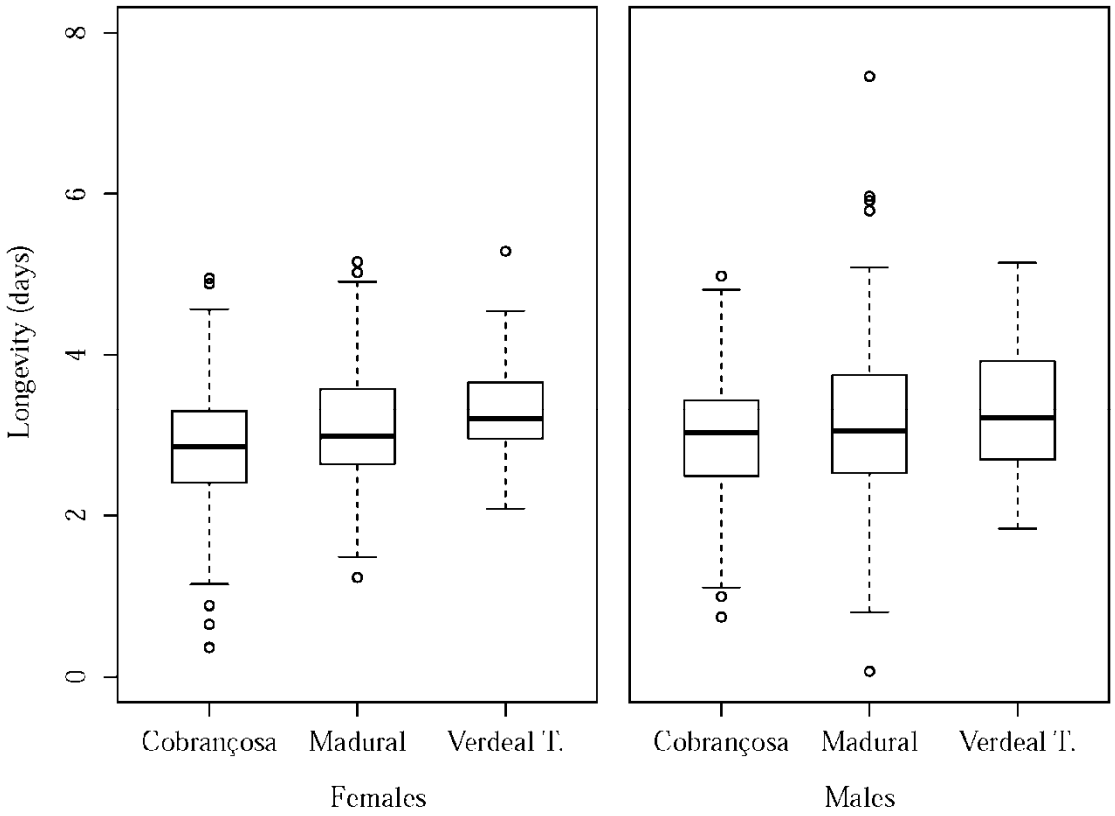

Figure 8.3. Boxplot of longevity (days; n = 100) of olive fly adults emerged from pupae developed under cvs. Cobrançosa, Madural and Verdeal Transmontana olives.

However, adults emerged from pupae developed in cv. Verdeal Transmontana olives lived longer than those from cvs. Cobrançosa and Madural. In average, a male lived for 3.31 days while females lived for 3.29 days from cv. Verdeal Transmontana. In cv. Madural, males and females lived for 3.20 and 3.08 days, while in cv. Cobrançosa they lived 2.98 and 2.89 days, respectively (Figure 8.3).

Males and females from cv. Verdeal Transmontana lived significantly more than those from cv. Cobrançosa ($P = 0.008$ and $P < 0.001$ respectively), approximately 11.1% more for males, while females live approximately more 13.8%. This is important information, since if olive flies emerge in a location without proper nutritional needs, they will be able to live longer to find those requirements if they emerge from cv. Verdeal Transmontana. Therefore, these olive flies may possess higher reserves of nutrients, mainly lipids. In fact the oil content in cv. Verdeal Transmontana olives is higher than in cv. Cobrançosa (Gonçalves *et al*., 2012). Since pupae feed on olive pulp, they may store higher amounts of fat in their body, which will influence adults' longevity, providing them higher energetic values.

The main conclusions of the present study are that olive cultivar and maturation are crucial parameters in the oviposition preference of olive fly females. Both in field and in laboratory bioassays, olive fly females have a clear preference for olives from cv. Verdeal Transmontana, followed by cv. Madural and the less preferred was cv. Cobrançosa. Maturation index influence olive fly oviposition preference, being observed a clear reduction in oviposition from green (MI 2) and reddish (MI 3) to black olives (MI 4). Advanced maturation indexes may cause high levels of mortality of eggs/larvae and absorptive ovipositions.

Since each olive cultivar has different maturation pathways, the slower process in olives from cv. Verdeal Transmontana have a highly attractive action over olive fly females, since olives stays greener for longer periods. It was also concluded that olive fly adults live longer if their larvae develop in olives from cv. Verdeal Transmontana, a fact probably related to fat content of the drupes.

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CHAPTER 9.

Physico-chemical characteristics of *Olea europaea* L. olives and leaves and *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) cultivar oviposition preference

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Abstract

Olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) oviposition preference to Portuguese olive cultivars (cvs. Cobrançosa, Madural, and Verdeal Transmontana) was studied by physical and chemical parameters.

Physical parameters (biometrical data and color) of both olives and olive leaves of the three cultivars were recorded. In olives, maturation indices, infestation levels, and fatty acids profiles were also monitored through time.

Olives from cv. Verdeal Transmontana revealed higher susceptibility to olive fly attack, followed by cv. Madural, while cv. Cobrançosa was the less affected cultivar. Different maturation patters were also observed, with faster maturation in cv. Cobrançosa and a slower process in cv. Verdeal Transmontana. Olives from cv. Verdeal Transmontana reported higher fruit volume and lower elongation, while both olives and leaves reported always higher lightness (*L**), all possible attractive cues for olive fly. Fatty acids composition revealed also a characteristic profile in each cultivar with constant differences during crop season.

Overall, maturation process influenced biometrical data and color of olive cultivars. The slower maturation process characteristic from cv. Verdeal Transmontana could modulate the composition and physical appearance of olives, therefore interfering with olive fly females oviposition preference.

Keywords: olives; olive cultivar; color; physical parameters; olive fly; oviposition

Introduction

Olives production is steadily increasing in the last years worldwide, with about 20.3 million tons in 2013, the second highest production level ever accomplished (FAOSTAT, 2015). Since ancient times, the olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), has been one of the key pests of olive crops (Daane and Jonhson, 2010) and a simultaneous increase and widespread attacks are being reported. Indeed, a significant drop in olives production is expected in the 2014/2015 campaign, mainly attributed to olive fly attack. In the case of olive oil, a 27% reduction comparatively to the 2013/2014 campaign is expected (IOC, 2014). This will lead to a general increase of olive oil prices for consumers in 2014/2015, as well as a certain reduction in quality due to olive fly attack.

Olive fly females lay their eggs on fruits and, once hatched, the larvae feeds on olive pulp. The galleries created inside the olive and the exit holes opened to allow pupae and/or adult to exit cause severe quality drop in olive oils (Gómez-Caravaca *et al*., 2008; Pereira *et al*., 2004). Therefore, the impact of this pest in olive crop is substantial, has been increasing in the recent years, requiring efficient strategies to prevent these losses.

Interestingly, olive fly attack does not affect all olive cultivars similarly. Certain olive cultivars present systematically lower levels of infestation comparatively to others cultivars inserted in the same agro-climatic niche. Therefore, the study of the olive fly-olive tree interaction may give us important information on how or which are the aspects that may influence the olive fly oviposition preference. The main aspects studied so far respect to chemical, physical and molecular ones. In respect to physical factors parameters related to the hardness and elasticity of olives epidermis (Gonçalves *et al*., 2012), volume (Rizzo *et al*., 2012), and color (Katsoyannos *et al*., 1985) reported to be important in the oviposition preference of olive fly. Gonçalves *et al*. (2012) verified that olive fly prefer olives with harder pulp, corresponding to lower elasticity, which facilitate ovipositor penetration in the drupe. By other hand higher fruit volumes are also highly preferred by olive fly, with higher odds of infestation being attributed to these olives (Rizzo *et al*., 2012). Another important factor is the olive color, intrinsically related with maturation, with greener and yellow colors attracting more olive flies than reddish and black colors ones (Katsoyannos *et al*., 1985).

Once physical factors could give us information on the oviposition preference of olive fly, in the present work we intend to study the influence of physical parameters of both olives and olive leaves in the oviposition preference of olive fly in three Portuguese olive cultivars with different susceptibility degrees towards this pest: Cobrançosa (less

susceptible), Madural (intermediate susceptibility), and Verdeal Transmontana (high susceptibility). To achieve such goal, biometric data was taken from olives (weight, maximum and minimum diameters, length, volume, and elongation) and olive leaves (length, width, and weight), as well as their color during crop season evolution. Simultaneously, infestation levels and maturation indices were also monitored in the three olive cultivars while the fatty acids profile lipid profile was included to verify their possible action in the attraction of olive fly females.

Material and methods

In the present study, all biological material used was collected from an organic olive grove located in Paradela (Mirandela - 41º32'35.72''N; 7º07'27.17''W) Trás-os-Montes region (Northeast of Portugal) in 2013. The study focused in three of the main cultivars of this region, Cobrançosa, Madural and Verdeal Transmontana. From each olive cultivar, five trees were selected and marked in order to collect all biological material needed for the necessary parameters, as detailed below.

Infestation level and maturation index determination

In previously selected and marked olive trees the infestation level and maturation index were determined. Both parameters were assessed fortnightly from 27th Aug to 6th Nov., the last possible date prior to olives harvest.

To assess infestation level, 40 random handpicked fruits were collected from each olive tree (5 trees per cultivar; 200 fruits) in the mentioned periods and inspected in a binocular stereomicroscope for signs of infestation (oviposition sites or exit holes). Infestation level was expressed as the percentage of infested olives.

Simultaneously, in the same olives collected for infestation level determination, maturation index was assessed as described by Hermoso *et al*. (2001). Briefly, olives were classified into 8 maturation indexes (MI) based on epidermis and pulp color (0 to 7). Therefore, the fruit is classified as "MI 0" if the epidermis is green; "MI 1" for yellowish green; "MI 2" if the epidermis shows red spots in less than half fruit; "MI 3" if the epidermis is red or purple in more than half fruit; "MI 4" for black epidermis and white pulp; "MI 5" if the epidermis is black and less than half pulp is purple; "MI 6" if the epidermis is black and more than half pulp purple (without reaching the stone); "MI 7" if the epidermis is black

and total pulp purple (reaching the stone). The maturation index was calculated as follows: MI = (*a* × 0 + *b* × 1 + *c* × 2 + *d* × 3 + *e* × 4 + *f* × 5 + *g* × 6 + *h* × 7) / n, where the letters are the number of fruits in each MI of classification considered, and n the number of olives assessed.

Olives and leaves biometric parameters

For biometric parameters measurements only healthy olives and leaves were considered. Per olive tree and cultivar 40 olives and leaves (200 in total) were collected during six sampling dates (27th Aug; 12th Sep; 24th Sep; 10th Oct; 23rd Oct; and 6th Nov). Parameters evaluated in leaves were: length, width and weight. Parameters evaluated in olives were: weight, maximum diameter (D_{max}), minimum diameter (D_{min}), and length. From these parameters, olives volume (according to Burrack *et al*., 2011) and elongation (according to Rizzo *et al*., 2012) were calculated.

Olives and leaves color determination

Color from healthy olives and leaves from cvs. Cobrançosa, Madural and Verdeal Transmontana was measured with a Konica Minolta model CR-400 colorimeter. From each olive tree and cultivar 40 olives and leaves were collected (total of 200) at the same six sampling dates (27th Aug; 12th Sep; 24th Sep; 10th Oct; 23rd Oct; and 6th Nov). For each olive and leave 4 measurements were taken (with 90º of distance each in olive). The monochromatic variables *L**, *a**ª and *b**ª obtained from CIELAB method were measured. *L** is a measure of luminance or lightness component, which ranges from 0 to 100 (black and white); *a** ranges from negative to positive (green to red respectively); *b** also ranges from negative to positive (blue to yellow).

Fatty acids determination

For the fatty acids profile healthy olives from cvs. Cobrançosa, Madural and Verdeal Transmontana were separated by maturation indices from 0 to 3, according to the methodology of Hermoso *et al*. (2001) previously described. Olive fat was extracted in triplicate per cultivar and maturation index by a Soxhlet apparatus: 5 g of olive pulp

smashed under sodium sulphate anhydrous, and extracted with petroleum ether during a minimum period of 24 h (AOAC, 2000). Fat extracted was then stored at -20 ºC for further fatty acids profile determination according to Malheiro *et al*. (2012) and European Community Regulation EEC/2568/91. The fatty acid profile was determined with a Chrompack CP 9001 chromatograph equipped with a split-splitless injector, a FID detector, an autosampler Chrompack CP-9050 and a 50 m x 0.25 mm i.d. fused silica capillary column coated with a 0.19 µ film of CP-Sil 88 (Varian). Helium was used as carrier gas at an internal pressure of 110 kPa. The temperatures of the detector and injector were 250 ºC and 230 ºC, respectively. The oven temperature was programmed at 120 ºC during the first 3 min with an increase of 4 ºC/min until 220 ºC. The split ratio was 1:50 and the injected volume was of 1 µL. The results are expressed in relative percentage of each fatty acid, calculated by internal normalization of the chromatographic peak area eluting between myristic and lignoceric methyl esters. A control sample (olive oil 47118, Supelco) and a fatty acids methyl esters standard mixture (Supelco 37 FAME Mix) was used for identification and calibration purposes (Sigma, Spain).

Statistical analysis

Analysis of variance

An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov-Smirnov with Lilliefors correction (if n>50) or the Shapiro-Wilk`s test (if n<50), and the Levene´s tests, respectively. All dependent variables were analysed using a one-way ANOVA with or without Welch correction, depending if the requirement of the homogeneity of variances was fulfilled or not. The main factors studied were: the effect of cultivar and sampling date on the physical parameters (biometrical parameters and color) of olives and leaves, and fatty acids profile of olives from cvs. Cobrançosa, Madural, and Verdeal Transmontana. If a statistical significant effect was found, means were compared using Tukey´s honestly significant difference multiple comparison test or Dunnett T3 test also depending if equal variances could be assumed or not. All statistical tests were performed at a 5% significance level.

Principal component analysis

Principal components analysis (PCA) was applied for reducing the number of variables in the color parameters of olives and olive leaves (upper page) of the three olive cultivars to a smaller number of new derived variables (principal component or factors) that adequately summarize the original information, i.e., the effect of maturation in color parameters of olives and olive leaves from cvs. Cobrançosa, Madural and Verdeal Transmontana. Overall 6 variables corresponding to the *L**, *a** and *b** from olives and olive leaves were used in PCA. Moreover, it allowed recognizing patterns in the data by plotting them in a multidimensional space, using the new derived variables as dimensions (factor scores). PCA was performed by using SPSS software, version 21.0 (IBM Corporation, New York, U.S.A.).

Results and discussion

Infestation levels and maturation

Olives from cvs. Cobrançosa, Madural and Verdeal Transmontana were monitored during crop season 2013 for their infestation levels and maturation indices. Olive fly infestation levels in the three cultivars are represented in Figure 9.1.

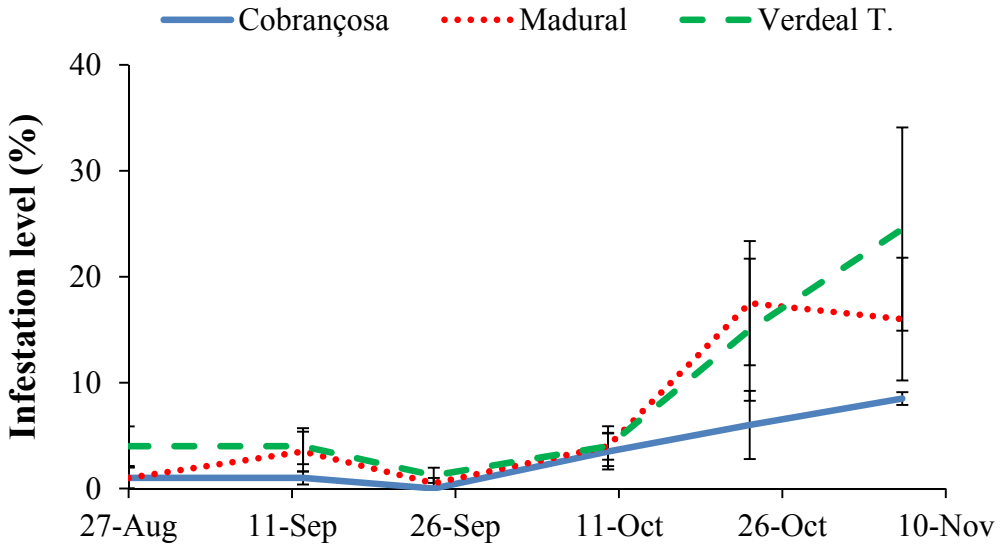

Figure 9.1. Infestation levels (%) of olives from cvs. Cobrançosa, Madural and Verdeal Transmontana.

From a general analysis it is possible to infer that cv. Verdeal Transmontana is the most susceptible olive cultivar, reporting maximum infestation levels of 24.5% at the end of the study. Madural reported maximum infestation levels of 17.5% (23rd Oct), while cv. Cobrançosa was the less susceptible to olive fly, reporting maximum infestation levels of 8.5%. The infestation levels in the three olive cultivars were similar from 27th Aug until 10th Nov. After that infestation levels raised more intensely, mainly in the two most susceptible olive cultivars. In cv. Cobrançosa olives the infestation levels also increased but in lower extent. At the end of the study (6th Nov) the infestation levels were significantly different between cv. Verdeal Transmontana and Cobrançosa (P = 0.03), while at the remaining sampling dates no statistical differences were observed (P > 0.05). The results obtained clarify that with the time course, olives infestation level among cvs. Cobrançosa and Verdeal Transmontana increases, and that from the latter is increasingly preferred by olive females. These results were also observed by Gonçalves et al. (2012) and Malheiro et al. (2014) by studying the infestation levels of these three olive cultivars, however with much higher infestation levels in previous seasons. In the mentioned works, olive fly preference towards cvs. Verdeal Transmontana and Madural, in detriment of cv. Cobrançosa, was also verified.

Oviposition preference of olive fly may be related to physical parameters of olive tree (both olives and leaves), some of which directly related to maturation process, that inflict considerable changes in olives physical parameters (biometrical parameters and color as well). Figure 9.2 displays the maturation indices from the three olive cultivars. Visually, is easily perceived that cv. Cobrançosa has a faster maturation process, with olives becoming reddish in the last week of October (MI = 2.1), and being completely reddish soon, in the first week of November (MI = 3.0).

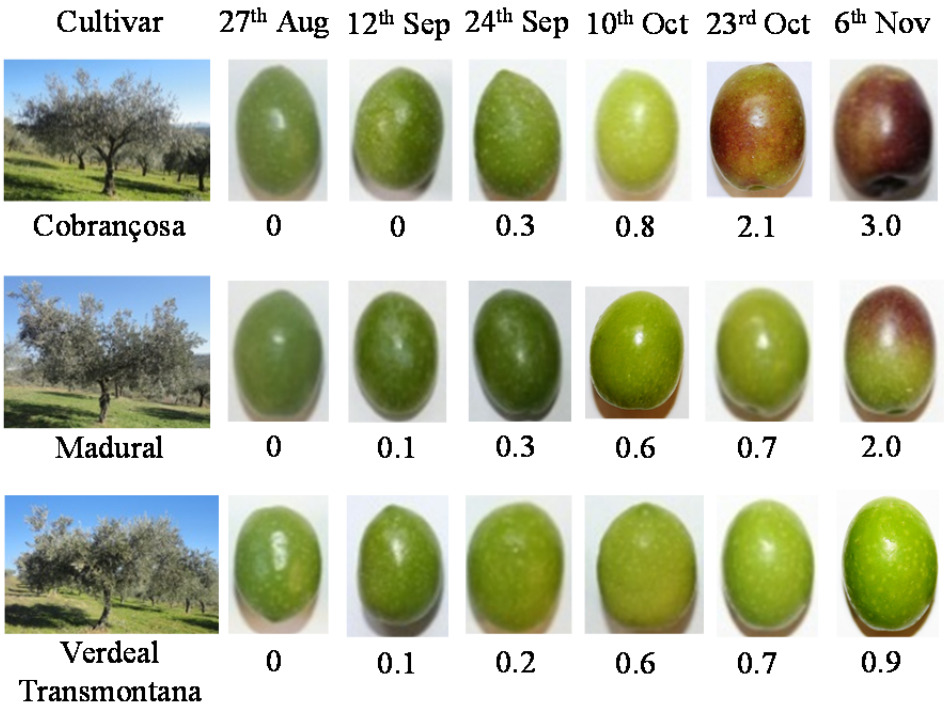

Figure 9.2. Maturation index of olives from cvs. Cobrançosa, Madural and Verdeal Transmontana during olive crop season.

Comparatively, cv. Verdeal Transmontana olives remained green or yellow-green during the entire study and up to harvest, with always a 0 < MI < 1 (Figure 9.1). Olives from cv. Madural revealed an intermedium maturation process, with a MI of 2.0 verified at the end of the study (Figure 9.2). Nevertheless, cv. Madural fruits didn't follow their pattern, normally the fruits of this cultivar become mature before cv. Cobrançosa fruits (Gonçalves *et al*., 2012). Therefore physical changes as well as appearance of olives are considerably changed during maturation. In the following section olives and leaves color is discussed regarding olive fly oviposition preference.

Color of olives and olive leaves

Olives and olive leaves color (both upper and down pages) was assessed at different sampling dates to observe if it could influence olive fly oviposition preference. The monochromatic variables *L**, *a** and *b** are detailed in Table 9.1.

Table 9.1. Color parameters (*L**, *a**, and *b**) in olive leaves (upper and down page) and olives from cvs. Cobrançosa, Madural, and Verdeal Transmontana during crop season (mean ± standard deviation; n = 200).

Olives	***L****			***a****			***b****		
	Cobrançosa	**Madural**	**Verdeal T.**	**Cobrançosa**	**Madural**	**Verdeal T.**	**Cobrançosa**	**Madural**	**Verdeal T.**
27th Aug.	50.8±0.9 bC	50.2±0.7 aA	52.1±1.4 cA	-17.6±0.5 cA	-18.6±0.2 bA	-19.3±0.1 aB	32.2±0.8 aD	32.5±0.4 bB	34.8±0.6 cA
12th Sep.	52.8±0.2 bD	52.4±0.5 aB	53.9±1.6 cB	-16.6±0.7 cB	-18.3±0.8 bA	-18.8±0.6 aC	30.6±1.1 aC	32.5±1.7 bB	34.7±1.7 cA
24th Sep.	53.2±1.3 bD	52.8±0.6 aB	53.7±0.5 cB	-16.3±1.3 cB	-18.1±0.7 bAB	-18.6±1.6 aC	32.5±1.5 aD	33.2±1.8 bB	35.4±3.6 cA
10th Oct.	58.0±0.6 bE	55.3±0.8 aC	57.9±2.1 bC	-17.9±1.3 cA	-18.7±0.6 bA	-19.9±0.1 aA	37.3±1.9 bE	36.5±0.9 aD	39.1±2.1 cD
23rd Oct.	46.0±5.9 aB	54.9±2.0 bC	58.9±1.6 cD	1.7±0.3 cC	-17.5±0.3 bB	-20.0±0.6 aA	18.1±2.3 aB	34.6±3.4 bC	38.3±1.4 cC
6th Nov.	35.2±6.8 aA	50.7±2.2 bA	59.8±1.1 cE	6.8±0.9 cD	-8.7±3.5 bC	-18.0±2.3 aD	7.5±4.5 aA	27.9±3.3 bA	37.8±0.9 cB
Leaves Down page	***L****			***a****			***b****		
	Cobrançosa	**Madural**	**Verdeal T.**	**Cobrançosa**	**Madural**	**Verdeal T.**	**Cobrançosa**	**Madural**	**Verdeal T.**
27th Aug.	67.0±8.4 bB	67.9±2.6 abBC	67.5±1.7 aBC	-7.1±1.3 bB	-6.4±1.7 cC	-7.5±0.8 aA	15.0±2.2 bD	14.6±2.4 aD	14.8±1.4 abD
12th Sep.	68.3±1.7 aA	69.3±2.2 cE	67.1±2.2 bBC	-7.3±0.8 aA	-7.1±1.3 bA	-7.6±0.7 aA	14.6±1.2 aCD	14.6±2.0 aD	14.7±1.4 aCD
24th Sep.	68.5±1.6 aB	68.4±2.0 bD	69.2±2.3 bC	-7.1±0.9 aBC	-6.5±1.2 bB	-6.9±1.0 abBC	14.9±1.6 bBC	13.2±1.6 aC	14.6±2.0 bCD
10th Oct.	66.2±2.7 aA	67.8±2.2 bCD	66.7±6.7 aB	-7.4±1.2 aAB	-6.8±0.8 bB	-7.2±1.2 aB	14.2±1.5 bB	13.1±1.2 aB	14.2±2.3 bC
23rd Oct.	66.6±1.6 aA	66.6±2.5 aA	66.5±1.6 aB	-6.8±0.8 aD	-6.5±1.0 bC	-6.9±0.7 aC	13.5±0.9 bA	12.8±1.8 aAB	13.8±1.1 bB
6th Nov.	66.8±1.4 bA	67.0±2.0 bAB	65.9±2.1 aA	-6.8±0.6 aCD	-6.4±0.7 bC	-6.3±0.9 bD	13.5±0.9 bA	12.4±1.1 aA	12.7±1.7 aA

Leaves Upper page	***L****			***a****			***b****		
	Cobrançosa	**Madural**	**Verdeal T.**	**Cobrançosa**	**Madural**	**Verdeal T.**	**Cobrançosa**	**Madural**	**Verdeal T.**
27th Aug.	42.6±2.5 bC	41.7±4.2 aD	44.1±3.3 cBC	-9.0±1.8 cA	-9.8±2.2 bB	-10.9±1.6 aA	12.4±2.6 aD	13.9±3.4 bD	15.3±2.9 cD
12th Sep.	43.3±3.7 bD	42.2±2.5 aD	43.6±3.9 bBC	-8.5±1.5 cB	-10.7±1.8 aA	-9.2±1.6 bB	11.7±2.3 aD	14.7±2.7 cE	12.1±2.3 bB
24th Sep.	42.6±3.1 bC	40.4±3.2 aC	46.7±5.0 cD	-8.2±1.6 bC	-9.3±2.2 aB	-9.8±2.0 aB	11.5±2.3 aC	12.7±2.7 bC	14.6±3.3 bC
10th Oct.	41.2±4.1 bB	39.6±2.4 aB	43.9±6.7 cB	-7.4±1.4 bD	-9.1±1.1 aB	-9.6±2.0 aB	9.6±1.7 aB	11.6±1.5 bB	13.3±3.3 cB
23rd Oct.	39.8±2.0 bA	38.0±1.9 aA	40.6±2.4 cA	-6.0±1.0 bE	-7.2±1.1 aC	-7.3±0.9 aC	7.6±1.2 aA	9.0±1.4 cA	8.9±1.2 bA
6th Nov.	40.1±2.1 bAB	37.3±1.9 aA	41.9±4.5 cCD	-6.0±1.1 bE	-7.2±0.8 aC	-6.3±1.3 bD	7.7±1.3 Aa	8.7±1.0 bA	7.7±1.7 abA

In the same line, for each parameter and date studied, mean values with different minor letters differ significantly ($P < 0.05$); In the same column, for each parameter and cultivar studied, mean values with different capital letters differ significantly ($P < 0.05$).

Regarding the olives color, both cultivar and sampling date influenced the results obtained. Regarding lightness (*L**; 0 and 100 corresponding respectively to black and white coloration), olives from cv. Cobrançosa increase continuously until 10th Oct, when olives are yellow-green (*L** = 58.0). After that, when maturation start to advance towards reddish tones, lightness reduced significantly to 46.0 at 23rd Oct, and to 35.2 at 6th Nov (Table 9.1). Olives from cv. Madural also reported an increment in lightness values until 23rd Oct (*L** = 54.9) decreasing then to 6th Nov (*L** = 50.7), corresponding to the time in which olives also started to change color to reddish tones (Figure 9.2). Nevertheless, olives from cv. Verdeal Transmontana reported always higher lightness values, and it increased during the entire study from 52.1 at first sampling date (27th Aug) to 59.8 at the last sampling date (6th Nov). From the results obtained, and interpreting them according to the results from Figure 9.1, lightness increased during olives maturation from green (MI = 0) to yellow-green olives (MI = 1), reducing sharply when olives start changing to reddish tones (MI = 2 and 3).

The other two monochromatic parameters are also in accordance with Figure 9.1. Concerning *a** values (negative and positive values representing respectively green and red colorations) cv. Cobrançosa olives are less green than cv. Madural and Verdeal Transmontana during the entire study. Olives from cv. Cobrançosa loss their green coloration at 23rd Oct (*a** = 1.7) and become clearly red at 6th Nov (*a** = 6.8). Olives from cv. Madural lost their green coloration sooner, with a* values ranging between -18.6 (27th Aug) and -8.7 (6th Nov), corresponding this last value to the changing color stage. Olives from cv. Verdeal Transmontana maintained a green coloration during the entire study (Figure 1), therefore with higher *a** values comparatively to both Cobrançosa and Madural olives (Table 1). A curious observation was that *a** values from cv. Verdeal Transmontana olives at the last sampling date were higher than those reported by cv. Cobrançosa olives at the first sampling date, attesting the characteristically slow maturation process of this cultivar.

Concerning *b** values (negative and positive values representing respectively blue and yellow colorations), they decreased in olives from cvs. Cobrançosa and Madural, with higher loss of yellow coloration in Cobrançosa olives (Table 1). Fruits from cv. Verdeal Transmontana maintained their yellow-green coloration (Figure 9.1) until the end of the study, up to olives harvest time.

Olive leaves color was recorded on both upper and down pages. Down pages have a lighter green, while upper pages are greener. Small *L** values variations were found in the three olive cultivars during crop season in the leaves down pages, varying between 65.9 and 69.3. Small variations were also observed in the *a** values during crop

season, ranging between -6.3 and -7.6. Values of *b** decreased consistently during the entire studied season, from 15.0 to 13.5 in cv. Cobrançosa, from 14.6 to 12.4 in cv. Madural, and from 14.8 to 12.7 in cv. Verdeal Transmontana (Table 9.1). In the leaves upper pages all *L**, *a** and *b** values decrease during crop season. In a general way, olive leaves from cv. Verdeal Transmontana reported always higher values of the three color parameters measured during the entire study. Again, like in olives, *L** values, corresponding to lightness, were higher in the most susceptible olive cultivar. These observations, both in olives and olive leaves could be related to the epicuticular waxes composition of each olive cultivar. Epicuticular waxes could intervene in a decisive way in olive fly preference towards cv. Verdeal Transmontana. Neuenschwander *et al*. (1985), a pioneer in the study of susceptibility of olive cultivars to olive fly, reported that surface covering could decisively influence olive fly. Later, Kombargi *et al*. (1998) verified that superficial waxes of olives reduced by half the number of eggs laid in olives from Greek cultivars. Furthermore, the total amount and profiles of epicuticular waxes in both olives and leaves are cultivar dependent (Guinda *et al*., 2010) and during maturation they decrease mainly in olives (Peragón, 2013). Therefore the loss of lightness in both olives and leaves could be related to epicuticular waxes, and this aspect could influence olive fly oviposition, being interesting to explore in future studies. Significant differences were found among cultivars in some color parameters, but a higher impact of cultivar was observed in the upper pages of olive leaves.

The color parameters evaluated on both olives and leaves presented different characteristic on each olive cultivar at a determined date assessed. By applying a PCA to the data related to olives and olive leaves (upper page) color parameters at the last assessed date and we verify that three main groups are created according to the olive cultivar (Figure 9.3).

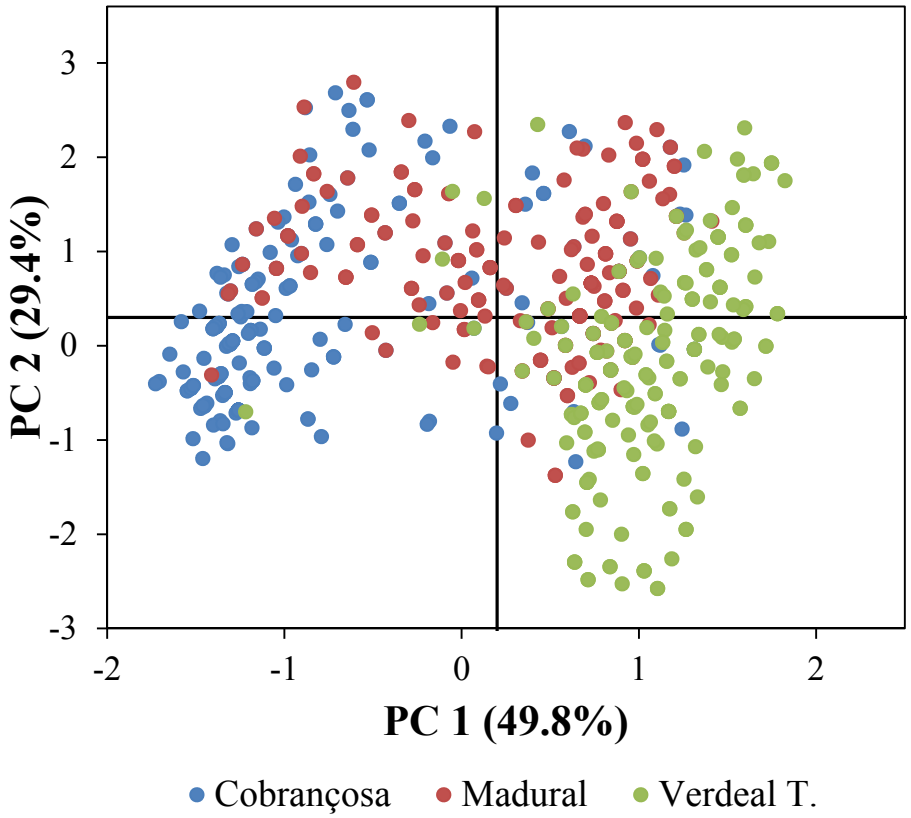

Figure 9.3. Principal component analysis obtained from the color parameters (*L**, *a**, and *b**) of olive leaves upper page and olives from cvs. Cobrançosa, Madural, and Verdeal Transmontana at the last date studied (6th Nov). Principal components explain 79.2% of the total variance.

In Figure 9.3 is possible to observe that cv. Verdeal olives and leaves (positive region of PC 1) are clearly different from those of cv. Cobrançosa (negative region of PC 1). Olives and leaves from cv. Madural are placed among the more and less susceptible olive cultivars.

Therefore, in field conditions, when olive fly attacks become more intense, olive fly has at the same time olives with different colors, from cultivars with different maturation rates. Indeed, when all olives were green the infestation levels observed were quite similar in the three cultivars. Infestation levels started to differentiate among cultivars at the two last sampling dates, corresponding to the progress of maturation (Figure 9.1). Color parameters corroborate results verified in Figure 9.1, therefore olive fly may be attracted to green and green-yellow fruits, while olives at higher maturation stages (MI = 3 or superior) may be less preferred to oviposit if in the presence of green or yellow-green ones. This could justify the high preference of olive fly to cv. Verdeal Transmontana olives, and less preference towards cv. Cobrançosa. In fact, it was already observed in

other cultivars that infestation odds decrease from green to reddish fruits (Rizzo *et al*., 2012). Even more, olive fly females are attracted to yellow and green colorations to oviposit rather than red or dark colors (Katsoyannos *et al*., 1985). Nevertheless, some other physical parameters can also be correlated with oviposition preference, as discussed in the next section.

Physical parameters of olives and olive leaves

Olives and olive leaves physical parameters were evaluated at different sampling dates. For olive leaves, three main parameters were measured: length, width, and leaf weight. In general, leaves from cv. Cobrançosa presented a slightly lower length, varying between 7.01 and 7.30 cm, while cv. Madural varied from 7.00 to 7.45 cm, and those from cv. Verdeal Transmontana ranged from 7.05 and 7.36 cm. Regarding width, cv. Madural leaves are wider than the other two cultivars (1.13-1.35 cm). Cobrançosa leaves reported a width of 1.02-1.11 cm while cv. Verdeal Transmontana reported only 0.93-1.03 cm. Regarding weight, again, cv. Madural reported the heaviest leaves with mass between 0.25-0.30 g, while cvs. Cobrançosa and Verdeal Transmontana ranged between 0.20-0.22 and 0.19-0.21, respectively. Apparently, olive leaves physical parameters evaluated may not be decisive in the oviposition preference of olive fly. However, the lower leaves width observed in Verdeal Transmostana could constitute favorable parameters for olive fly penetration into the olives branches. However, in future works the curvature of leaves and their shape should be considered since it may reflect light in a different way that could cause any change in the behavior and choice of olive fly. The leaves density per branches could also be a parameter to take into account, since they could offer shelter and protection to olive flies.

The biometrical data collected on olives are reported in Table 9.2.

Table 9.2. Biometrical parameters (weight, D_{max}, D_{min}, length, volume, and elongation) of olives from cvs. Cobrançosa, Madural and Verdeal Transmontana during crop season (mean ± standard deviation; n = 200).

	Weight (g)			**D_{max} (cm)**		
	Cobrançosa	**Madural**	**Verdeal T.**	**Cobrançosa**	**Madural**	**Verdeal T.**
27th Aug	2.04±0.14 bB	1.91±0.42 aAB	1.96±0.24 abA	1.34±0.04 aB	1.33±0.08 aAB	1.33±0.11 aA
12th Sep	1.98±0.21 aAB	2.03±0.43 aB	1.95±0.27 aA	1.31±0.07 aAB	1.34±0.11 bB	1.35±0.11 bA
24th Sep	1.87±0.20 aA	1.89±0.27 aA	1.92±0.43 aA	1.28±0.06 aA	1.31±0.09 abA	1.32±0.15 bA
10th Oct	2.86±0.74 cC	2.35±0.29 aC	2.54±0.37 bB	1.55±0.15 bD	1.43±0.07 aC	1.51±0.11 bB
23rd Oct	2.76±0.20 bC	2.39±0.33 aC	2.65±0.12 bB	1.51±0.04 bC	1.45±0.08 aC	1.52±0.05 bB
6th Nov	2.86±0.15 bC	2.67±0.33 aD	2.84±0.31 bC	1.51±0.02 aCD	1.49±0.07 aD	1.56±0.09 bC
	D_{min} (cm)			**Length (cm)**		
	Cobrançosa	**Madural**	**Verdeal T.**	**Cobrançosa**	**Madural**	**Verdeal T.**
27th Aug	0.70±0.02 aB	0.74±0.05 bD	0.74±0.09 bC	1.97±0.04 Ab	1.89±0.09 aA	1.92±0.06 aA
12th Sep	0.61±0.04 aB	0.69±0.07 cC	0.63±0.07 bB	1.96±0.05 bA	1.90±0.11 aA	1.94±0.05 bA
24th Sep	0.56±0.04 aB	0.62±0.07 bA	0.57±0.09 aA	1.94±0.04 bA	1.86±0.04 aA	1.95±0.08 bA
10th Oct	0.67±0.12 aB	0.63±0.03 aAB	0.64±0.03 aB	2.11±0.11 aB	1.97±0.07 cB	2.07±0.05 bB
23rd Oct	0.63±0.02 aB	0.64±0.03 aB	0.64±0.04 aB	2.10±0.75 bB	1.96±0.09 aB	2.09±0.04 bBC
6th Nov	0.60±0.04 aB	0.63±0.03 bAB	0.62±0.04 bAB	2.12±0.03 bB	2.03±0.06 aC	2.13±0.05 bC
	Volume (cm^3)			**Elongation**		
	Cobrançosa	**Madural**	**Verdeal T.**	**Cobrançosa**	**Madural**	**Verdeal T.**
27th Aug	1.86±0.15 aA	1.79±0.30 aAB	1.81±0.33 aA	1.47±0.04 aC	1.43±0.04 bB	1.45±0.10 bB
12th Sep	1.79±0.21 aA	1.83±0.39 aB	1.88±0.34 aA	1.50±0.05 cCD	1.42±0.04 aB	1.45±0.12 bB
24th Sep	1.67±0.20 aA	1.71±0.26 aA	1.82±0.50 bA	1.52±0.04 bD	1.42±0.07 aB	1.49±0.13 bC
10th Oct	2.75±0.70 bC	2.15±0.28 aC	2.51±0.42 bB	1.37±0.07 aA	1.37±0.02 aA	1.38±0.08 aA
23rd Oct	2.52±0.20 bB	2.19±0.33 aC	2.56±0.17 bB	1.40±0.02 cAB	1.36±0.03 aA	1.38±0.07 bA
6th Nov	2.57±0.12 bBC	2.40±0.29 aD	2.78±0.34 cC	1.41±0.01 bB	1.37±0.03 aA	1.37±0.06 aA

In the same line, for each parameter and date studied, mean values with different minor letters differ significantly ($P < 0.05$); In the same column, for each parameter and cultivar studied, mean values with different capital letters differ significantly ($P < 0.05$).

Weight, maximum and minimum diameters (D_{max} and D_{min} respectively), and weight were recorded. After that, volume and elongation were calculated as described previously. All the biometrical parameters increased as function of the maturation progress and fruit development. Concerning olives volume, cv. Verdeal Transmontana reported significant higher volume in the two dates assessed, 24th Sep and 6th Nov (Table 9.2). This is an important observation since a direct relation between olives volume and infestation level has already been clearly established (Rizzo *et al*., 2012). Another important information recovered from biometrical data is olives elongation, which is negatively correlated with infestation level (Rizzo *et al*., 2012). In our cultivars the two most susceptible cultivars (Madural and Verdeal Transmontana) reported also lower elongation values than cv. Cobrançosa.

Olives fatty acids profile

Olives from the three cultivars were separated from MI = 0 to MI = 3, being their oil extracted and the fatty acids profile analyzed. Regardless of olive cultivar and maturation index, oil was mainly composed by oleic acid ($C_{18:1}$) as characteristic from olive oil. This monounsaturated fatty acid was present in higher amounts in cv. Verdeal Transmontana, fruits with values ranging from 78.0% (at MI = 0) and 80.4% (at MI = 3) in an opposite tendency to cvs. Cobrançosa and Madural, where oleic acid decreased during maturation, from 75.0 to 73.7% and from 73.5 to 70.2%, respectively (Table 9.3).

Table 9.3. Fatty acids profile of olives from cvs. Cobrançosa, Madural and Verdeal Transmontana at different maturation indices (mean values; n = 3).

	Cobrançosa				**Madural**				**Verdeal Transmontana**			
	MI 0	**MI 1**	**MI 2**	**MI 3**	**MI 0**	**MI 1**	**MI 2**	**MI 3**	**MI 0**	**MI 1**	**MI 2**	**MI 3**
$C_{14:0}$	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
$C_{16:0}$	11.6	11.8	11.8	11.7	11.7	12.0	12.1	12.2	11.6	11.8	10.9	10.9
$C_{16:1}$	0.67	0.75	0.75	0.77	0.54	0.59	0.53	0.66	0.71	0.78	0.67	0.60
$C_{17:0}$	0.20	0.18	0.16	0.17	0.11	0.11	0.05	0.10	0.19	0.17	0.21	0.22
$C_{17:1}$	0.26	0.26	0.25	0.24	0.15	0.14	0.07	0.13	0.29	0.28	0.31	0.32
$C_{18:0}$	4.52	4.29	4.54	4.50	3.47	3.50	2.65	3.34	3.54	3.16	3.43	3.36
$C_{18:1}$	75.0	74.8	74.2	73.7	73.5	72.0	70.3	70.2	78.0	78.7	79.0	80.4
$C_{18:2}$	5.57	5.86	6.36	7.04	8.20	9.70	12.39	11.53	3.25	3.13	3.50	2.34
$C_{18:3}$	1.01	0.86	0.87	0.84	1.18	0.98	1.00	0.92	1.02	0.84	0.81	0.64
$C_{20:0}$	0.58	0.51	0.51	0.51	0.46	0.43	0.36	0.38	0.60	0.51	0.52	0.53
$C_{20:1}$	0.26	0.23	0.23	0.23	0.30	0.26	0.29	0.26	0.34	0.31	0.30	0.29
$C_{22:0}$	0.15	0.12	0.13	0.13	0.12	0.11	0.10	0.10	0.18	0.14	0.16	0.17
$C_{24:0}$	0.07	0.06	0.06	0.05	0.07	0.05	0.04	0.04	0.11	0.08	0.07	0.07
SFA	17.1	17.0	17.2	17.1	16.0	16.2	15.3	16.2	16.2	15.9	15.3	15.2
MUFA	76.2	76.1	75.4	74.9	74.5	73.0	71.2	71.2	79.3	80.0	80.3	81.6
PUFA	6.58	6.72	7.23	7.88	9.38	10.7	13.4	12.4	4.27	3.97	4.30	2.97
***Trans* isomers**	0.18	0.17	0.17	0.15	0.18	0.16	0.15	0.15	0.18	0.13	0.15	0.14

Palmitic acid ($C_{16:0}$) was the second main fatty acid, with constant values during maturation in cv. Cobrançosa, a slight increase in cv. Madural, and a more pronounced decreased in cv. Verdeal Transmontana. Important differences were observed in linoleic acid ($C_{18:2}$) relative amounts, increasing considerably during maturation on both cvs. Cobrançosa and Madural, while in cv. Verdeal Transmontana it decreases. In cv. Verdeal Transmonta linoleic acid varied between 3.25 and 2.34%, far below the minimum values of cv. Cobrançosa 5.57%. In cv. Madural linoleic acid reported high values ranging from 8.20 and 11.53%. Globally, the polyunsaturated (PUFA) fatty acids content was significantly lower in cv. Verdeal Transmontana.

The characteristic fatty acids composition found in olives of the three olive cultivars may play an important role in olive fly oviposition. When olive fly females oviposit, the penetration of ovipositor in olive pulp may perceive the medium in which they will lay their eggs, since ovipositor is composed by chemosensilla (Zhang *et al*., 2012). This specific perception organ is composed by receptor neurons. In this particular case the amount of fat, higher in cv. Verdeal Transmontana, as well as its composition, particularly the lower PUFA content, may decisively influence olive fly to lay their eggs. Furthermore, fatty acids composition also could influence the survival of adults of olive fly. Since cv. Verdeal Transmontana report higher fat content, larvae developed in their olives could accumulate higher amounts of fat in their organism, reserves that will pass to adults and that could satisfy the nutritional requirements of adults for longer periods. The composition of the fat is other aspect that could also influence survival of adults and for other functions. For instance, fatty acids are precursors in the synthesis of pheromones, being therefore important in insects reproduction and survival (Arrese and Soulages, 2010). Regarding fatty acids, future works could be carried out to study the impact of fatty acids composition in the longevity and behavior of olive fly adults. This could lead to a higher longevity by adults, and an important ecological aspect to this pest.

Conclusions

The results obtained in the present work allowed to conclude that both physical and chemical parameters may be taken in consideration by olive fly for oviposition purposes. We concluded that the maturation process, which influences olives color and biometrical data, may contribute for oviposition preference as well. Olive leaves color is characteristic in each cultivar, with higher lightness being exhaled by the most susceptible olive cultivar, Verdeal Transmontana. Fatty acids profile is also characteristic in each cultivar and it could be an internal factor in fruits for olive fly oviposition.

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PART III

General discussion and Conclusions

CHAPTER 10.

General discussion

Olive fly infestation monitoring in the field revealed that olives from cv. Verdeal Transmontana were those preferred by olive fly to oviposit, followed by cv. Madural, while cv. Cobrançosa reported lower susceptibility. These observations were verified in two different crop seasons: season 2011/2012; and season 2013/2014 (Figure 10.1).

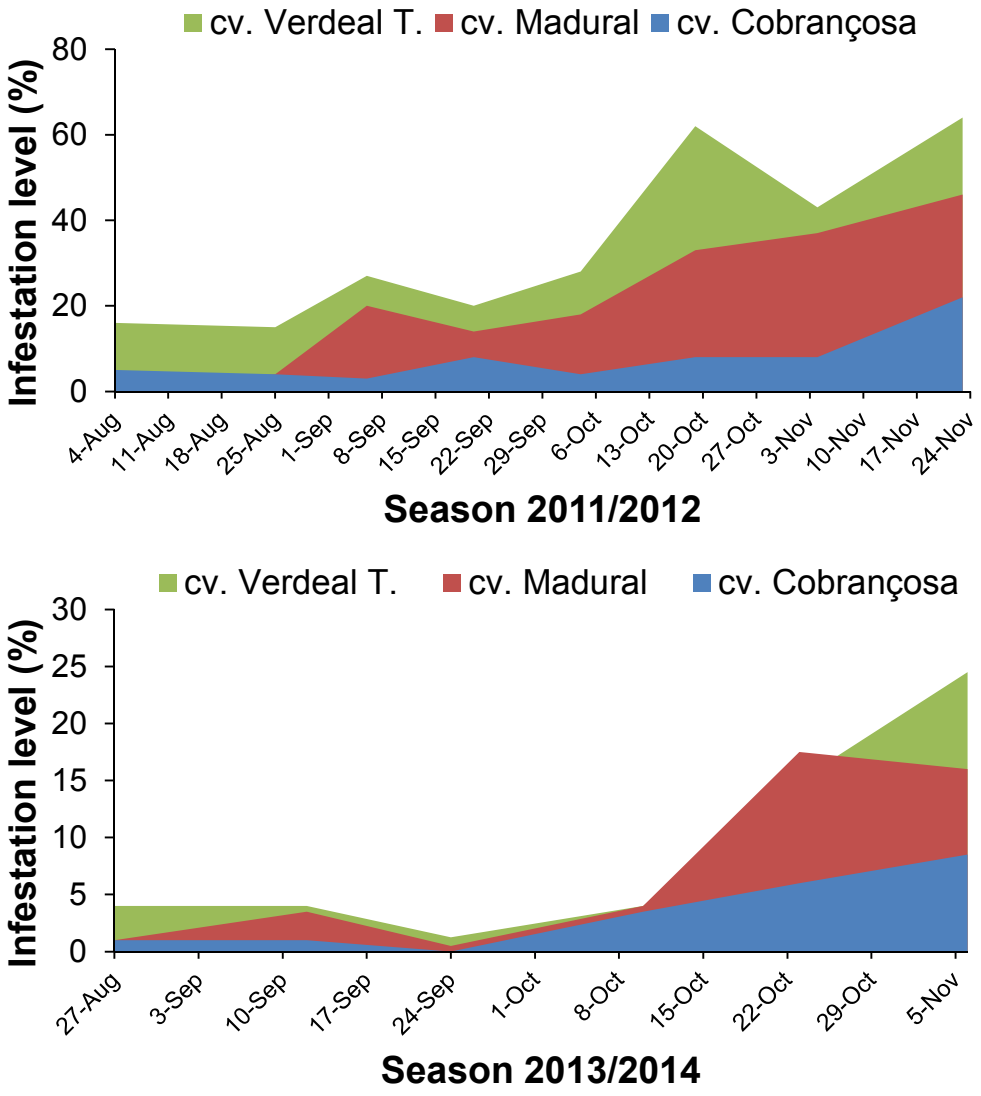

Figure 10.1. Infestation levels caused by olive fly in the field (organic olive grove located in Paradela, Mirandela – Trás-os-Montes) during crop seasons 2011/2012 and 2013/2014 in cvs. Cobrançosa, Madural, and Verdeal Transmontana.

Higher infestation levels were observed during the 2011/2012 crop season than those in 2013/2014 (Figure 10.2), similar to those predicted for the present 2014/2015 season. In 2011/2012, cv. Verdeal Transmontana reported a maximum and minimum infestation level of 15% and 64%, respectively, while in 2013/2014 those values were between 2.5% and 24.5%. Similar observations were recorded for cvs. Madural and Cobrançosa, with maximum infestation values of 22% and 46% for cvs. Cobrançosa and Madural, respectively, in 2011/2012, while in 2013/2014 the maximum infestation recorded were of 8.5% and 17.5%, respectively. Nevertheless, all infestation levels recorded in these two crop seasons are below those reported by Gonçalves *et al*. (2012) in an olive grove located in the same region for the same cultivars in 2007/2008, when these authors reported maximum values of infestation of 88%, 70%, and 58%, respectively in cvs. Madural, Verdeal Transmontana, and Cobrançosa. Despite the high differences observed among crop seasons regarding total infestation of olives in the same olive grove, the relative susceptibility remains similar. These differences are mainly related to climatic conditions. In the crop season 2013/2014 (low infestation levels) summer temperatures were high, followed by a rigorous winter with low temperatures. The high temperatures in summer may have caused high mortality rates in pupae in the soil. Those adults who effectively emerged from pupae also had difficulties to survive and reproduce at high temperatures (Wang *et al*., 2009). During autumn and winter temperatures were very low, with minimum temperatures around zero degrees Celsius and sometimes even below at night period. Eggs and larvae inside olives could not survive at such conditions, since eggs don't hatch below 7.5-10 ºC and larvae can't develop under 10-12.5 ºC (Tsitsipis 1977). Therefore, natural causes controlled olive fly populations and maintained the infestation levels below those commonly observed in organic olive orchards.

Despite the differences found in the infestation levels between crop seasons, it is clear that olive fly females have a preference for cv. Verdeal Transmontana olives and a lower preference for cv. Cobrançosa. Based in this information, several works were developed in this thesis relying on physical and chemical aspects that could influence olive fly oviposition preference.

Since volatiles are recognized as influencing insects host recognition and location (Bruce *et al*., 2005), volatile emissions were analyzed from olive the three olive cultivars (both olives and leaves). The results obtained reveal that olive cultivar and harvest date assessed clearly influenced the volatiles composition in both leaves and olives (Chapters 5 and 6).

Olive leaves were mainly composed by GLV's (green leaf volatiles), alcohols and aldehydes. Nevertheless, during olives maturation, leaves reported a significant increase in toluene content. This aromatic hydrocarbon was present in higher relative abundance in cvs. Madural and Verdeal Transmontana than in cv. Cobrançosa. In fact, a positive correlation between this aromatic hydrocarbon and infestation levels reported in the field was established.

In the case of olives, GLV's were also present in high amounts in the beginning of maturation, mainly esters, decreasing considerably at further maturation stages. In olives, toluene was also in high relative amounts, with special importance in cv. Verdeal Transmontana, reaching nearly 82% of the volatile profile analyzed. Similarly to what was observed in leaves, this compound was extremely correlated with infestation levels. Therefore, this aromatic hydrocarbon may act as an olive fly attractant, since its content is higher in the more susceptible olive cultivars and is present in lower abundance in cv. Cobrançosa, the less susceptible one. In Figure 10.2, the patterns created by toluene in olives and leaves during maturation, and the relation to olive fly infestation levels is notorious.

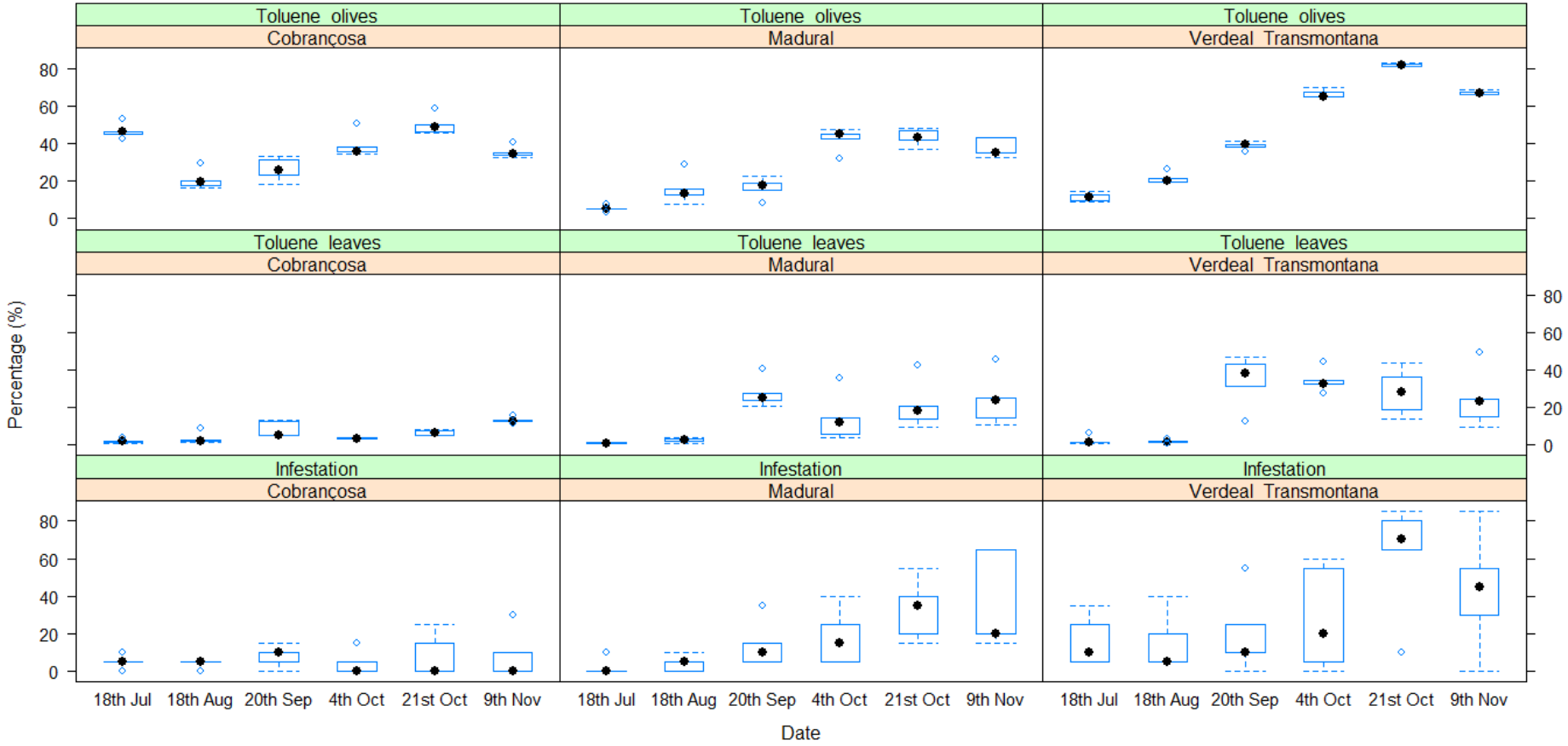

Figure 10.2. Emission of toluene (%) in olives and leaves, and infestation levels (%) of cvs. Cobrançosa, Madural and Verdeal Transmontana during olives maturation in crop season 2011/2012.

Besides toluene, important variations among cultivars and during olives maturation were observed in α-copaene. This sesquiterpene was also correlated with infestation levels in cvs. Madural and Verdeal Transmontana, while no correlations were established for cv. Cobrançosa.

These two volatiles are chemical cues with important influence in the olive fly oviposition preference. Toluene is an attractant of olive fly (Scarpati *et al*., 1993), while α-copaene is also associated to olive cultivars with high susceptibility to olive fly (Alfonso *et al*., 2014). Since toluene is present in high relative abundance in leaves and olives and α-copaene also in olives, these components could decisively attract olive fly to the most susceptible olive cultivars. Once in the olive orchard, olive tree volatiles may act as oviposition preference factors at short distance, guiding olive fly females in their quest.

Other secondary metabolites with high volatility, the essential oils (EO's), are also among the chemical cues with effects in insects behavior, mainly with repellent and toxic properties (Isman, 2000; Srivastava *et al*., 2015). Therefore, these metabolites should not be forgotten as potential biocontrol agents in pest management. In order to verify the potential effects of EO's in olive fly oviposition preference, the EO's from the leaves of cvs. Cobrançosa, Madural and Verdeal Transmontana were analyzed and the antenna sensitivity towards them was evaluated (by EAG assays) with olive flies of both sexes at different ages (Chapter 7). A simple and clear observation was removed from the EAG trials in both sexes, at all ages and concentrations tested: an inverse relation between EAG signal and olive cultivar susceptibility, reporting cv. Cobrançosa higher EAG signals (Figure 10.3).

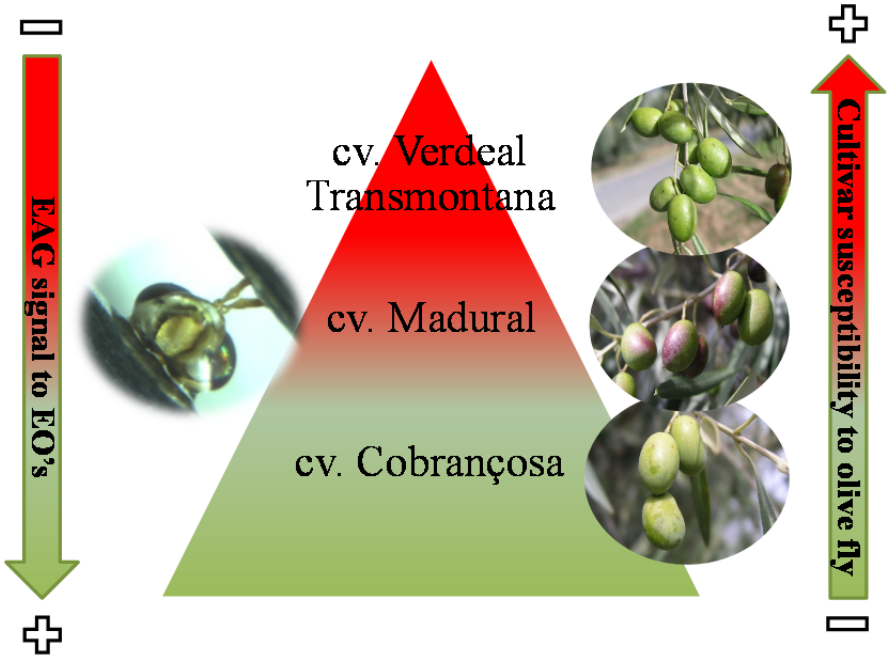

Figure 10.3. Relation between cultivar susceptibility and adults EAG signal in the antenna of olive flies to EO's from leaves of the different olive cultivars.

At the light of the EAG results obtained, the hypothesis that EO's from olive leaves may exert a repulsive action towards both sexes of olive fly is strong. The signal obtained from EO's from cv. Cobrançosa leaves is significantly higher comparatively to cvs. Madural and Verdeal Transmontana in both sexes, for all ages and concentrations tested. The results obtained are intrinsically related to EO's chemical composition that proved to be characteristic according to each cultivar (Chapter 7). Since olive leaves essential oil composition is highly influenced by cultivar (Campeol *et al*., 2001; Campeol *et al*., 2003) and harvest date (Flamini *et al*., 2003), further studies should be carried out to obtain maximized effects. In order to test the potential repulsive action of leaves EO's towards olive flies, *in vivo* laboratory bioassays as well as semi-field and field trials should be developed. Also, GC-EAD (gas-chromatography with an electroantennographic detector) should also be carried out to possibly elucidate if a single component of EO's is responsible for the observed effect or if it could be a synergetic action from several EO components. The extracted information from these assays could lead to the development of new semiochemicals with repulsive action to olive fly. Furthermore, olive leaves are an abundant and cheap sub-product from olive oil industry that could be exploited as a new strategy to valorize them as pests control agents. This could be also a sustainable and eco-friendly way to control olive fly.

Besides EO's, selected olive tree volatiles ((*E*)-2-hexenal, nonanal, α-pinene, farnesene, xylene) and semiochemicals ((*Z*)-9-tricosene and spiroketal) were tested at

EAG assays (Chapter 7). The results obtained allowed to verify that volatiles from olive tree have an effect on olive fly, being recognized in the antenna of males and females. Regarding olive tree volatiles (*E*)-2-hexenal and nonanal were those that elicited higher EAG response to olive flies. (*E*)-2-hexenal is an aldehyde that is exhaled from olives if they are injured or with open wounds. Higher EAG response was reported in females when they reach sexual maturity ([5-10[days old), and thereafter. Nonanal was more active in males also when they reach sexual maturity. In the case of (*E*)-2-hexenal, this aldehyde is an olive fly repellent (Scarpati *et al*., 1993), and its content in olives is highly dependent on olive cultivar and maturation stage. Since the mechanisms involved in its formation are gradually reduced during maturation, due to the loss of activity from the enzymes responsible for the different steps (lipoxygenase pathway), its repellent action is reduced as well. Regarding nonanal, this aldehyde was identified as a minor component of olive fly sexual pheromone (Botsi *et al*., 1995), attractive to males. When males reached their sexual maturity the EAG signal increased considerably, being this volatile an attractant to olive fly males. Furthermore, nonanal is a common component of lures and attractive food sources to olive flies, reporting as well strong EAG signals in both sexes of olive fly (Seris, 2011). Nonanal is present in olives and in small amounts in leaves of olive tree (Chapters 5 and 6), as well as in the EO's of leaves of the three cultivars (Chapter 7). In olives and leaves EO's nonanal is present in considerable higher abundance in cv. Cobrançosa comparatively to cvs. Madural and Verdeal Transmontana. Therefore, the less susceptible olive cultivar may cause a higher attraction of males for feeding purposes, but males have no direct action in olives infestation, whose role is exclusive from females.

In the EAG trials conducted, higher response was always obtained by males comparatively to females. However, according to age, a higher decrease in antenna sensitivity was observed in males, while in females the response also decreased but in a slower pace. Besides the differences found among sexes in EAG signals, there are no differences in the structure and number of sensillas in the antennas of both sexes (Liscia *et al*., 2013).

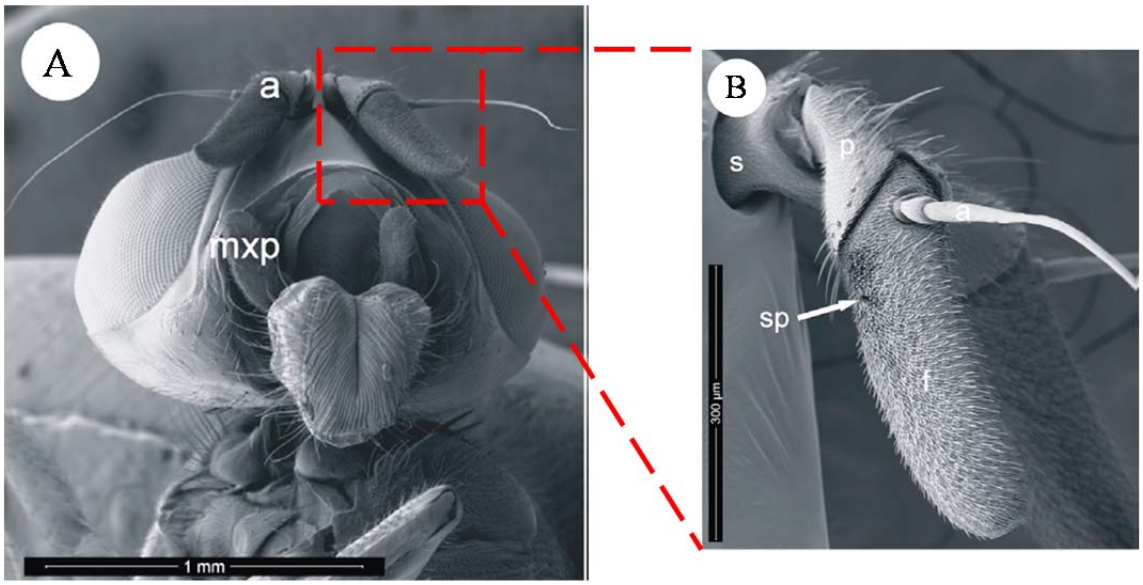

Figure 10.4. Head (A) and antenna (B) of olive fly (Liscia *et al*., 2013).

Oviposition bioassays were conducted under laboratory conditions to evaluate the influence of maturation and olive cultivar in the olive fly females preference. In the two types of bioassays conducted to determine the influence of cultivar, the three-choice bioassays results were inconclusive. The simultaneous presence of olives from the three cultivars may dilute the options of olive flies to oviposite, confusing them, with similar results observed in the three cultivars, in opposition to the results verified systematically under field conditions (Chapter 5, 6, 7 and 8). Therefore, one-choice oviposition bioassays were carried out, and the results obtained were in accordance to those verified in the field, with a higher preference for cv. Verdeal Transmontana, followed by cv. Madural, while cv. Cobrançosa was the less preferred. The results obtained are resumed in Figure 10.5.

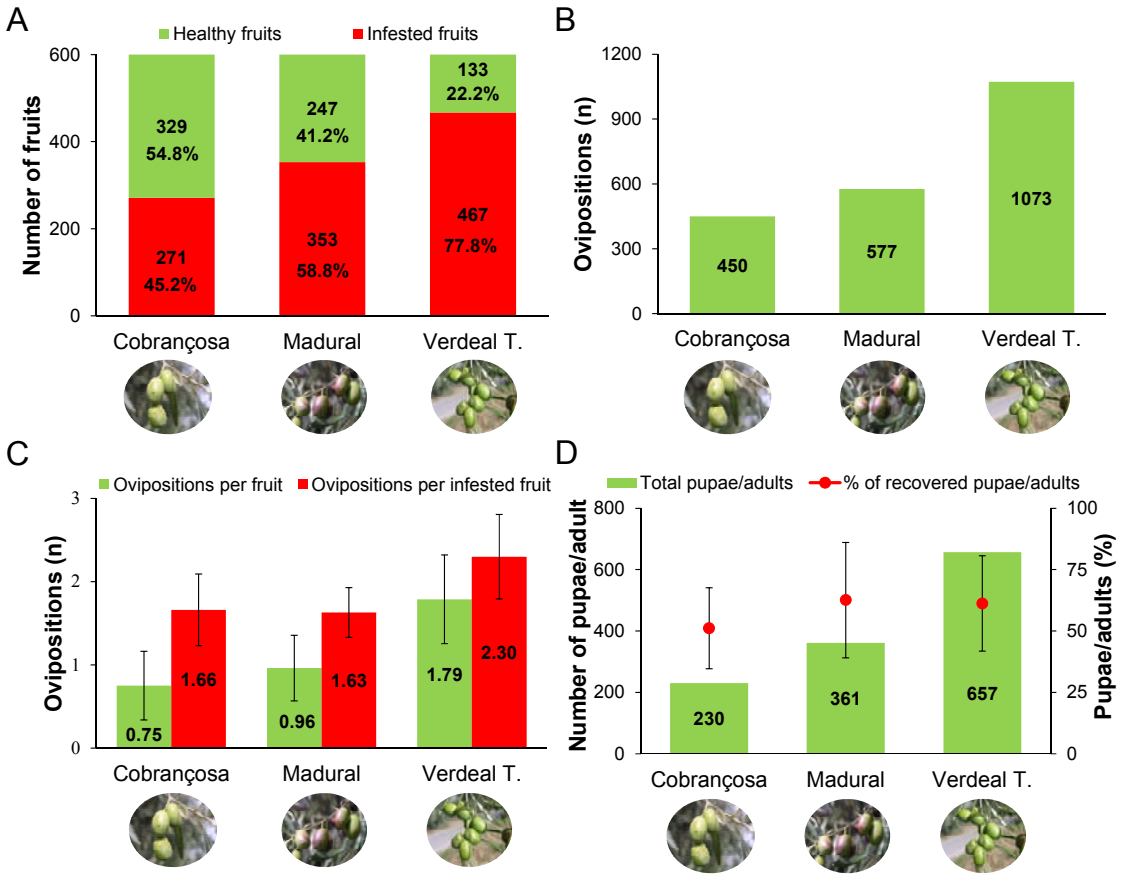

Figure 10.5. Results from one-choice oviposition bioassays conducted with cvs. Cobrançosa, Madural and Verdeal Transmontana: A – healthy vs. infested olives; B – total number of ovipositions; C – ovipositions per fruit and infested fruit; D – percentage of pupae/adults recovered according to total number of ovipositions.

In this oviposition bioassay important information was retained: cv. Cobrançosa showed to be less preferred by olive fly females to oviposit, and the percentage of pupae/adults collected was also lower. Therefore, besides the contribution of external factors for olive fly preference, internal factors, linked to olives inherent composition, may contribute for the mortality or survival of larvae and subsequent adults. As already discussed in Chapter 8, the lower number of pupae/adults recovered from cv. Cobrançosa olives comparatively to cvs. Madural and Verdeal Transmontana might be related to two main causes: i) a high number of abortive ovipositions; and ii) a high rate of mortality of eggs and larvae inside olives pulp. The first hypothesis is mainly related to the perception of olive fly at the oviposition moment, being its chemo-sensilla and antenna sensilla sensitive to the olives composition and volatiles released from the oviposition attempt. One of the main volatiles exhaled during oviposition is (*E*)-2-hexenal, a compound already proven to be repellent to olive fly (Scarpati *et al*., 1993), and that was found to act over olive fly females antenna at sexual maturation and beyond (Chapter 7). The release of

(*E*)-2-hexenal during oviposition could be higher in cv. Cobrançosa olives, obliging olive fly females to leave and abort the oviposition, leaving no egg inside olive pulp. Nevertheless, the volatiles exhaled from each olive cultivar at oviposition should be determined in order to test this hypothesis.

Concerning the possibility of a high number of eggs and larvae dead inside cv. Cobrançosa olives could be related to phenolic composition of olives and enzymatic activity triggered due to olive fly aggression. The main phenolic compound in unprocessed olives is oleuropein, a secoiridoid. The amounts of this phenol as well as total phenols in olives from cvs. Cobrançosa, Madural, and Verdeal Transmontana are reported in Table 10.1.

Table 10.1. Oleuropein and total phenols content (mg.kg-1 of olive pulp, fresh weight) of olives from cvs. Cobrançosa, Madural, and Verdeal Transmontana during maturation (data from Sousa *et al*., 2014; Sousa *et al*., 2015).

	cv. Cobrançosa	**cv. Madural**	**cv. Verdeal Transmontana**
Oleuropein			
29th September	32.9 ± 0.2	36.4 ± 3.4	13.1 ± 0.2
13th October	3.7 ± 0.2	1.0 ± 0.2	0.6 ± 0.1
27th October	0.25 ± 0.02	0.33 ± 0.06	1.0 ± 0.0
10th November	0.13 ± 0.06	0.30 ± 0.07	0.9 ± 0.0
18th November	n.q.	0.26 ± 0.12	0.4 ± 0.0
Total phenols			
29th September	33.8 ± 0.2	39.0 ± 4.0	14.8 ± 0.2
13th October	4.6 ± 0.2	1.4 ± 0.2	1.3 ± 0.1
27th October	1.2 ± 0.0	0.7 ± 0.1	2.0 ± 0.0
10th November	1.0 ± 0.1	0.7 ± 0.1	1.4 ± 0.0
18th November	1.0 ± 0.0	0.5 ± 0.1	0.7 ± 0.1

n.q. – not quantifiable, below the limit of quantification.

Until mid-October, the three olive cultivars are at the same maturation level (Chapters 8 and 9), and toleuropein content is comparatively low in cv. Verdeal Transmontana (Table 10.1). While maturation advances, oleuropein content decrease considerably, but remain in higher amounts in cv. Cobrançosa, eleven times more that cv. Verdeal Transmontana at 13th Oct, being both cultivars at the same maturation level, near MI = 1. This information is very important since oleuropein and one of its derivatives, demethyloleuropein, are hydrolyzed by β-glucosidase. This reaction is enhanced when olives are attacked by olive fly (Spadafora *et al*., 2008) and it leads to the formation of highly toxic molecules, as glutaraldehyde-like structures (Koudonas *et al*., 2015). Due to the higher amount of substrate in cv. Cobrançosa, high levels of these molecules could be released and accumulate around eggs and larvae, killing them. The accumulation of these molecules in olive pulp (Sivakumar *et al*., 2007), could increase the mortality at advanced

stages of maturation. Such fact was observed in cvs. Cobrançosa and Madural when tested to verify the impact of maturation in the olive fly preference (Chapter 8). Olive fly females clearly prefer olives at early maturation stages (MI = 2 and 3) than olives at advanced maturation stages (MI = 4). At MI = 4 the ratio of recovered pupae/adults is considerably low, 7.0% and 23.2% in cvs. Cobrançosa and Madural.

Regarding the preference for early maturation stages, the main factors related could be of physical nature, namely color. During maturation (from MI = 2 to MI = 4) olives turn red and then black, losing a considerable part of their lightness (*L** values; Chapter 9). Olives of cv. Cobrançosa at 6th Nov and those from cv. Madural at 23rd Oct possess the same maturation index, 2.1 and 2.0 respectively. However *L** values for cv. Cobrançosa were of 46.0 and for cv. Madural were of 50.7 (Chapter 9).

Returning to the initial observation that olives from cv. Cobrançosa are less preferred and cause higher mortality and/or higher number of abortive ovipositions, a third aspect should join this premise, adults live less. Those ovipositions made in olives from cv. Cobrançosa, which eggs hatch, larvae are able to correctly develop and form a pupae, later giving an olive fly adult, give birth to adults that lived considerably less than those developed under cvs. Madural and Verdeal Transmontana pulp (Chapter 8). This observation is valid for both sexes, with higher longevity in males. Regarding these results, a chemical factor should be implicated, namely the fatty acid composition, characteristic in each olive cultivar. Fatty acids profile at different maturation indices was determined from olive of the three cultivars (Chapter 9), and major differences were found. Olives from cv. Verdeal Transmontana have more fat content (Gonçalves *et al*., 2012) and are richer in oleic acid, while those from cv. Madural are considerably richer in linoleic acid. The differences found in the fatty acids profile of the three olive cultivars could play an important role in olive flies longevity. This means that adults developed under cv. Verdeal Transmontana olives will possess higher reserves of fat, which will provide them higher amounts of energy to move, search for food sources and living longer. This is an important ecological data. The question now is: do olive flies know that their eggs and adults are likely to have higher chances of survival in one cultivar in detriment of other? Does this fact influence olive fly oviposition preference? Answers worthy to support further studies.

Regarding physical factors and their influence in oviposition preference, color, as already discussed, is an important aspect. However, olives color do not work alone, since the results obtained also point out that olive leaves could influence oviposition preference. Similarly to olives, lightness values from leaves are significantly higher in leaves from cv.

Verdeal Transmontana (Chapter 9). The effect may resides in the upper page of olive leaves, being their coloration also used by human eyes in order to help identifying olive cultivars by leaves color and shape. Leaves can also aid olive fly in their pursuit for olives, creating a green-dark background, turning easier the localization of olives by olive flies. In this aspect olive volume is also an important aspect, since olive flies prefer bigger fruits than smaller ones (Neuenschwander *et al*., 1985). Olives from cv. Verdeal Transmontana besides reporting a slower maturation process comparatively to the other two cultivars, also exhibits higher volume (Chapter 9). Regarding elongation, cv. Cobrançosa report higher elongation values, but olive flies prefer less elongated olives (Rizzo *et al*., 2012).

Overall, oviposition preference is influenced by several aspects related to olive tree. Physical factors of olive leaves and olive tree itself are crucial for host recognition at long distance, playing color a preponderant role. Inside the olive orchard, olive fly is driven by aspects of chemical and physical nature. Biometric parameters (volume and elongation) and leaves and olives color together with their volatile emissions are certainly implicated in olive fly preference. When already set to oviposit, again, chemical factors could lead olives to abort oviposition. Physical factors like firmness, elasticity and energy necessary to perforate olive epidermis may also remove olive fly from oviposition (Gonçalves *et al*., 2012). Once inside olive fruits, immature stages of olive fly, eggs and larvae, may suffer the action of defense mechanisms of chemical and molecular nature, influencing olive fly next generations as well as their adults survival. Beyond all these factors influencing oviposition preference is the maturation process. Since maturation process is characteristic in each cultivar, olives suffer internal and external modifications that maturation molds in each cultivar, leading to their differentiation at physical and chemical level, assisting olive fly in its oviposition preference.

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CHAPTER 11.

Conclusions

The results obtained in this thesis revealed that the study of the interaction between pests and hosts can give important information about host susceptibility. It was concluded that olive fly has a preference by cv. Verdeal Transmontana, and that cv. Cobrançosa, although not being resistant, is clearly a less susceptible olive cultivar.

Olive tree volatiles, both from olives and leaves, are dependent on olive cultivar and harvest period assessed. Cultivars with higher susceptibility report higher abundance of attractant volatiles to olive fly.

Leaves essential oils are recognized by olive flies antenna, and higher electroantennographic responses were reported from the essential oils extracted from the less susceptible olive cultivar. Composition of leaves essential oils is characteristic of each cultivar and a possible repellent action of leaves essential oil may be found in this work.

Olive tree volatiles influence olive fly behavior at different stages of adults development, with higher electrophysiological responses at sexual maturity and after, at oviposition period. Both olive tree volatiles and essential oils report higher electrophysiological response than recognized olive fly semiochemicals.

Olives from cv. Cobrançosa, besides being less preferred by olive fly, also caused higher mortality/abortive ovipositions, and lead to a lower longevity of adults of both sexes, comparatively to cvs. Madural and Verdeal Transmontana. Olive fly prefers olives at earlier maturation stages to oviposit than those at advanced maturation stages. The mortality/abortive ovipositions rate increased considerably in olives at advanced maturation stages, regardless of olive cultivar.

Physical parameters, mainly olives and leaves color and lightness, olives volume and elongation, favor oviposition in more susceptible cultivars. Olive pulp composition, namely fatty acids profile, and phenolic composition, are might be directly implicated in the longevity and death of olive fly adults, respectively. Olives maturation process, responsible for internal and external changes in olives, is characteristic of each olive cultivar, and it is the most influencing factors of all, since it molds all characteristics of olives over time.

Physical and chemical parameters of cvs. Cobrançosa, Madural and Verdeal Transmontana, altogether, act over olive flies oviposition preference. These parameters

exert activity at four levels: long distance; short distance; at oviposition; and at immature development and adult's survival.

The results obtained in the present thesis may open new lines of investigation and applied research in order to develop new strategies, sustainable and eco-friendly, to manage one of the key pests of olives worldwide, the olive fly, *Bactrocera oleae* (Rossi).