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PhD course in Plant Protection
- XXIII CYCLE -

DOCTORAL THESIS

Development of a continuous small-rearing technique on natural host for the olive fruit fly *Bactrocera oleae* (Rossi) and study on volatile organic compounds (VOCs) emitted by *Olea europaea* L. as potential host allelochemics.

AGR/11

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Abstract

The olive fruit flies *Bactrocera oleae* (Rossi), as most of the *Tephritidae* species ("true fruit flies"), is a pest of great economic importance. It's among the most serious pests of *Olea europaea* L. in the Mediterranean Basin and in olive growing regions worldwide.

Longstanding control programmes as well as current research efforts emphasize the development of long-term management practices, focusing on classical biological control for sustainable management. As part of a classical biological control programme, several attempts have been done in the past in order to rear olive fly *ex-situ*. Undue efforts are often directed toward producing the largest possible number of flies at the least possible cost, but the flies produced on artificial diets or on factitious hosts may not be effective in carrying out the mission for which they were intended. In the case of *B. oleae*, genetic changes seem to occur when olive flies are reared on artificial diet causing adverse effect on their performances and fitness if compared to wild individuals. For this reason, researchers are now oriented to obtain laboratory colonies maintaining them on their natural host: developing rearing protocols for olive fruit fly on its natural host would allow bioecological and behavioural studies and successful field-releases of olive fruit fly biological control agents.

The present study establishes fundamental guidelines for a small-scale and cost-effective rearing of *B. oleae*, for the first time successfully culturing year-round the olive fruit fly on its natural host basing upon the continual availability of fresh olive fruits, suitable to oviposit.

In line with IPM and biological management purposes, ending the use of non-selective long-lasting poisons and beginning the use of insects attractants, not just pheromones, could potentially become a powerful management tool, interfering with plant and insects at vulnerable steps. At this regard, the second purpose of the present study was the characterization of the broadest possible range of volatile compounds and to investigate the possible changing trends of biological volatile emissions during development stages. The return of feral olive fly populations to the olive groves after the first abundant summer rains it must certainly be due to volatile compounds emitted by the plants and active on the olive fly. These results involve that host-plant odours play the fundamental ecological role of olfactory attractants and oviposition stimulants interacting with *B. oleae.*

Olea europaea L., the common olive, is considered to be a low emitting species: its terpenoids emissions are generally found to be zero or close to zero. A number of authors have already attempted the identification of volatile components deriving from various parts of the olive plant or dissolved in the oil, by means of distillations, solvent extractions or head-space samples from plant detached portions, mainly focusing on the compounds bioactivity against pathogens and entomo-pathogens. At this regard there is a confuse variety of roles attributed to the plant compounds.

During the last few decades, much has been done in order to understand chemical communications in insect-insect interactions and about the insect responses to specific volatiles compounds. Research emphasis is now directed towards insect-plants interactions: it needs to properly investigate and recognize the volatiles released by living plants first of all, testing bioactive aspects of such compounds and correlating insect responses in the second place.

The present study reports on the original results of VOCs detection from whole, living olive trees (*Olea europaea* L.). For the first time, an early comparison of the volatile organic compounds emitted from plants in different phenological stages (vegetative and fruiting stage) was performed.

The method developed appears to be functional for a true detection of biogenic compounds from whole olive trees, monitoring the changing trend of volatile profile characteristics during time and obtaining the corresponding volatile profile characteristic for the different phases. For the first time, an early comparison of the volatile organic compounds emitted from plants in different phenological stages (vegetative and fruiting stage) was performed.

Despite the usual low emission rates of the specie, the variations of VOCs emission between different phenological stages of *Olea europaea* L. were clearly displayed and a broad range of emitted volatile compounds were successfully characterized.

The information accumulated forms overall an excellent base of knowledge for studying plant-insect interactions and are likely to improve the current strategies against the olive fruit fly *Bactrocera oleae* Rossi, for the first time enabling its continuous lab-rearing on the natural host and better clarifying natural occurrence and the ecological role of host allelochemicals, i.e. attractants of the pest.

1. The olive fruit fly (*Bactrocera oleae* Rossi): a continuous small-scale rearing technique on its natural host (*Olea europaea* L.) in laboratory.

1.1. Introduction

The olive fruit flies *Bactrocera oleae* (Rossi), as most of the *Tephritidae* species ("true fruit flies"), is a pest of great economic importance. It's among the most serious pests of *Olea europaea* L. in the Mediterranean Basin and in olive growing regions worldwide.

In the last century the olive fruit fly have followed the expansion of the olive cultivation to North, Central and South America, South Africa, China and Australia, and infestations have been reported everywhere olive grows except Australia.

Both longstanding control programmes and current research efforts emphasize the development of long-term management practices, focusing on classical biological control for sustainable management. European biological control programmes for the olive fruit fly have relied almost exclusively on *Psyttalia concolor* (Szépigeti) (*Hymenoptera: Braconidae*), also thanks to the ease of its mass-rearing on the Mediterranean fruit fly *Ceratitis capitata* (Wied) (*Diptera: Tephritidae*) on artificial diet. Nevertheless, this African species has not proved particularly successful, being not a narrow host specialist and failing to establish in most regions. Moreover, using the Medfly as a factitious host in artificial media may contribute to the poor performance of *P. concolor* in the field (Kimani-Njogu *et al.*, 2001). Other parasitoids of the olive fruit fly have proved more difficult to rear (Wharton, 1989; Sime *et al.*, 2007).

In order to develop mass-rearing systems, undue effort is often directed toward producing the largest possible number of flies at the least possible cost, but the flies produced on artificial diets or on factitious hosts may not be effective in carrying out the mission for which they were intended. In the case of *B. oleae*, genetic changes seem to occur when olive flies are reared on artificial diet causing adverse effect on their performance and fitness if compared to wild individuals (Tsakas & Zouros, 1980; Loukas, 1985; Kostantopoulou *et al*., 1986). The lab-produced flies were found to differing in certain biological traits such as longevity, reproductive pattern and capacity, male competitiveness, flight ability, field dispersal, eye colour and vision, pheromone production. For this reason, researchers are now oriented to obtain laboratory colonies maintaining them on their natural host (Sime *et al.*, 2007; Genç & Nation, 2008).

As part of a classical biological control programme, to develop and improve rearing protocols for olive fruit fly on its natural host would permit the study and comparison of biological parameters and successful field-release of olive fruit fly biological control agents (Sime, 2007). In this regard, set up a simple and affordable small-scale rearing technique to supply olive fruit fly (*Bactrocera oleae* Rossi) instars continuously throughout the year, even when fresh fruits are not available naturally to oviposit, is essential to optimize biological studies as a whole.

Several attempts have been done in the past in order to rear olive fly *ex-situ* with the aim of studying different bioecological and behavioural aspects. Most of the studies carried out under laboratorial conditions referred to only one generation (Economopoulos *et al*., 1976; Remund *et al.*, 1977; Pucci *et al.*, 1982; Tzanakakis & Koveos, 1986; Koveos & Tzanakakis,

1990; Raspi *et al.*, 1997). Other investigations on multiple generations relied on artificial diets (Tsitsipis, 1977).

The rearing technique developed within the research activities of the doctoral course is a small-scale, practical and cost-effective method that succeeds in culturing the olive fruit fly (*Bactrocera oleae* Rossi) on its natural host in the laboratory, continuously throughout the year, even when olive fresh fruits, suitable to oviposit, are not naturally available in the field.

1.2. The olive fruit fly *Bactrocera oleae* (Rossi)

Within the order *Diptera, Bactrocera oleae* (Rossi), commonly known as "olive fruit fly", belongs to the suborder *Brachycera*, superfamily *Tephritoidea*, family *Tephritidae* (Norrbom *et al.*, 1999;). The olive fruit fly is one of the smaller species in the genus: the adult is about 5 mm long and has a wing expanse of ±10 mm (Fig. 1).

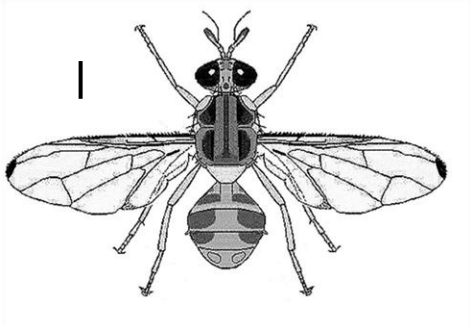

Fig. 1 *Bactrocera oleae* (Rossi) adult male.

Fig. 2 *Bactrocera oleae* (Rossi) adult male (Natasha Wright, *Florida Department of Agriculture and Consumer Services*, Bugwood.org).

The head, thorax, and abdomen are dark coloured with markings and several white or yellow patches on the top and sides of the thorax (Fig. 2). The thorax is black and has a silvery pubescence dorsal surface stripped with three narrow parallel black lines. The inner portion of the scutellum is black, the posterior portion is yellow. The black abdomen is covered with a scattered grey pubescence, the basal segments are marked with transverse pale bands and an irregular parallel reddish-brown bar or blotch in the centre of the apical segments. The

terminal segment is reddish-yellow. The sheath of the ovipositor is black, with the ovipositor reddish in colour. Usually only one egg is laid per fruit. However, multiple eggs may be laid in olive varieties that produce large fruit; females prefer large-fruited varieties to smaller-fruited varieties for egg laying.

The wings, transparent and marked in brown, are positioned horizontally and are held away from the body. Olive fruit flies may be distinguished from related fruit flies by the presence of black spots on the wing tips and the lack of banding across the wings that occurs in most other related species such as the Mediterranean fruit fly.

Larvae are yellowish white, legless maggots with pointed heads. When first hatched, they are tiny and hard to see. The larval stage is spent entirely within the fruit

1.2.1. Life cycle

The life cycle of the olive fruit fly includes the stages of egg, three larval instars, *pupa* (or *puparium*) and adult, and it is closely linked to the seasonal development of its main host, the cultivated olive and wild relatives (*Olea europaea* L.), and to the local climate. The *larvae* are frugivorous, strictly monophagous, feeding exclusively on olive fruits, while adults feed on different substrates like fruit juices, honeydew, yeast, bacteria, nectar, pollen and other opportunistic sources of liquid or semi-liquid food.

The species could be uni-, bi- o multi-voltine. In the Mediterranean region, 2 to 5 generations of flies occur yearly. In the hottest regions, adult flies remain active year-round and the eggs and maggots can be found throughout the year in unharvested fruit.

The winter is spent usually in the pupal stage few centimetres below the soil and leaf litter; the newly generation emerge from March to May depending upon the latitude and temperature.

A preoviposition period of 6 to 10 days elapses before mating, maturing ovaries, with longer time required at low temperature. Courtship and mating occur at dusk, near the end of the daylight period. Females of the olive fly produce a multicomponent pheromone, its major component is 1,7-dioaxpiro[5.5]undecane, a long-range attractant for males. Males also product this compound in little amounts, but there are evidences that females are not attracted to the compound, from either sex. In June females begin to seek actively and oviposit in early maturing olive fruits. A dozen of eggs may be laid daily, usually one egg per fruit, and about 200 to 250 are laid in a lifetime. Feral females are oligogamous and mate few times during their lifetime, while male olive flies are polygamous.

During summer as temperatures and day length increase and few mature fruits remain on trees, female flies enter a state of reproductive diapause (the so-called “white period”) in which few or no eggs are produced. Nevertheless, the adults remain active, and may disperse to other crops. Adult olive flies have been collected in wild olives as well as in citrus, walnut and several fruit plantings (cherry, plum, avocado, loquat, nectarine, etc.) and may congregate wherever sucrose is available: from aphid infestations, from fruit juices, from honeydew, and so on.

As the new olive crop develops, adult females are attracted to the olive fruits and come back to the olive orchards, beginning to lay eggs. They puncture the ripening fruit when the

pits of the olives begin to harden, just under the fruit's skin, often creating a dimple or brown spot. Stings fresh marks look like moon shaped blemishes on the olive skin.

Under laboratory conditions, an individual female olive fruit fly may lay 10 to 40-60 eggs per day and, from 200 to 500 eggs in her lifetime. The newly hatched *larvae* (maggot) feed and develop entirely into the fruits, upon the pulp, causing the fruits withering and dropping.

Larvae that develop during summer pupate mostly within the fruit and emerge later in the season. Last generations larvae (late fall) pupate in the soil, 3 cm beneath the surface on average, where they spend the winter; however, some maggots could overwinter in fruits on trees and pupate in spring.

Multiple generations occur throughout summer and fall. In summer the flies can complete a generation in as little as 30 to 35 days, given optimum temperatures (20°C to 27°C). The activity threshold for the adult olive fruit fly is about 14°C; below this temperature they are not very active.

Eggs hatch from 2-4 days at 28-30°C to 3 weeks at lower temperatures (thermal thresholds 6°C to 33-34°C); larvae develop in about 15-20 days during summer and fall, to 150 days during winter (thermal thresholds 10-12°C to 32-34°C). There are evidences that larval development undergo different *stimuli* apart from temperature, i.e. photoperiod, cultivars, ripeness and size of the olive fruits.

Pupal development requires 8 to 10 days during summer but may take as long as 6 months in winter (thermal thresholds 10°C-32°C). Duration of the life cycle varies from one to six or seven months. Hot (35-40°C) and dry conditions reduce the build-up of olive fruit fly populations. Fruit fly maggots can experience relatively high mortality during hot, dry weather.

1.2.2. Distribution

The olive fly is distributed all around the Mediterranean basin, but reports of its occurrence are accumulating from various parts of the world, including South and Central Africa, Near and Middle East, California and Central America (Rice *et al*. 2003; Nardi *et al.*, 2005), therefore the fly is apparently widespread wherever cultivated olive trees (gen. *Olea*) are grown extensively, as well as where wild olive trees are indigenous.

The pest has recently moved into California and Mexico: the presence of the olive fly in the New World is most likely associated with the introduction of olive trees for agricultural purposes (Nardi *et al.*, 2005). Because of the olive fly rapid dispersal and establishment, every country where olives are grown is thought to be virtually at risk of invasion (USDA 2001). The specie is not currently found in New Zealand and Australia.

1.3. Material and methods

Olive fruits came from a typical olive orchard of the Northern Lazio (Cura di Vetralla, VT, Central Italy), organically managed. All trees are of Canino cv, aging 40 years old.

To provide fresh fruits year-round, some fruitful branches were wrapped into special "muffs" of straw and tulle, assembled directly on fruitful branches starting from the late July-early August (as soon as the fruits reach the right size and before the pit's hardening) (see Fig. 5). Securing both olives' soundness and slow ripening, this method usually provides fresh, healthy fruits through 13 months and over.

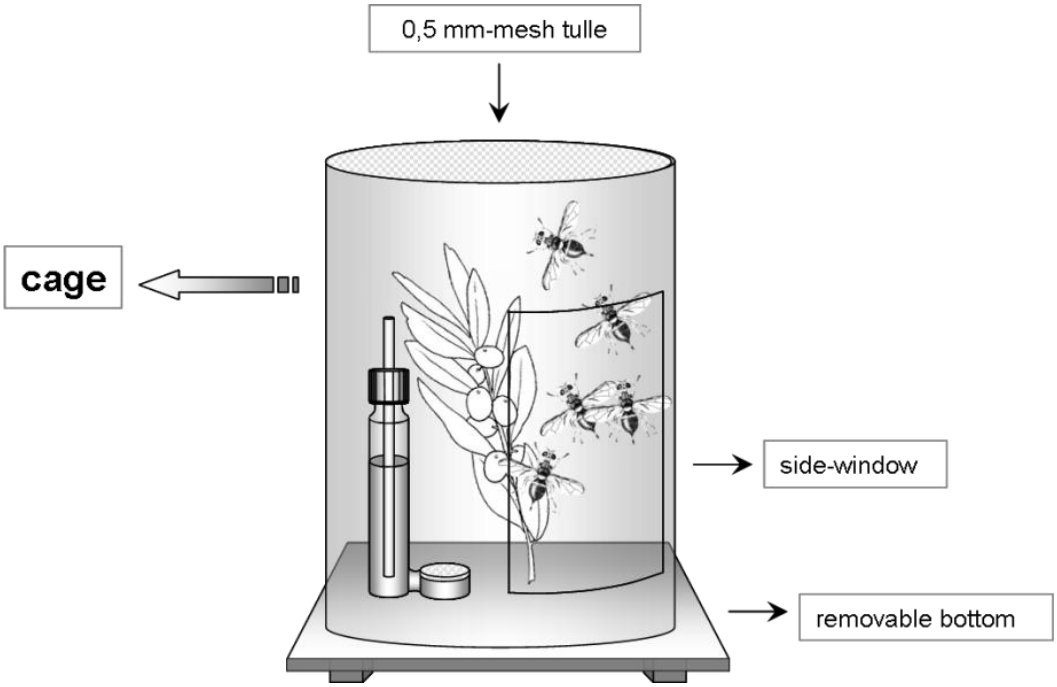

Fig. 3 Scheme of the rearing cage (different components and flies are not in scale)

Every 10 days some branches were picked from the "muffs", deprived of leaves to avoid a quick dehydration of fruits, then disposed into the cages.

Cages, feeders and instruments were expressly designed. Rearing cages are cylindrical (Ø = 30 cm, h = 40 cm), made of Plexiglas with a removable bottom and a little side-window. The cage top is closed with a 0,5 mm-mesh tulle (Fig. 3). Feeders are made up of a cylindrical glass container (Ø=3 cm and h=10 cm) stoppled with a plastic screw top. As soon as the container is filled, the nutritional solution pours, through a bottom connection, into a little tray (Ø=4 cm, h=1 cm) (Fig. 4). The tray is plugged up with a porous material to let solution surfacing slowly and adults feeding comfortably. To avoid overflowing, a pipe (not capillary) must traverse the container's cap, with a free end outside the container (see Fig. 4). Feeders were washed once a week using sodium hypochlorite, then soaked into water for 3 hours, and again washed in plenty of water. Cages were cleaned when needed, using a common detergent. A proper displacement of the adults from a rearing cage to others (shifting cages) allowed us to separately maintain individuals of each generation. At least 3 cages are necessary to maintain population in every generation.

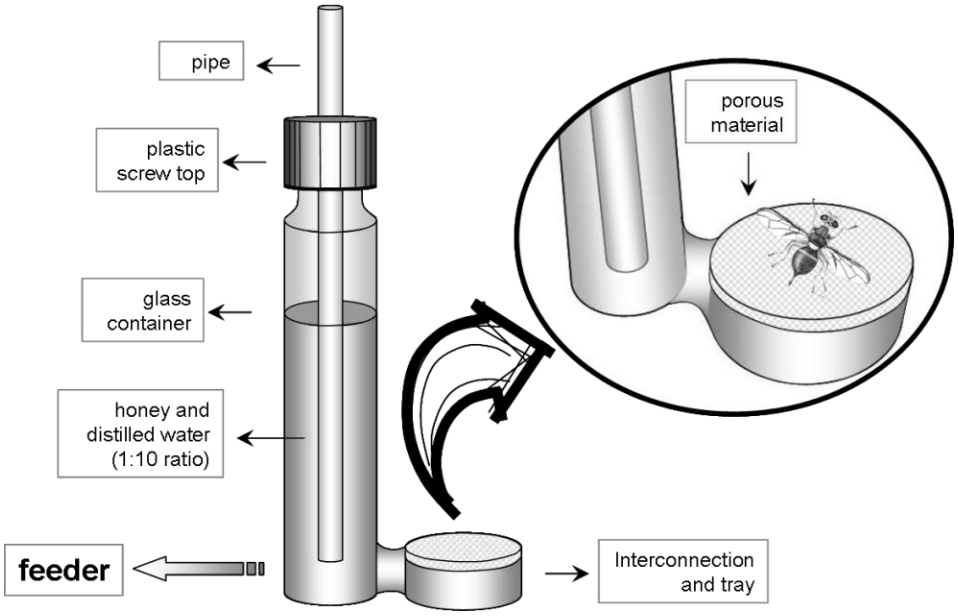

Fig. 4 Scheme of the feeder (olive fly not in scale).

The olive fly culture was permanently maintained into a climatic chamber to easily control temperature T, relative humidity RH and photoperiod LD.

To perform the effective permanence of a *B. oleae* population over time, this rearing method essentially base upon the year-round availability of fresh olive fruits, suitable to oviposit.

The olive fly larvae do not develop on fruit before the pit hardening stage, and picked fruits tend easily to rot and in any case olive fruits cannot be held in storage without affecting their suitability for oviposition and larval feeding and development. Even using different olive cultivars with varying seasonal periods of ripening, and collecting olives at different times following the geographic progression of ripening from south to north, it is not possible provide olive fruits of an acceptable quality for more than some months of the year. Besides, it becomes a too much expensive method of fruits collection (Sime *et al.*, 2007).

The method developed, supplying permanently fresh fruits to the adult population, allows starting a potential indefinite number of different life cycles in sequence.

During the late July-early August 2008, when in orchards the olive fruits reached the right size but not still the pit's hardening, an adequate amount of "muffs" were arranged on fruitful olive branches, to assure the fresh fruits availability for the entire year.

Fig. 5 "Muffs" of straw and tulle on fruitful branches of *Olea europaea* L., Canino cv, July, 2009

Fig. 6 Branches and olive fruits just unclothed of the muff, Agust 2010

Fig. 7 Muff-unclothed ripening fruits on a nearly flowering olive tree, Spring 2008

Fig. 8 Feeder: the tray is plugged up with a porous material to let adults feeding comfortably on a solution of distilled water and honey, 1:10.

The rearing started the 21st of November 2008, when wild adults of *B. oleae* were collected and moved to the climatic chamber. Two populations, each one of about 30 adults (sex ratio 1:1), were placed into two different cages whit bunches of sound, fresh fruits to allow eggs laying. After 10 days, the punctured fruits were separated from the adults population and kept apart into a different cages, where the development cycle from egg to newly adults could proceed independently (*n* cycle). At the same time, bundles of little branches with fresh fruits were picked from the 'muffs', deprived of leaves to avoid a quick dehydration of fruits, then disposed into the adults cages to let new ovipositions occur (*n*+1 cycle).

Every 10 days, the punctured olives were segregated into different cages (*n* cycle) and adults laid eggs on fresh fruits (*n*+1 cycle): following the decades, within the laboratory culture different cycles occur, at different development times, coexisting and overlapping.

To separate the development of every life cycle, it needs obviously the use of several cages simultaneously (from a minimum number of 3 cages on).

The adult populations were sustained through immigrations of the newly adults coming from the different cycles: every 10 days, concurrently with the shifting of the rearing cages, the newly adults were counted separately per sex and cycle of origin, and placed into the ovideponing cages.

The supplying of nutrients to adults (sugar, water and proteins) was regular and abundant, to avoid food competition that may have long-term effects on several adult fitness traits (longevity, fecundity) without reducing the immediate pre-imaginal survival. The liquid diet was a solution of distilled water and sugar (1:8) or honey (1:10), while the proteic source was water soluble extract of autolyzed yeast cells (a mixture of amino acids, peptides, water soluble vitamins and carbohydrates).

To provide a more comfortable *media* to pupate instead of the plastic countertop of the cages, we tested successfully moistened paper towels, nebulized with distilled water when needed (Navrozidis, 2005; Genç, 2008) (Fig. 9).

Fig. 9 Pupae between paper towels nebulized with distilled water

The parameters of T, RH and photoperiod LD were monitored daily through the chamber detectors and digitally recorded with a datalogger (Fig. 10).

Fig. 10 Datalogger

To avoid a quick shrivelling of the fruits, the chamber humidity must never reach the lower, threshold value of 65% RH. Water stress, resulting in shrivelled fruits, will impede both female oviposition and larval development, as it happened in some accidental faults of the chamber hygrometer. On the other hand, because of mildews proliferation, RH shouldn't overcome the precautional value of 85%. Hence, the chamber humidity was setted at 75% RH.

With the purpose of recognize the better combination of climatic variables and the ecophysiological responses of the specie, two temperature values of 16°C and 27°C were combined in 4 different arrangements with the photoperiod values of 8:16 and 16:8 LD.

1.4. Results

The rearing method appears to be both practical and cost-effective, for the first time enabling the continuous lab rearing of the olive fruit fly on its natural host, without interruptions thanks to the year-round availability of olive fruits.

The main issue of developing *B. oleae* rearing protocol on natural host is the storage method for olive fruits. The olive fly larvae do not develop on fruit before the pit hardening stage, and picked fruits tend easily to rot and in any case olive fruits cannot be held in storage without affecting their suitability for oviposition and larval feeding and development. Using fresh fruits from different olive cultivars with varying seasonal periods of ripening doesn't provide olive fruits of an acceptable quality for more than some months of the year. Besides, it becomes a too much expensive method of fruits collection (Sime *et al.*, 2007).

To perform the effective permanence of a *B. oleae* population over time, the technique developed essentially bases upon the year-round availability of fresh olive fruits, suitable to oviposit. Supplying permanently fresh fruits to the adult population makes possible to start a potential indefinite number of different life cycles in sequence.

The olive fly culture lasted ceaselessly for more than one year, from November 2008 until the summer of 2010. Some accidental malfunctioning of the climatic chamber caused temporary relocations of the culture. Nonetheless, during the rearing progression different combinations of temperature and photoperiod values were successfully tested (16°C and 8:16 LD, 27°C and 8:16 LD, 27°C and 16:8 LD, 16°C and 16:8 LD), with the aim to determine the most suitable combination of climatic parameters.

This rearing methodology, forcing the adults ovipositions within 10 days per each bunch of fresh fruits, helps to synchronize the development time of the instars and therefore to concentrate the emergences of newly adults. The subsequent segregations of punctured olives and the consequent separate development of each life cycle, lead to the overlapping of multiple lab-generations: every development cycle start regularly after 10 days from the previous one, lasting and therefore overlapping for a certain time depending on its development time.

Temperature is the single most important factor determining development rates of the immature stages and the adult maturation rates of the majority of insects. For the optimization of rearing procedures, numerous attempts have been made to determine the effect of temperature and development rate relationships from tephritid fruit flies. Eggs of *B. oleae* don't hatch at the temperature threshold values of 7,5°C and 35°C, although some development occurs at these temperatures (Tsitsipis, 1977). Complete larval and pupal stages development could occur at temperatures between 12 and 35°C (Tsitsipis, 1977; Tsitsipis, 1980; Moore, 1960; Tzanakakis *et al.*, 1968; Manikas, 1968; Girolami, 1979; Fletcher, 2002).

Girolami (1979) noticed the complete development of immature stages reared on olive fruits: eggs complete their development at temperatures between 7.5°C and 37.5°C, larvae at temperatures 10°C to 32.5°C, pupae at temperatures 10°C to 30°C.

Two levels of temperature (16°C and 27°C) were evaluated in their effects on both fly physiology and management issues of the culture, in combination with the short-day (8:16) and the long-day (16:8) photoperiod.

The ecophysiological responses of the fruit fly were evaluated by measuring the following biological traits:

- *pupation and emergence periods*, expressed as average time of occurrence (days);
- *total life span* of generations, from oviposition to adult death;
- *number of pupae,* the number of puparia collected;
- *rate of emergence*, expressed as number of eclosions on total pupae.
- *sex ratio*, expressed as females on males;

Results are tabulated (Tab. 1) and graphically presented as means per treatment (±SD). To determine treatment effect, we used analysis of variance (one-way ANOVA, Levene's homogeneity-of-variance test), and post-hoc Tukey's HSD test or Thamana's T2 test to compare treatment means.

Tab. 1 Culture statistical output of rearing records (T, RH and photoperiod are specified): mean, standard deviation SD, min and max values (range), nr of replicates, population mean confidence level. Significance of mean differences by one-way ANOVA test, * highly significant differences (p < 0.001), * significant differences (p < 0.05), ns not significant**

	pupal period (days) ***	*emergence period (days)* ***	*puparia nr* ***	*emergence rate* *	*total life span (days)* ***	*sex ratio* *ns*
			16°C 8:16 LD 75% RH			
mean	38,69	37,00	95,6	0,93	103,53	1,00
SD	3,09	6,20	41,9	0,03	8,59	0,14
min	32,00	28,00	25,0	0,88	91,00	0,81
max	42,00	50,00	188,0	0,98	114,50	1,18
repl. nr	16,00	16,00	16,0	1600	16,00	16,00
p=0.05	1,65	3,30	22,3	0,02	4,58	0,08
			27°C 8:16 LD 75% RH			
mean	18,81	19,81	53,3	0,70	79,61	1,00
SD	4,32	4,13	30,0	0,25	9,23	0,17
min	12,00	12,00	20,0	0,20	59,00	0,67
max	26,50	26,00	125,0	0,97	93,50	1,33
repl. nr	16,00	16,00	16,0	16,00	16,00	16,00
p=0.05	2,30	2,20	16,0	0,13	4,92	0,09
			27°C 16:8 LD 75% RH			
mean	14,00	15,21	27,5	0,65	61,08	1,29
SD	4,63	5,02	24,9	0,27	5,66	0,48
min	9,00	8,00	8,0	0,00	55,00	0,75
max	23,00	21,00	79,0	1,00	70,00	2,33
repl. nr	12,00	12,00	12,0	12,00	12,00	12,00
p=0.05	2,94	3,19	15,8	0,17	3,60	0,31
			16°C 16:8 LD 75% RH			
mean	26,46	25,13	42,5	0,82	87,17	1,55
SD	6,99	6,52	30,8	0,12	13,96	0,81
min	13,00	13,00	1,0	0,68	56,00	0,75
max	36,00	36,00	86,0	1,00	100,00	4,00
repl. nr	12,00	12,00	12,0	12,00	12,00	12,00
p=0.05	4,44	4,14	19,6	0,07	8,87	0,51

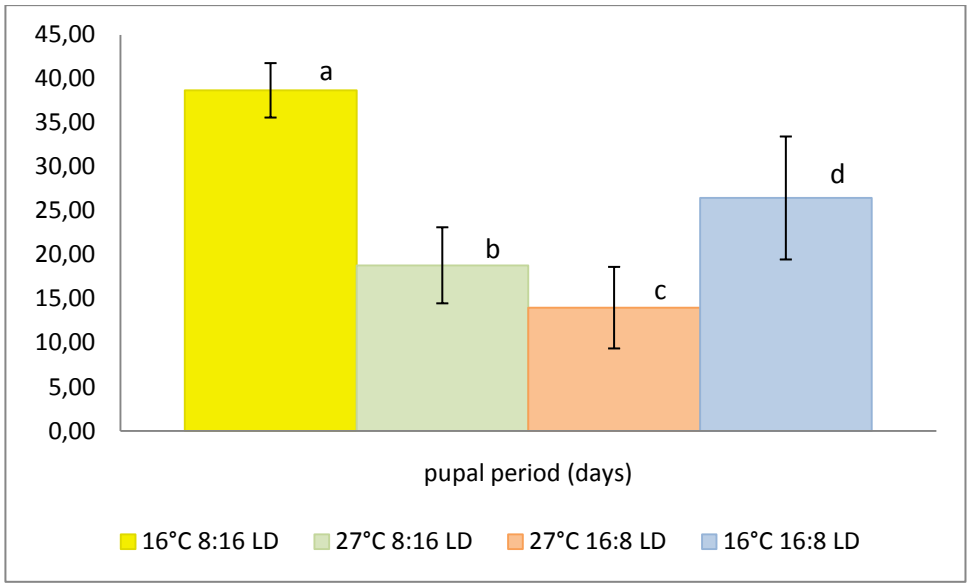

Fig. 11 Pupal period, mean time of occurrence (days) per treatment: different letters above each bar represent significant differences (Tukey's HSD multiple comparison test). (Error bars indicate SD).

The period of pupal occurrence exhibits the longer duration (38,7 days on average) and the less variability (±3,1 days) at the temperature of 16°C, photoperiod 8:16 LD. The combination of T 16°C and long photoperiod (16:8 LD) displays the greater variability (26,5±7 days) (Fig. 11)

Testing the difference between each pair of means (pairwise multiple comparisons, HSD-Tukey), the treatment 16°C, 8:16 LD proves to be statistically highly different ($p<0.001$). Likewise, highly significant difference ($p<0.001$, HSD-Tukey) exist between the temperatures of 27°C and 16°C at the photoperiod 16:8. The means relative to 27°C, 8:16 LD and 16°C 16:8 are statistically significant ($p<0.05$, HSD-Tukey). Evaluating the temperature of 27°C, the difference between photoperiods (8:16 and 16:8) is significant at $p=0.05$.

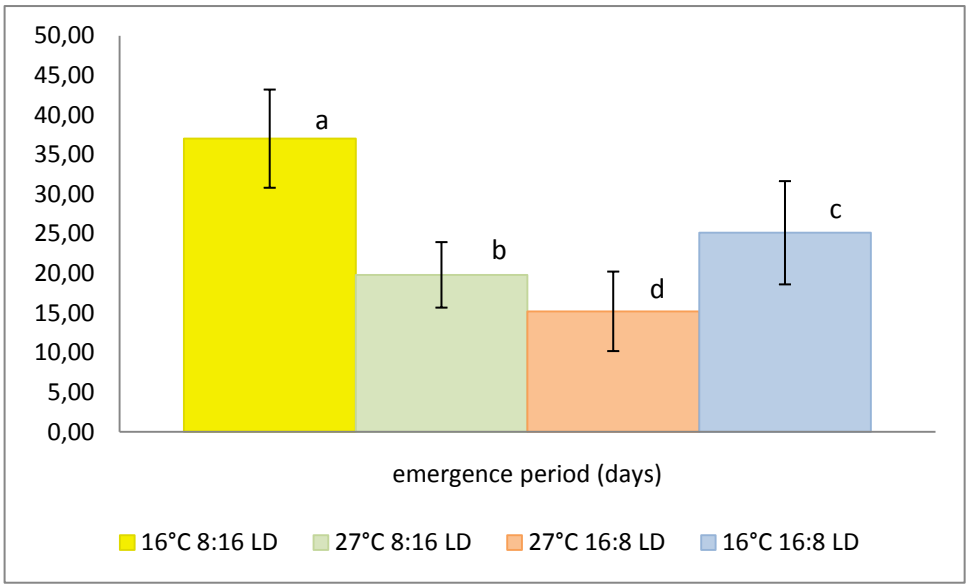

Fig. 12 Eclosion period, mean time of occurrence (days) per treatment: different letters above each bar represent significant differences (Tukey's HSD multiple comparison test). (Error bars indicate SD).

As expected, similarly to the pupation responses, the mean duration of emergences reaches the maximum values at the lower temperature of 16°C, both with short photoperiod 8:16 LD (37±6,2 days) and long photoperiod 16:8 LD (25,1±6,5 days). The treatment 16°C, 8:16 LD shows highly significant differences ($p<0.001$, HSD-Tukey) compared to the other means. Likewise, highly significant difference exists between 27°C and 16°C of temperature at the photoperiod 16:8 LD ($p<0.001$, HSD-Tukey). The difference between treatment 27°C, 8:16 and 16°C, 16:8 is significant at $p=0.067$.

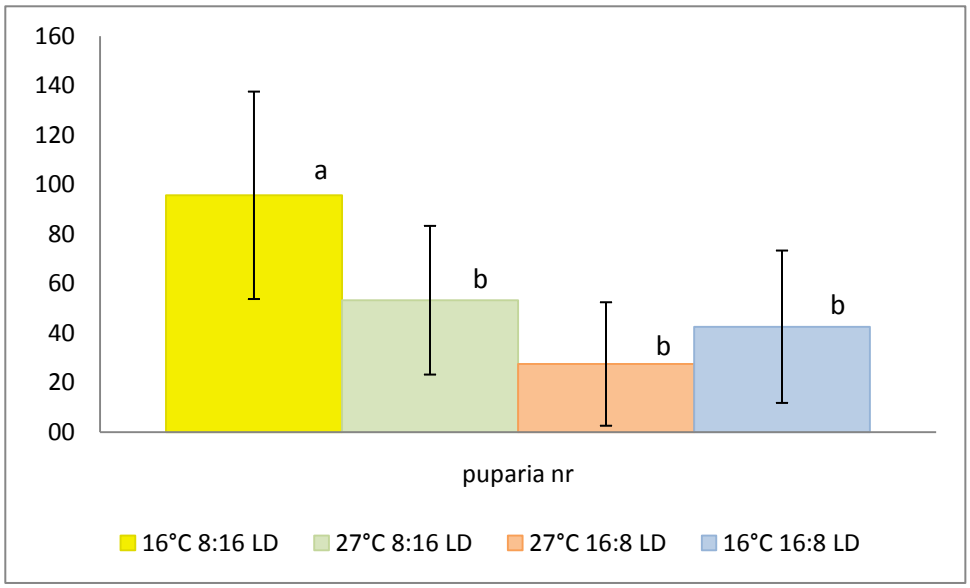

Fig. 13 Mean numbers of pupae, different letters above each bar represent significant differences (Tukey HSD). (Error bars indicate SD).

Number of puparia is largely affected by great variability, the maximum detected values are 95,6±41,9 days and 53,3±30 days for treatments 16°C, 8:16 LD and 27°C, 8:16 LD, respectively (Fig.). The combination 16°C, 8:16 LD shows highly significant differences ($p\leq0.001$, HSD-Tukey) compared to the means of 27°C, 16:8 LD and 16°C, 16:8 LD. There is statistical difference at $p<005$ between the temperature of 16°C and 27°C, photoperiod 8:16 LD. The other means are not statistically significant from each other.

The amount of pupae collected from the culture express the effect of temperature and photoperiod on both adult reproductive potential and larval survival, *ceteris paribus*. The expected influence of temperature is once more quite evident, in addition it's possible to observe a certain effect of the photoperiod, clearly noticeable at the temperature of 16°C.

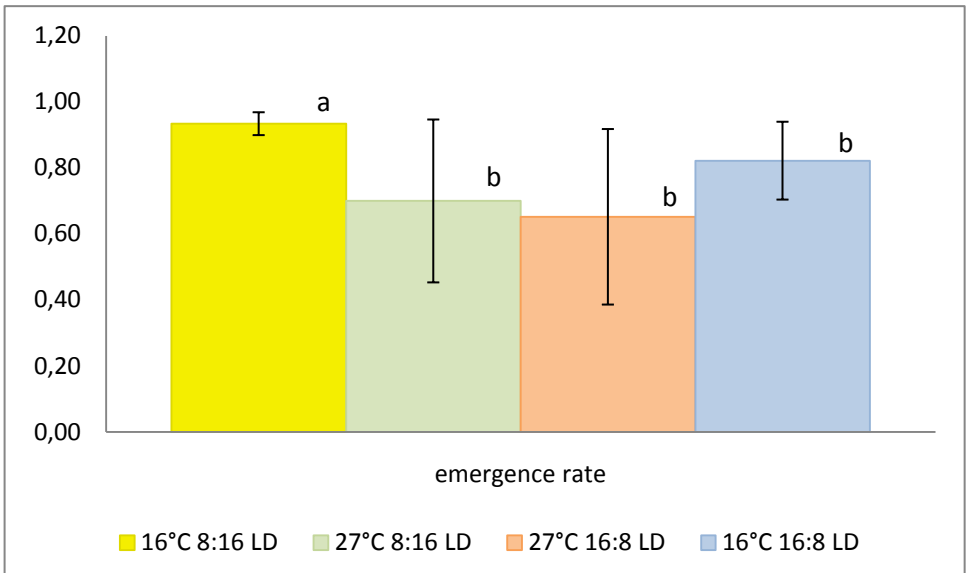

Fig. 14 Rate of emergence, different letters represent significant differences (Thamane-T2 test). (Error bars indicate SD).

Evaluation of eclosion rates shows significant differences ($p<0.05$, Thamane-T2) among the treatment 16°C, 8:16 LD and the other means.

Pupae vitality mostly undergoes the influence of temperature: it could be noticed the marked increase of the variability along with the temperature. The emergence ratio ranges from 0,65±0.27 at 27°C, 16:8 LD, to 0,93±0.03 at 16°C, 8:16 LD (Fig. 14).

Besides, the influence of temperature and photoperiod appears to determine the site of pupation (into or outside the fruits): a few of the newly adults emerged directly from fruits at high values of temperature and long-day photoperiod, although some occurrences were noticed at short-day photoperiod.

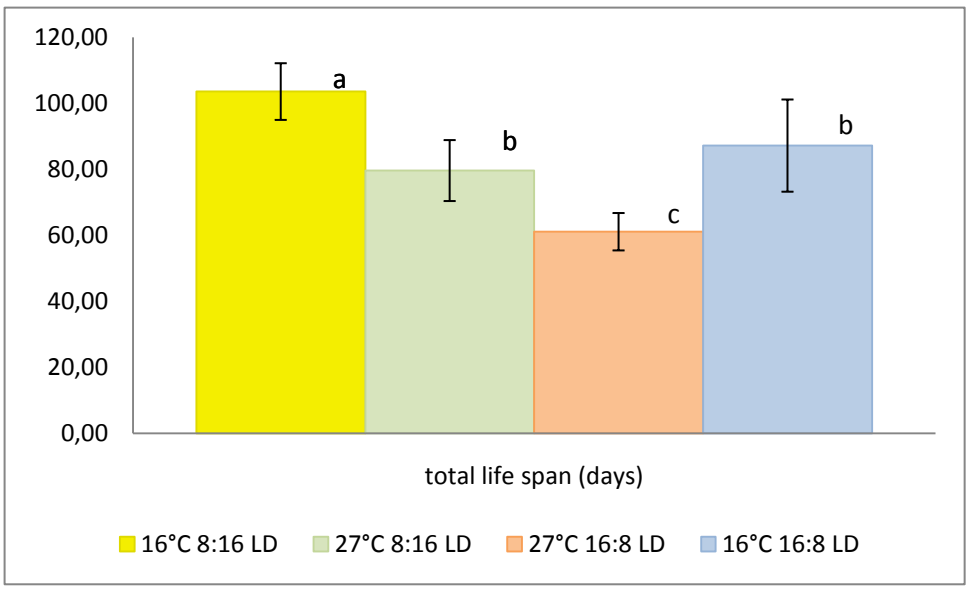

Fig. 15 Life span, from oviposition to adult death: different letters above each bar represent significant differences (Tukey's HSD multiple comparison test). (Error bars indicate SD).

Across all treatments, the higher values of the total life span could be detected at 16°C, 8:16 LD and 16°C, 16:8 LD (103,5±8,6 days and 87,2±14 days, respectively). At the temperature of 27°C, the life span reached the minimum values of 80±9.2 days (27°C, 8:16 LD) and 61.1±6 (27°C, 16:8 LD) (Fig. 15).

Hence, the life extent was primary a negative function of tested temperatures, ranging from 61 days at 27°C to 103 days at 16°C. The photoperiod influence can be noticed as well, with highly significant differences of means ($p<0.001$, HSD-Tukey) between the long and the short day, both at 16°C and 27°C of temperature.

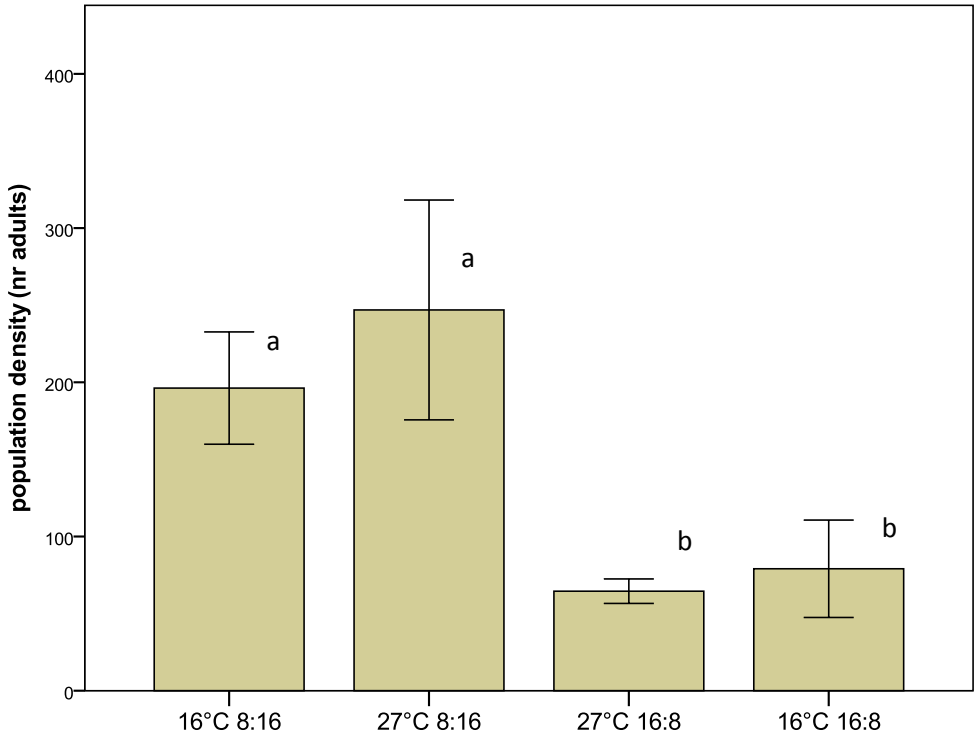

Fig. 16 Population density, mean number of adults per each treatment (ANOVA highly significance of mean differences, $p<0.001$). (Error bars indicate SD). Different letters above each bar represent significant differences (Thamane-T2 test).

The mean number of adults in ovideponing populations was greater at the 27°C, 8:16 LD (247±71.3 flies) and 16°C, 8:16 LD (191,2±39.3), and reached the minimum values at 16°C, 8:16 LD (79.1±31.6 adults) and 27°C, 16:8 (64,5±8) (2 replicates). The phenomenon variability, to some extent expression of the population unsteadiness, clearly increase at the higher density, mainly because of the overcrowding respect to both the volume of rearing cages and the (constant) amount of healthy fruits available for oviposition.

1.5. Discussion and conclusions

Data mostly confirm the marked influence of the temperature, as principal factor determining development rates of the immature stages. As expected, the mean time of occurrence of pupation and emergence reaches the maximum values at the lower temperature of 16°C. Besides, a certain effect of the photoperiod can be noticed, especially at the lower temperatures, resulting in better performances of the specie at the short-day photoperiod 8:16.

The length of the pupal period is directly correlated to the larval stage: both undergo predominantly the influence of the temperature. At the high temperature of 27°C, as expected, data report the shortening of the pupal stage, which must be linked to a more rapid development of larvae. Nevertheless, duration of the larval stage can be affected also by the host quality. Neuenschwander and Michelakis (1979) noticed faster larval developments attributed to increasing nutritional quality as olive fruits ripened. On the other hand, the reduction of the larval stage might be caused by the high mortality affecting larvae on poor quality host: only rapid developing larvae survive.

The rearing technique developed assures the year-round supplying of fresh, ripened olive fruits, hence increases in the speed of larval development could not be attributed to differences of host quality. Though, at 27°C the suitability of fruits to safely host larvae and pupae decrease more rapidly than at 16°C, because of the quicker shrinking and drying of fruits at high temperatures. Hence, the actual differences in development times could be partially enhanced by the rapid ripening and shrivelling of fruits.

The amount of pupae collected from the culture express the effect of temperatures, and to some extent the effect of the photoperiod on both adults reproductive potential and larval survival, *ceteris paribus*. Temperature mostly affects pupae vitality, with a clear increase of eclosion rate variability. The influence of temperature and photoperiod together appears to determine the site of pupation (into or outside the fruits): a few of the newly adults emerged directly from fruits at high values of temperature and long-day photoperiod, although some occurrences were registered at short-day photoperiod. It's not clear, however, if the preference for the pupation site directly undergoes the rates of temperature and/or photoperiod, or if there could be indirect influences, for instance on the suitability of the fruits to safely host larvae and pupae (shrinking of drying fruits).

In nature the females of the olive fruit fly deposit only one egg in a host. Under rearing conditions or in the case of a very high infestation rate, the average number of larvae feeding in a fruit may be greater than one. Nevertheless, the average number of larvae per fruit was always lower values above which the deleterious effects of competition appear.

If larval competition was always very low, overcrowding of adults occurred in some cases at the higher temperatures, leading to scramble aggressiveness particularly between females that become too numerous respect to the volume of rearing cages and to the number of healthy fruits available to oviposition. At this regard, it's worth considering that fruits quality (size, ripening and shrivelling) affects the performances of females and the survival of larvae more than short supplying of fruits.

The adults life extent was primary a negative function of tested temperatures, ranging from 61 days at 27°C to 103 days at 16°C. The photoperiod influence can be noticed as well, with highly significant differences of means between the long- and the short-day, both at 16°C and 27°C of temperature. The higher level of temperature tested (27°C), although not directly

influencing the flies, appeared to be excessive both for fruits and diets storing, thus ultimately affecting the specie performances.

On balance, the optimal combination of climatic parameters to make effective the rearing method was at 16°C of temperature and photoperiod 8:16 LD, that led to the better performances of the biological traits evaluated. Nonetheless, to accelerate slightly the development time could be profitable for a more comfortable management of the culture, particularly in case of undersupplying of cages and feeders. Adopting a temperature level roughly of 20°C could be a reasonable conciliation between productive and management needing.

The rearing techniques developed appears to be both practical and cost-effective, for the first time enabling the continuous laboratory rearing of *B. oleae* on its natural host, without interruptions thanks to the year-round availability of olive fruits.

Several attempts have been done in the past in order to rear olive fly *ex-situ* with the aim of studying different bioecological and behavioural aspects.

Most of the studies carried out so far referred to only one generation (Economopoulos *et al*., 1976; Remund *et al.*, 1977; Pucci *et al.*, 1982; Tzanakakis & Koveos, 1986; Koveos & Tzanakakis, 1990; Raspi *et al.*, 1997). The main issue of developing *B. oleae* rearing protocol on natural host is the storage method for olive fruits. The olive fly larvae do not develop on fruit before the pit hardening stage, and picked fruits tend easily to rot and in any case olive fruits cannot be held in storage without affecting their suitability for oviposition and larval feeding and development. Using fresh fruits from different olive cultivars with varying seasonal periods of ripening doesn't provide olive fruits of an acceptable quality for more than some months of the year. Besides, it becomes a too much expensive method of fruits collection (Sime *et al.*, 2007).

Other investigations on multiple generations relied on artificial diets (Tsitsipis, 1977). The quality of lab-produced olive fruit flies, both for use as insectaria breeders and for field releases, is of great importance. When olive flies are reared on artificial diet, adverse effect on performance and fitness occur if compared to wild individuals (Tsakas & Zouros, 1980; Loukas, 1985; Kostantopoulou *et al*., 1986). To overcome the quality problems of lab-produced olive flies, the renewal of the stock flies at various intervals has been practiced by many laboratories, but the genetic tradeoff of lab-populations could intervene in a few generations making the renewal of limited value.

To perform the effective permanence of a *B. oleae* population over time, the technique developed essentially bases upon the year-round availability of fresh olive fruits, suitable to oviposit. Supplying permanently fresh fruits to the adult population makes possible to start a potential indefinite number of different life cycles in sequence.

Furthermore, the rearing technique developed in this study allows to eventually and profitably replenishing the culture with additional wild flies, simply collecting naturally infested fruits in orchards and introducing them, after careful inspection, into the rearing progression. This approach could make easier to prevent or escape the population genetic tradeoff. After standardized, it appears to be very promising for optimizing further studies, *i.e.* physiological, biological and behavioural studies, parasitoids rearing and release.

In conclusion, this study establishes basic guidelines for rearing *B. oleae* on its natural host, for the first time obtaining year-round olive fly generations. Both longstanding control programmes and current research efforts emphasize the need for research towards

development of rearing protocols for olive fruit fly on its natural host. In this regard, this is the first, small-scale, practical and cost-effective method that succeeds in culturing year-round the olive fruit fly on its natural host in the laboratory, even when olive fresh fruits, suitable to oviposit, are not naturally available in the field.

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2 Study on volatile organic compounds (VOCs) emitted by *Olea europaea* L. as potential host allelochemics.

2.1 Introduction

The biogenic volatile compounds (bVOCs) can mediate a wealth of interactions, being perceived at a distance from their emitter by a number of other organisms of the same or different species. Identity, quantity and ratios of such chemicals convey specific information to the receivers about the identity and physiological state of the releasing organism and are influenced by abiotic and biotic factors such as nutrient availability, light intensity or pathogen infection (Visser, 1986; Gouinguene & Turlings, 2002; Rostas *et al*., 2006).

The studies on plant volatile emission could provide useful information on plant interactions with other organisms, their physiological status and the level of stress they are experiencing. Early works identified isoprene and monoterpenes as the predominant chemical species emitted by vegetation, whereas other VOCs seem to play a minor role. Nevertheless, despite isoprene and monoterpenes are known to be the most prominent compounds, recent studies have identified more than 70 hydrocarbons, including isoprene, mono- and sesqui-terpenes and a substantial number of oxygenated organics, as being emitted from ornamental, agricultural and natural plant species (Winer *et al.*, 1992; Niinemets *et al.*, 2004).

Many VOCs are constitutively emitted by undamaged plants, but biotic factors and the interaction between biotic and abiotic factors can induce plants to emit much larger amounts of these compounds and may also trigger the *de novo* synthesis of several VOCs (Pare & Tumlinson, 1999; Hilker & Meiners, 2006; Heil &Ton, 2008; Dicke, 2009). In particular, VOCs emission consequently upon herbivoure attack may have a role in plant indirect defences, attracting natural enemies (induced synomones), as recorded for several, different tritrophic systems (Parè&Tumlinson 1999, Walling, 2000). Synomones, acting over long (volatile) or very short range (volatile or through contact) seem could be induced not only by feeding but also by oviposition (Meiners&Hilker, 2000; Hilker&Meiners, 2006; Colazza *et al.*, 2004).

Olea europaea L., the common olive, is considered to be a low emitting species: its terpenoids emissions are generally found to be zero or close to zero (Arey *et al.*, 1991; Winer *et al.*, 1992; Benjamin *et al.*, 1996; Kesselmeier and Staudt, 1999; Owen *et al.*, 2001; Karl *et al.*, 2008). A number of authors have already attempted the identification of volatile components deriving from various parts of the olive plant or dissolved in the oil, by means of distillations, solvent extractions or head-space samples from plant detached portions, mainly focusing on the compounds bioactivity against pathogens and entomo-pathogens. At this regard there is a confuse variety of roles attributed to the plant compounds (Girolami *et al.*, 1981; Scarpati *et al.*, 1993; Rotundo *et al.*, 2001; Campeol *et al.*, 2001). In this regard, the return of natural *B. oleae* populations to the olive groves after the first abundant summer rains it must certainly be due to volatile compounds emitted by the plants and active on the olive fly (Girolami et al, 1982; Scarpati *et al.*, 1993).

To date there is still inadequate knowledge of the biogenic emissions from living olive trees, nor sufficient investigations into the possible correlation of VOCs emissions with phenological growth stages as well as physiological status of the plant.

The present study reports on the original results of VOCs detection from whole, living olive trees (*Olea europaea* L.). For the first time, an early comparison of the volatile organic compounds emitted from plants in different phenological stages (vegetative and fruiting stage) was performed.

2.2 Biogenic Volatile Organic Compounds, an overview.

The biogenic Volatile Organic Compounds (bVOCs) are "*organic atmospheric trace gases other than carbon dioxide and monoxide*", including isoprenoids (isoprene and monoterpenes) as well as alkanes, alkenes, carbonyls, alcohols, esters, ethers and carboxylic acids (Kesselmeier and Staudt 1999).

Plants are known to release more than 30.000 different VOCs (Niinemets *et al.*, 2004), some compounds appear to be taxon-specific whereas others are common to several, different plant families. The most prominent compounds, therefore the most investigated ones, are isoprene and monoterpenes, followed by alcohols and carbonyls.

Isoprenoids and other VOCs can be quite reactive into the atmosphere, where they can take part in tropospheric ozone and particle formation chemistry, therefore their chemical lifetime could ranges between some minutes and hours.

The large number of different compounds emitted from vegetation, together with sampling and analysis problems, could make difficult to understand their biosynthesis, emission rates, regulation and biological functions.

From a physiological point of view, the most important biogenic VOCs can be categorized into three basic chemical classes according to their biosynthetic pathways: the fatty-acid derivatives (green leaf volatiles GLV and jasmonates) produced by the lipoxygenate pathway, the terpenoids deriving from the isoprenoid pathways (via mevalonate or deoxyxylulose), and aromatic compounds, such as methyl salicylate and indole, produced by the shikimic acid pathway (Niinemets *et al.*, 2004).

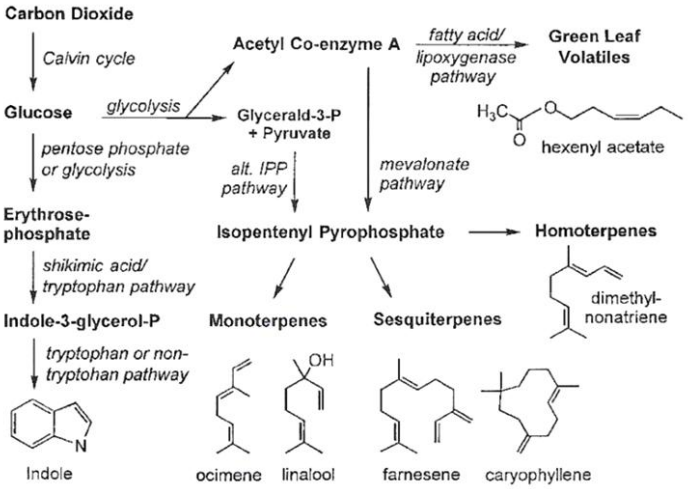

Fig. 17 Biosynthetic pathways leading to the release of plant volatiles (from Parè&Tumlinson, 1999).

An overview of the main volatile biogenic compounds, grouped into classic chemical classes, is detailed hereafter.

2.1.1 Terpenoids or isoprenoids

Terpenoids are made of a carbon skeleton composed of C_5 units of isoprene (C_{10} monoterpenes, C_{15} sesquiterpenes, C_{20} diterpenes, C_{30} triterpenes, and so on).

They are strong smelling and hardly water-soluble, could be detected in plants as well as in animals and micro-organisms. The group of monoterpenes comprises hydrocarbons with acyclic, mono-, bi- and tricyclic structures: myrcene, ocimene, pinene, sabinene, terpinene, limonene, and so on. (Fig. 18), with or without the inclusion of oxygen (monoterpenoids like menthol, camphor, linalool, geraniol).

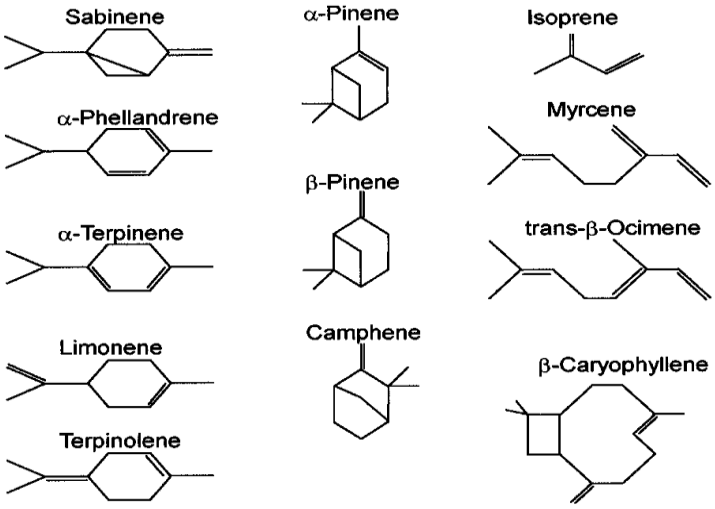

Fig. 18 Molecular structures of the volatile hydrocarbons isoprene (C_5), some monoterpenes (C_{10}) and the semivolatile sesquiterpene caryophyllene (C_{15}). (from Kesselmeier&Sstaudt, 1999).

Monoterpenes constitute the main fraction of terpenic (essential) oil produced and stored in plant secretory organs (trichomes, resine ducts); usually, monoterpenes-storing plants are monoterpenes emitters, but in contrast there are species, like some oaks, that emit high amount of monoterpenes, not storing them. Stored monoterpenes and other bVOC are released depending on the temperature, and this emission behaviour is usually referred to as "pool emissions". Instead, the monoterpenes emissions that exhibit a strong dependence on light is termed "newly synthesized emissions" (Karl *et al.*, 2008).

Isoprene is never stored but immediately released and volatilized, therefore the rate of isoprene emission equals the biosynthetic rate and its volatilization depends on both temperature and light . It's emitted from many species, from woody plants species rather than herbaceous ones, sometimes without regards to the taxonomical correlations (e.g. oak species).

Both isoprene and monoterpenes are produced by the mevalonic acid pathway (Fig. 17).

Physiologically, isoprenoids synthesis by plants is ruled by several controls within the plastids, whereas sesquiterpenes are synthesized within the cytosol. All isoprenoids are synthesized as an early step of the mevalonic acid pathway in mature chloroplasts, which converts two molecules of acetyl CoA into the C_5 precursor "active isoprene" IPP and its isomer DMAPP (isopentenyl pyrophosphate IPP and dimethylallyl pyrophosphate DMAPP) by a chloroplastic isoprene synthase enzyme. By adding another IPP unit to DMAPP (head-to-tail

addition), the monoterpene precursor geranylpyrophosphate GPP is formed, that is the starting unit for other monoterpenes through activity of different monoterpene cyclases; further additions of IPP units produce sesquiterpene and diterpene species. Different biochemical pathways could lead to the isoprenoid precursor IPP, requiring energy (ATP, NADPH and NADH) and a carbon substrate like pyruvate, glyceraldehydes 3-phosphate or acetate.

2.1.2 Alkanes and alkenes.

Trees, crops, grasslands are C_2-C_4 alkanes biogenic sources, but a sufficient understanding of the plant physiological production is available for the synthesis of ethene only. The emission of ethene appears to be stimulated after injury or stress like arid conditions, extreme temperatures or air pollution. However, presumably all plant tissues produce ethene under normal conditions (the synthesis proceeds from the amino acid methionine).

Tab. 2 Biogenic alkanes and alkenes (from Kesselmeier&Staudt, 1999)

ethane	ethene
n-pentane	propene
hexane	1-butene

2.1.3 Organic acids

In the gas phases, the more important organic acids are formic and acetic acids. Indirectly, vegetation contributes to the organic acids tropospheric budget by emitting isoprene and other NMHC (non methane hydrocarbons), which can be oxidized to deliver formic and pyruvic acids, or by oxidation of formaldehyde and acetaldehyde in the liquid phase.

There is also growing evidence that formic and acetic acids are directly emitted by vegetation: trees are generally regarded as direct emitters of both organic acids, and crops were found to act as a significant sink (Kesselmeier and Staudt, 1999). There are several metabolic pathways involving formic and acetic acids, particularly acetic acid and its activated form acetyl-CoA (the main product after decomposition of carbohydrates and fats) (Fig. 19). Acetate units serve for the synthesis of fatty acids, carotenoids, terpenes and so on. Additionally, plants use free acetate for many anabolic pathways.

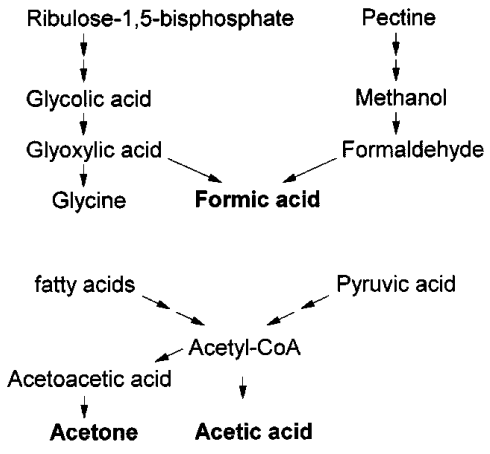

Fig. 19 Pathways for formic acid and acetic acid production, enclosing also methanol and acetone production (from Kesselmeier and Staudt, 1999).

2.1.4 Carbonyl compounds

Biogenic carbonyls (directly or indirectly released from vegetation) are listed hereafter (Tab. 3). Their contribute to tropospheric ozone is significant.

Tab. 3 Biogenic carbonyls (from Kesselmeier and Staudt, 1999 and Arey *et al.*, 1991).

- formaldehyde	- pentanone-2	- 1-phenyl-ethanone
- acetaldehyde	- 2-methyl-2-propenal	- octanone-3
- propanal	- 3-methyl butanal	- nonanal
- acetone	- 2-methyl-2-pentenal	- decanal
- butanal	- hexanal	- benzaldehyde
- i-butanal	- (e)-2-hexenal	- 6,6-dimethyl-bicyclo[3.1.1]-heptane-2-carboxaldehyde
- butenal	- (z)-3-hexenal	- methyl-isopropyl-ketone
- i-butenal	- heptaldehyde	- methyl-vinyl-ketone
- butanone-2	- pentanal	- 2-methyl-6-methylene-1,7-octadien-3-one
- butanone	- 2-pentanone	- 6,6-dimethyl-2-methylenebicyclo[3.1.1]heptan-3-one
- 2,3-butanedione	- 4-methyl-2-pentanone	
- crotonaldehyde	- 2-heptanone	- verbenone

The main biogenic precursors are secondary reactions of biogenic hydrocarbons with radical OH, NO_x and ozone as well as in photolysis, e.g. ozonolysis at vegetation surfaces is a source of acetone and other carbonyl compounds. Some reactive terpenes and isoprene may produce high aldehyde concentrations. (Z)-3-hexene-1-ol and (Z)-3-hexenylacetate, that are emitted by a large number of plants, could be transformed to aldehydes (i.e. propanal) or to formaldehyde and acetaldehyde (in presence of OH radicals and NO_x). Also plant emissions of methanol could contribute to the formaldehyde budget (Kesselmeier and Staudt, 1999). However, plants appear to emit directly a large spectrum of aldehydes, producing a high number of aldehydes, for instance long-chain compounds present in the cuticular layer of leaves and fruits, or low-molecular compounds like hexanal, cis-3-hexenal, trans-2-hexenal called “leaf aldehydes” (Green Leaf Volatiles, GLVs) because responsible for the characteristic fragrance of fresh leaves. A stress-induced release of acetaldehydes is also known.

2.1.5 Alcohols, esters and ethers

Even if probably of minor importance when compared to the terpenoids, alcohols are emitted in great number from vegetation, representing the major part of emission spectra of several plant species.

(Z)-3-hexene-1-ol and (Z)-3-hexenylacetate are named leaf alcohol and leaf ester, respectively. Initially assigned to be essential parts of flower odour, leaf alcohols and esters could range from few percent of VOC emission up to 50-100% for some species (i.e. almond, apricot, nectarine, peach, plum). Some of the alcohol species of biogenic origin identified so far are listed, together with the partially oxygenated monoterpenes linalool, isopulegol, borneol, menthol and p-cymene-8-ol:

Tab. 4 Alcohol species, from Kesselmeier and Staudt (1999), Arey *et al.* (1991).

- methanol	- 2-methyl-3-butene-2-ol	- 2-pentene-1-ol
- ethanol	- 3-methyl-1-butanol	- 1-hexanol
- propanol	- 3-methyl-2-butene-1-ol	- (Z)-3-hexene-1-ol
- 2-methyl-1-propanol	- 3-methyl-3-buten-1-ol	- 1-octanol
- 1-butanol	- 1-pentanol	- 1-octene-3-ol
- 2-butanol	- 3-pentanol	
- 2-methyl-2-butanol	- 1-pentene-3-ol	

Tab. 5 Partially oxygenated monoterpenes (from Kesselmeier and Staudt, 1999).

- linalool	- menthol	- p-cymene-8-ol
- isopulegol	- borneol	

Tab. 6 Esthers (from Kesselmeier and Staudt, 1999).

- ethylacetate	- methycapronolate	- ehylhexylacetate
- butylacetate	- hexylacetate	- methylsalicylate
- methylbutirate	- (Z)-3-hexenylacetate	- bornylacetate

Tab. 7 Ethers (from Kesselmeier and Staudt, 1999).

- p-dimethoxy-benzol	- p-menthylanisol	- 3-methyl-furane
- 1,8-cineol	- Furane	- ethyl-furane
- estragol	- 2-methyl-furane	- vinyl-furane

Methanol appears to have emission rates very similar to those of isoprene and monoterpenes in several plants.

The development stage appears to have significant influence on alcohols emission behaviour, e.g. linalool emissions by orange (Arey 1991, Seufert 1997).

2.1.6 Other VOCs

The issues in sampling and analysis techniques, that need to be focused on each group of chemical species, make the data set still insufficient to reliably describe the emission of VOCs other than isoprenoids.

Isoprene and monoterpenes are known to be the most prominent compounds, whereas other VOCs seem to play a minor role. Nevertheless, the available emission data for many OVOCs are still scarce and more data are needed. Despite early works identified isoprene and monoterpenes as the predominant chemical specie emitted by vegetation, recent studies have identified more than 70 hydrocarbons, including isoprene, mono- and sesqui-terpenes and a substantial number of oxygenated organics, as being emitted from ornamental, agricultural and natural plant species (Winer *et al.*, 1992). However, relatively few studies have directly measured the emission rates of hydrocarbons from vegetation.

The emission rates of some of the OVOCs appear comparable to those of the better investigated isoprenoids, so that new investigations are strongly encouraged.

2.2 Environmental factors and VOCs emission from vegetation

Factors influencing VOCs release from vegetation, i.e. light, temperature, development stage, stress, injuries, air pollution, have been poorly investigated so far.

Apart from the natural, genetical variability in volatiles emission among diverse plant species and vegetation types, the VOCs release exhibits important temporal and spatial variations resulting from complex interactions with the environment, only partially understood. Light, temperature, development stage, stress such as injuries, air pollution and other biotic or no biotic factors in the plant environment, are known to have a considerable influence on VOCs release.

The plant gas and water vapour exchanges are modulated via stomata, depending on various internal and external factors, so that the stomatal aperture is of basic interest to understand trace gas exchanges. Some gas species can diffuse through the hydrophobic cuticle (cuticular diffusion) or glands or hairs on the outside as well.

Trace gases could be distinguished into two groups: some gas species are stored after synthesis into special cells or organs and hence released from storage pools, while for other trace gases synthesis and release are directly connected.

The interrelation between isoprene and monoterpenes diffusion and the stomatal closure is still not clear: monoterpenes are potentially able to permeate through cuticles also, as can other gases such as CO_2, SO_2 and O_2 and evidence exists that monoterpene emissions from leaves are strongly influenced by cuticula hydratation (Kesselmeier and Staudt, 1999). Anyway, other trace gases appear to undergo different mechanisms affecting accumulation, concentration gradients and release, than isoprenoids.

The short-term effects (=the emission response occurs within minutes up to one hour) of light and temperature are the best-described environmental factors influencing plant emissions, and the emission behaviour of monoterpenes and isoprene is the best investigated so far. All studies on the emission of isoprene and monoterpenes show clear temperature dependence (Kesselmeier and Staudt, 1999).

Isoprene emissions appear to be triggered by light, being biochemically linked with photosynthetic synthesis and, because the lack of storage pool, isoprene synthesis and release will cease within minutes under dark conditions (consequently CAM species could have light-independent isoprene emissions). The emission response to radiation shows saturation behaviour, similarly to the light saturation of CO_2 assimilation; the temperature response instead, leads to a maximum and to a subsequent decrease at higher temperatures due to enzyme inactivation. Mathematically it is possible to describe the interrelation between temperature and light with the isoprenoids emission using empirically designed algorithms, that take into account empirical factors derived from measurements on emitting plant species (Fig. 20) The values assigned to the empirical parameters determine the shape of each response curve: slopes, inflections, saturation and optimum levels of the functions. It's worth to point out that the so-called parameters differ among plant species and can even vary among shade- and sun-leaves within the canopy of an individual tree. Moreover, the temperature response appears to be influenced by the rate of increase itself.

Monoterpenes, being stored after synthesis in storage pools that are generally large compared to the emission rates, appear to be released quite independently of light. In many

plant species the monoterpenes emission is a blend of stored and neo-synthesized compounds, so that a part of their releases undergo light influence as well. Investigations on *Quercus spp.* give evidence that emission of high amounts of monoterpenes must not be strictly referred just to storing-plant species (Kesselmeier and Staudt, 1999). However monoterpenes emission is basically considered a volatilization out of storage organs, dependents mainly on temperature, vapour pressure and transport resistance. Hence their emission behaviour will show an exponential increase with temperature (Fig. 20).

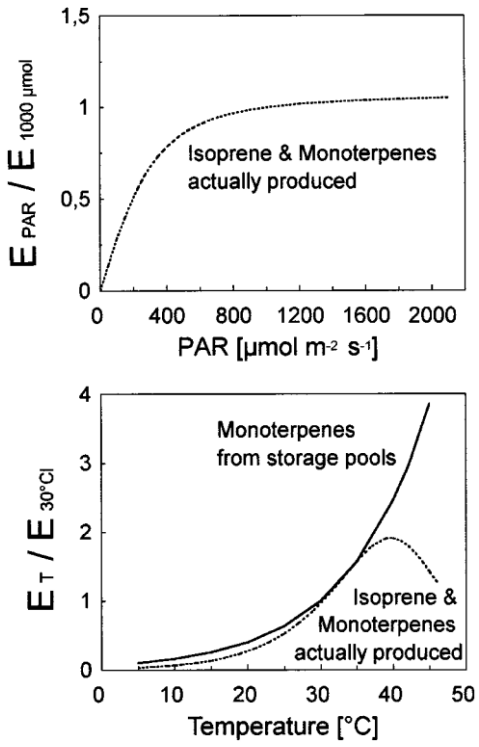

Fig. 20 Emission of isoprene and monoterpenes in relation to light (PAR) under constant temperature, and temperature under constant light. Note the principal drop of production due to enzyme inhibition under high temperatures in the case of actually produced compounds.

The short-term effects on VOCs emission of other factors apart from light and temperature have been less investigated. Obviously, any environmental factors in the plant habitat could potentially affect VOC emission, both in the short- and in the long-term.

Relative air humidity seems to affect the releases of monoterpenes and isoprene, triggering their emission probably through the hydratation of the cuticula.

Mechanical stress, injury and biotic disturbance (herbivore and pathogen attacks) can have multiple short- and long-term effects on VOCs emission. Damaged leaves commonly emit C_6 aldehydes, alcohols and their derivatives; after damages and mechanical stress, plants usually exhibit emission bursts of monoterpenes (that could easily leads to errors when studying the emission by short-term enclosure systems). Instead, isoprene emissions appear to be unaffected by plant damages.

The long-term effects (=over hours, days, months and even years) of environmental factors are more difficult to detect and to study in relation to specific causes and biochemical pathways.

Nonbiotic environmental factors long-term affecting the isoprenoids emissions could be generally indicated as “growth conditions” (e.g. canopy position and season). When plants growth is constrained by limited nutrient and water availability (like many Mediterranean ecosystems) the volatiles composition varies: the total amounts could be reduced, but the metabolic stress could provoke additional production or higher losses of particular volatiles, as it is known happening for ethylene, hexenal, 2-hexenal, hexanol, hexenol, hexyl-acetate, 2-hexenyl-acetate (GLVs), all compounds typically related to fatty acids breakdown. With regards to isoprene and monoterpenes, moderate drought conditions, simulated on young potted plants, seem to unaffect terpenes emission during CO_2 and H_2O gas exchange declines, like severe water stress do.

It’s worth remember that also the plant photomorphogenesis could determine significant differences in emissions quality and quantity, among sun and shade leaves within the same canopy.

Frost, heat, nutrient deficiencies, wounding, air pollution have all to be regarded as potential triggers for physiological changes as well as trace gas emission. Must be considered that plant seasonal development, water availability, light and temperature regime may have a significant influence on plant emissions, apart from the ecotype as a whole.

2.3 Detection

Nowadays the sampling techniques for biological VOCs are mainly solvent extraction (SE), steam distillation (SD), dynamic headspace (DH, also called dynamic purge&trap PT) and solid phase microextraction (SPME). Solvent extraction and steam distillation are classical and conventional method, requiring long extraction times, large amount of solvents and multiple steps. Moreover, solvent extractions are liable to ISOLATE non-volatile material from tissues, while steam distillation CAUSE heat-produced rearrangements and loss of unstable volatiles: in both cases artefacts are produced. Nevertheless, being simple procedures, they are still extensively applied (i.e. fragance-aroma characterization). DH and SPME have aroused much attention being the advanced and environment-friendly sampling techniques for biological VOCs.

Headspace method, in a broad sense the sampling of volatiles in the gaseous phase around fresh or living material, gives a true and natural picture of the emission composition, therefore we have concentrated on this method. Headspace techniques, through adsorption or cold trapping, are accumulating methods whereby volatile substances can be enriched in time, as they’re produced and emitted by the plants. Head-space sampling is a non-destructive technique, which can be used on living plants either in the laboratory or in the field, and samples can be repeated in time on the same individual.

Routinely, dynamic headspace analysis is employed, although static headspace analysis is sometimes used. In DH, the sample is confined in an entrainment chamber and an ultra-purified inert carrier gas (usually purified air) is passed over the sample. The volatile chemicals released by the sample are carried by the gas to a solid trap, usually a porous organic polymer or activated charcoal, where the analytes are adsorbed and preconcentrated. There are several sorbent traps, fitting the different properties of the chemical compounds, so that DH can achieve high selectivity and sensitivity. Properly prolonging sampling time could also improve the enrichment effect (Jakobsen, 1997).

The desorption of analytes from the solid trap for gas chromatography can be achieved

by either elution with a solvent (solvent desorption) or rapid heat treatment (thermal desorption). To desorb and identificate trapped volatiles, headspace sampling is usually coupled with gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS) or gas chromatography-flame ionization detector (GC-FID), that are excellent, sensitive detection techniques for analysis of biological VOCs.

In static headspace analysis instead, the sample is tightly closed into a vessel, where it comes into equilibrium with its vapours and volatiles can be trapped using a syringe or a similar device and directly injected into the GC.

To quantify the release of a trace gas species in relation to temperature and light it's commonly used the emission factor *Es* (standard conditions of temperature and light: 30°C and 1000 μmol m^{-2} s^{-1} photosynthetic active radiation PAR), given in relation to leaf biomass (leaf dry weight LDW) and time. When not determined under standard conditions, emissions must be adjusted to consider the different light and temperature influences, by using universal algorithms; to reduce errors it's recommended to measure emissions strictly close to standard conditions. Often, measures under controlled conditions are performed in laboratories on young plotted plants, whereas measures taken on mature plants in the field are under non-environmental controlled conditions. Thus, there is an experimental uncertainty associated with emissions factors reported in the literature.

There is an obvious large variability of emissions among plant species; moreover an important intraspecific variability could be detected among different authors' investigations. The experimental conditions are usually the main cause of bias, but there are cases of significant intraspecific variability observed under similar conditions of study. Nevertheless it's always possible to detect the quantitatively most important components of the volatile emission among several numbers of compounds identified in different studies.

3 Biogenic VOCs from *Olea europaea* L., state of the art.

Olea europaea L., the common olive, is considered to be a low emitting species: its terpenoids emissions are generally found to be zero or close to zero (Arey *et al.*, 1991; Winer *et al.*, 1992; Benjamin *et al.*, 1996; Kesselmeier and Staudt, 1999; Owen *et al.*, 2001; Karl *et al.*, 2008).

Early works identified isoprene and various monoterpenes as the predominant chemical specie emitted by vegetation, therefore by olive specie as well. Available OVOCs emission data for the specie are scarce and there are only few studies or early screenings.

In Benjamin *et al.* (1996), referring to Winer *et al.* (1992), the emission rates of monoterpene for *O. europaea* were normalized to standard conditions (30°C of temperature and light intensity level of 1000 μmol m^{-2} s^{-1}), resulting in the emission standard *Es*= 0,3 µg $g(LDW)^{-1}h^{-1}$.

Kesselmeier and Staudt (1999) report a number of normalized emission rates (isoprene and monoterpenes) from different botanical species, compiling available data from several investigations that have already been performed. Part of this table it's reported hereafter (Tab. 7) to focus on the emission factors relative to *O. europaea*.

Tab. 8 Reported normalized emission rates: isoprene and monoterpene emissions. Emission factors (*Es*) are given in relation to leaf biomass and time and describe the release of a trace gas species under standard conditions of temperature (30 °C) and light (1000 μmol m^{-2} s^{-1} PAR). Data refers to Arey (1991) and Winer *et al.* (1992). From Kesselmeier&Staudt, 1999.

Oleaceae	Isoprene [μg $g(LDW)^{-1}$ h^{-1}]	Monoterpenes [μg $g(LDW)^{-1}$ h^{-1}]
Fraxinus caroliniana	-	-
Fraxinus uhdei	+	+
Ligustrum lucidum	+	+
Olea europaea	+	0.5
Olea europaea	0	0.1
Phillyrea angustifolia		0.47

Owen *et al.* (2001) screened the emissions of 40 Mediterranean plant species, just searching for isoprene and monoterpenes. Emission samples were taken from living plant in natural habitats, using a portable *dynamic branch enclosure system* in Teflon (Street 1995, Owen 1998). A total of 32 volatile compounds were detected, varying among plant species from 19 emitted compounds (*Quercus ilex* L.) to a single compound emission, usually of isoprene. The authors normalized the emission rates to generate emission factors for each plant species, using Guenther algorithms (Owen *et al.* 2001, Guenther. *et al.* 1995). The most frequently emitted compounds were α-pinene, limonene, sabinene, β-pinene, myrcene and carene. The species were then categorized, according to their main emitted compound, in isoprene, α-pinene, linalool or limonene emitters. For *Olea europaea* L. only a single emission of α-pinene was recorded, with the very low rate of ≤ 0,1 µg g^{-1} dw h^{-1}.

In the European plant-specific emission inventory of Karl *et al.* (2008), zero monoterpene emissions are attributed to olive specie and olive groves, referring to Arey *et al.* (1991) and to EMEP CORINAIR (1999) (Tab. 9). Due to the lack of data for OVOCs the authors use a default standard emission potential.

Tab. 9 Terpenoids standard emission of *Olea europaea* L. (from Karl *et al.* 2008). Emission factors (*Es*) are given in relation to leaf biomass and time under standard conditions (30 °C, 1000 μmol m^{-2} s^{-1} PAR). Data refers to Arey *et al.* (1991) and EMEP CORINAIR (1999).

Standard Emission Potentials ***Es*** **[$\mu g_{DW}{}^{-1}$ h^{-1}]**	Isoprene	Monoterpenes	Monoterp. pool	OVOC (standard *Es*)	Sesquiterpenes
Olea europaea L.	0	0	0.3	1.7	0.06
Olive groves	0	0	0.11	1.7	0.06

A number of authors have already attempted the identification of volatile components deriving from various parts of the olive plant or dissolved in the oil, by means of distillations, solvent extractions or head-space samples from plant detached portions, mainly focusing on the compounds bioactivity against pathogens and entomo-pathogens. At this regard there is a confuse variety of roles attributed to the plant compounds.

Nevertheless, to date there is still inadequate knowledge of the biogenic emissions from living olive trees, nor sufficient investigations into the possible correlation of plant emissions with phenological growth stages.

The VOCs emitted by various detached part of *O. europaea* (Itrana cv) were analyzed through head-space collection (HD) and GC-MS in Scarpati *et al.*, 1993. The authors analyzed leaves, unripe, half-ripe and ripe fruits and crushed fruits (Tab. 10).

Tab. 10 VOCs from olive leaves and fruits (HS collection and GC-MS), Scarpati *et al.*, 1993.

leaves	*unripe fruits*	*half-ripe fruits*	*ripe fruits*	*crushed fruits*
- α-pinene - myrcenone - toluene - o-xylene - nonanal - p-cymene - ethylbenzene - p-xylene	- limonene - n-octane - β-pinene - myrcenone	- α-pinene - toluene - limonene - p-cymene - β-pinene - p-xylene - o-xylene - myrcenone - nonanal	- n-octane - α-pinene - 1-octene - nonanal - limonene - o-xylene - β-pinene	- α-pinene - hexanal - (E)-2-hexenal - β-pinene - nonanal - myrcenone

The (E)-2-hexenal released by crushed fruits is known to be generated from lipoxydases on linolenic acid and, as authors confirm via bioassay, it acts as a strong oviposition deterrent and olfactory repellent, together with hexanal. Olfactory-attractive compounds were found to be ethylbenzene (from leaves), toluene and α-pinene (leaves and fruits), n-octane (fruits).

During oviposition test carried out by the Authors, the most active compound on the olive fruit fly *Bactrocera oleae* (Rossi) was found to be α-pinene that is the most abundant components of leaves emission, also released by fruits. On the whole, the authors suggest that the most active compounds stimuling the oviposition may be leaf volatiles more than fruit volatiles (Scarpati, 1993).

These results, consistent with Girolami *et al.* (1981) involve that host-plant odours play the fundamental ecological role of olfactory attractants and oviposition stimulants interacting with *B. oleae*.

In Rotundo *et al.* (2001) 36 compounds have been identified from 3 different cultivars of *O. europaea* in 2 phenological stages, through hydrodistillation of crushed fruits and gas chromatography-mass spectrometry (GC-MS). Only 4 compounds were found to be released from all the cultivars: hexanal, 2-esenale, nonanale, 2-decenale (Tab. 11).

Tab. 11 Volatile compounds identified from crushed fruits of *O. europaea* in different phonological stages (steam distillation and GC-MS), Rotundo *et al.* (2001).

• = common to all cv

- hexanal •	- 2-ottenale	- 2,4-decadienale	- ftalato
- 2,4-dimetil-eptano	- nonanale •	- 2-undecenale	- acido esandioico, diottil estere
- octane	- 1,2-dicloro-cicloesano	- 2-tridecenale	- pregnenolone
- 2-etenil-2-butenale	- 2-etil-4-pentenale	- copaene	- 2,4-bis-dimetilbenzil-fenolo
- 2-esenale •	- 1-nonene	- 2,6,6-trimetil-2-butenone	- docosano
- 3-metil-pentenale	- 1,4-dimetil-ciclottano	- α-farnesene	- esatriocontano
- 1-esanolo	- metal-ciclottene	- 2,4-difenil-4-metil-2-pentene	- squalene
- eptanale	- 2-decenale •	- 1-metil-1-feniletil-4-fenolo	- esacosano
- 2-eptenale	- 2,4-nonadienale	- isopropyl-miristato	- antracene

Campeol *et al.* (2001) report the first investigation on the emission biodiversity among cultivars of *Olea europaea* L. (frantoio, leccino and cipressino) analyzing the volatile fraction of fresh leaves through hydrodistillation, GC and GC-EIMS analyses. The main constituents of the volatile fraction were monoterpenes and sesquiterpenes; 10 out of 41 identified compounds were common to all cultivars (•), 17 out of 41 compounds were common to at least 2 cultivars (+) (Tab. 10).

Tab. 12 Volatile composition of *O. europaea* emissions (frantoio, leccino and cipressino cultivars) via distillation of fresh leaves (from Campeol *et al.*, 2001).

● = common to all cv; $^{+}$=common to 2 cv

- (E)-2-hexenal ●	- (E)-2-octenal	- n-decanal	- β-selinene
- (E)-2-hexen-1-ol	- 1-undecene	- β-cyclocitral	- (E,E)-α-farnesene ●
- n-heptanal	- o-hydroxycumene	- (E)-3-caren-2-ol	- liguloxide
- benzaldehyde ●	- p-cymenene	- (E)-2-decenal $^{+}$	- (E)-nerolidol
- 2-heptenal	- linalool	- diidroedulan ●	- (Z)-3-hexenyl benzoate
- 2,3-dehydro-1,8-cineole	- n-nonanal ●	- theaspirane ●	- caryophyllene oxide $^{+}$
- 2-pentylfuran ●	- 4-terpineol	- (E)-β-damascenone ●	- (E)-2-hexenyl benzoate
- n-octanal $^{+}$	- (Z)-3-hexenyl butyrate $^{+}$	- (E)-β-damascone ●	- kongol ●
- (E,E)-2,4-heptadienal	- α-terpineol	- β-caryophyllene ●	
- phenylacetaldehyde $^{+}$	- hexyl butyrate	- α-humulene	
	- (E)-2-hexenyl butyrate	- germacrene	

The authors state it is possible to distinguish among cultivars according to their different leaf volatile compositions.

4 Volatile Organic Compounds (VOCs) emitted by *Olea europaea* L., an innovative approach.

Plant development stages could strongly affects quality and quantity of the plant emissions: there are clear evidences that phenological events, such as budding, flowering and fruiting, induce typical variations of the VOC pool, indirectly related to the growing seasons (Kesselmeier and Staudt, 1999). From bud break to a fully developed leaf, plants change their habitat as well as their physiology.

Spherical glass chambers with sealed joints (inert and transparent to photosynthetically active radiation, height adjustable from 1 to 2m and volume V=100l), were specifically designed and manufactured to collect comfortably volatiles emission from the whole trees. All connections were made with glass-glass or steel-glass contact secured with Teflon (Fig. 21). This fully enclosure system allows for the first time the collection of bVOCs from whole, living olive trees, avoiding the uncertainties commonly noticed among branches of the same tree when using ordinary branch-portable enclosure systems. Moreover, sampling different, whole plants at one time, it helps to minimize uncertainties due to the natural variability between specimens of the same specie.

The large volume of the chamber and the air flowing through the system prevent sharp increase in temperature and humidity within the chambers, minimizing stresses on the plant as well as potential bruising or disturbance of the foliage.

The glass chambers and all connections were carefully washed with using isopropilic alcohol, then let dry before sampling. A system made of an air-pump and an activated charcoal-plus-gauze filter was used to purify and pump air into the chambers. Air flow rate measurements were taken using a portable digital flowmeters.

All entrainments were carried out in a semi-opened green-house to better simulate natural light and temperature conditions: the mean value of temperature during the sample was 28°C and light intensity level of 1000PAR.

Fig. 21 Glass chambers with sealed joints, h=1 to 2 m, V=100 l, all connections were made with glass-glass or steel-glass contact secured with Teflon.

Fig. 22 Sorbent tubes.

4.1 Preliminary entrainments of bVOCs from *Olea europaea* L. in different phenological growth stages.

4.1.1 Material and method.

An early comparison of the volatile organic compounds emitted from plants in different phenological stages (vegetative and fruiting stage) was performed.

The aerial parts of four potted olive trees (4 years old, Canino cv), two fruiting and two in vegetative stage, were placed into the chambers in pairs.

Air, purified with an activated charcoal-plus-gauze filter, was pumped into the chambers and ejected through standard sorbent tubes (SKC, Anasorb 747 626-83) at a constant flow rate of ~600 ml/min. The bVOCs entrainments were replicate three times per each phenological stage.

Traps were eluted with 2 ml of CS_2 (1-Br-decane as inner standard), the extracts were concentrated to 1 ml under stream of N_2 (for an inner standard concentration of 2 ppm). The extracts were then analysed via gascromatography (GC) and gascromatography coupled with mass spectometry (GC-MS).

GC analyses (1 µl of extract) was performed on a Varian CP-3800 gaschromatograph equipped with FID detector and an electronic pressure control (EPC) (split mode 1:10). Compound were separated on a VF-1 ms fused silica capillary column (30 m, 0.32 mm i.d., 0.25 µm ft) (Varian, Palo Alto, CA, USA). The injector and detector operated at 260°C and 280°C, respectively. The oven temperature was programmed as follows: from 60°C (1 min) to 260°C (2 min) at 5°C/min. The carrier gas was ultrapure helium. Calibration graphs for the compounds were constructed by measuring peak heights vs concentrations. Individual components were identified by injection of pure standard compounds and comparison with their retention times.

The compounds present in the volatile profile were tentatively identified using coupled GC-MS. The components separated under the above conditions were identified using the NIST 98.1 mass spectral library.

The compounds were quantified as percentage GC peak areas, using integration data, normalized so that the total peaks of interest equalled 100. The estimation of the percentage was obtained by calculating the ratio between their concentration values and the total concentration of detected VOCs of each plant.

4.1.2 Results

Despite the usual low emission rates of the specie, the variations of VOCs emissions between different phenological stages of *Olea europaea* L. were clearly displayed (Fig. 23).

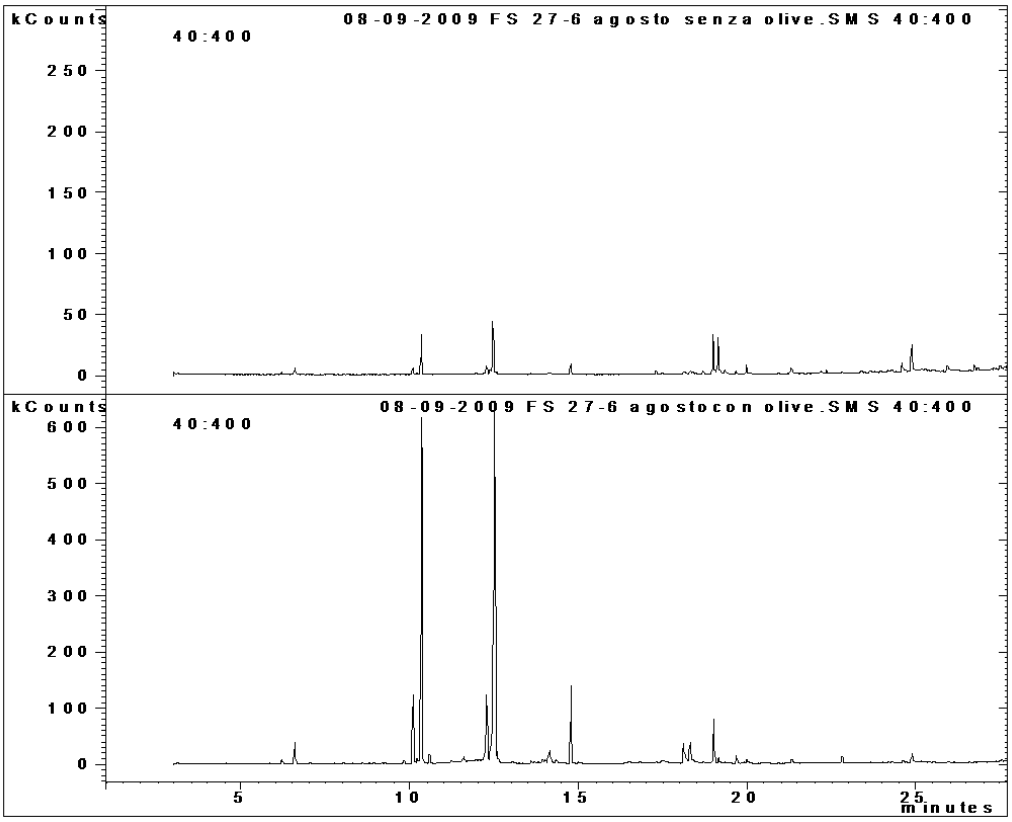

Fig. 23 Sample chromatogram: vegetative plants (on the top) vs fruiting plants; sample 27 July-06 Agust 2009.

The main compounds identified were terpenes and esters (Tab. 13):

- myrcene (7-methyl-3-methylene-1,6-octadiene)
- cosmene (2,6-dimethyl-1,3,5,7-octatetraene)
- allocimene (2,6-dimethyl-2,4,6-octatriene)
- p-1,3,8-menthatriene (1-isopropenyl-4-methyl-1,3-cyclohexadiene)
- 1,3,5,5-tetramethyl-1,3-cyclohexadiene
- β-cucubene
- verbenyl acetate (bicyclo[3.1.1]-hept-2-en-4 ol-2,6,6-trimethyl-acetate)
- p-mentha-2,8-dien-1-ol

It's fundamental, after obtaining the bVOC characteristics at different physiological status, to further clarify the difference of volatiles compositions at different metabolism phase: if there is a detectable quail/quantitative variation, and which are the volatiles typically characterizing the different stages.
Comparing the headspaces composition of fruiting and not fruiting trees it was clearly noticeable that the VOCs emissions were qualitatively similar, but the presence of fully grown fruits on plants remarkably increased the releases, as clearly noticeable for cosmene, p-1,3,8-menthatriene verbenyl acetate and (E)-p-mentha-2,8-dien-1-ol (Fig. 24).

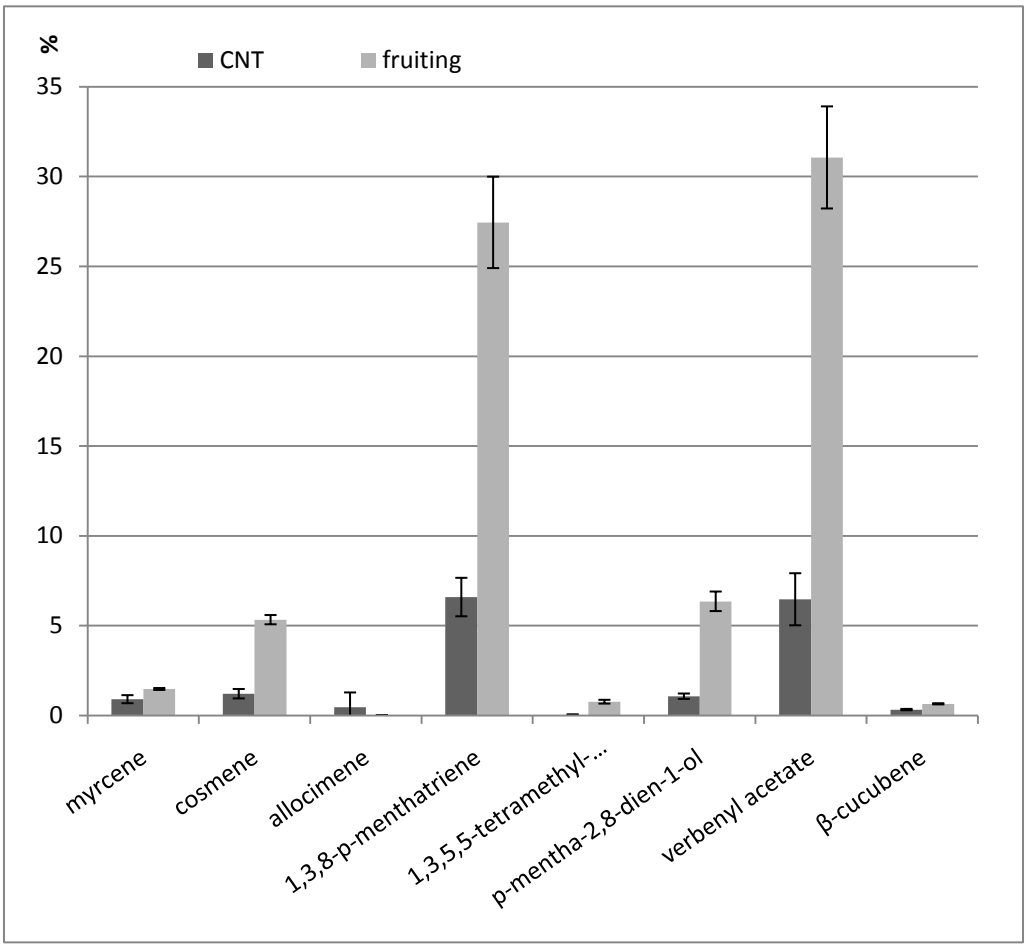

Fig. 24 Comparative diagram of headspaces composition of fruiting (in red) and not fruiting olive trees: the presence of fully grown fruits on plants increase the releases, as clearly noticeable for cosmene, p-1,3,8-menthatriene, verbenyl acetate and p-mentha-2,8-dien-1-ol. (Error bars indicate SD).

Tab. 13 Identified compounds and relative abundances (CNT = control; SMP = samples), significance of mean differences by paired t-test, * highly significant differences (P < 0.001), * significant differences (P < 0.05), ns not significant**

VOCs %/TOT	17-27 jul		27 jul-6 ago		6-17 ago		%/TOT		
	CNT	SMP	CNT	SMP	CNT	SMP	**CNT**		**SMP**
myrcene (7-methyl-3-methylene-1,6-octadiene)	1,106	1,451	0,934	1,424	0,662	1,527	0,901±0,224	*	1,467±0,053
cosmene (2,6-dimethyl-1,3,5,7-octatetraene)	1,507	5,557	1,075	5,049	1,032	5,374	1,205±0,263	***	5,327±0,257
allocimene (2,6-dimethyl-2,4,6-octatriene)	1,400	0,010	0,000	0,000	0,000	0,000	0,467±0,808	ns	0,003±0,006
1,3,8-p-menthatriene (1-isopropenyl-4-methyl-1,3-cyclohexadiene)	7,814	29,261	6,093	24,535	5,849	28,534	6,585±1,071	***	27,443±2,545
1,3,5,5-tetramethyl-1,3-cyclohexadiene	0,000	0,788	0,065	0,644	0,000	0,848	0,022±0,038	***	0,760±0,105
p-mentha-2,8-dien-1-ol	1,160	6,462	1,145	6,827	0,893	5,757	1,066±0,150	***	6,349±0,544
verbenyl acetate (bicyclo[3.1.1]-hept-2-en-4 ol-2,6,6-trimethyl-acetate)	8,112	34,059	5,395	30,707	5,879	28,409	6,462±1,449	***	31,058±2,841
β-cucubene	0,358	0,679	0,284	0,618	0,324	0,629	0,322±0,037	***	0,642±0,033

4.1.3 Discussion and Conclusions

Although there remains a lack of comprehensive knowledge concerning the exact function of biogenic VOCs, their distribution in plant tissues and their emission pathways, in many cases the fundamental role of monoterpenes in plant defence reactions has been well clarified. Some of the monoterpenes released from plants have an allelopathic function, for instance against seeds germination and growth of other species nearby; other monoterpenes act directly as defence compounds against pathogens and herbivores: β-pinene might limit fungal and microbial growth within the leaf tissues flooding the intercellular space to inhibit the respiration.

Many VOCs are constitutively emitted by undamaged plants, but biotic factors and the interaction between biotic and abiotic factors can induce plants to emit much larger amounts of these compounds and may also trigger the de novo synthesis of several VOCs (Parè&Tumlinson, 1999). VOCs emission consequently to phytophagous attack may have a role in plant indirect defences, attracting natural enemies of phytophagous (induced synomones) (Walling, 2000), as recorded for more than 15 different tritrophic systems.

The return of natural *B. oleae* populations to the olive groves after the first abundant summer rains it must certainly be due to volatile compounds emitted by the plants and active on the olive fly (Girolami et al, 1982; Scarpati *et al.*, 1993; Scarpati *et al.*, 1996). In a few days after the adults return, ovipositions follow: the reproductive behaviour seems to be regulated by chemical stimuli (attractants and oogenesis-stimulating substances) emitted from the host plant at different phenological stage (ripening of fruits) (Girolami, 1982). The Authors suggest that just before the pit-hardening stage, the volatile compounds emitted by olive plants could attract adult flies to the groves. The return may be anticipated by irrigation or rainfall, but nevertheless occur even in dry conditions. Likewise, the production of mature eggs in the ovaries could be due to olive volatiles compounds, and egg-laying may in addition undergo different stimuli, i.e. tactile (Girolami, 1982).

Biological VOCs emissions are usually related with the corresponding metabolic pathways and changes on bVOCs emission usually reflects different metabolic statuses and contains many useful bio-informations. Potential bio-markers could be distilled from the differences of bVOC emission at different metabolism phases or physiological status.

Knowledge about the natural occurrence and role of allelochemics opens up a huge, untapped potential for exploiting them as pest management tools.

The tested method appears to be functional for a true detection of biogenic compounds from whole olive trees and to monitor the changing trend of volatile profile characteristics during time and obtain the corresponding volatile profile characteristic for the different phases.

The information accumulated forms an excellent base of knowledge for studying plant-insect interactions. Despite the usual low emission rates of the specie, the variations of VOCs emission between different phenological stages of *Olea europaea* L. were clearly displayed. This approach could be effective to better clarify natural occurrence and the ecological role of host allelochemicals, i.e. as attractants of the olive fruit fly *Bactrocera oleae* Rossi, the major olive pest.

4.2 Study on bVOCs emission from *Olea europaea* L.: the qualitative point of view.

4.2.1 Materials and method.

Basing on the preliminary successful results, further samplings were carried on. The aim was the characterization of the broadest possible range of volatile compounds. Therefore, the volatile compound profile was obtained by GC coupled with GC-MS technique with thermal desorption, which gives a significantly enhanced sensitivity.

Different samplings were thus performed from July to October 2010. Pairs of potted olive trees (4 years old) were enclosed into the glass chambers to perform again the bVOCs entrainment from different phenological stages (vegetative and fruiting stage) and from different cultivars (Canino and Leccino cv) of the specie. However, to reach a better combination of efficient sampling and sensitive detection, purified air was pumped into the chambers and ejected at the lower rate of 10 ml/min through different sorbent tubes: 1-stage traps filled with Carbopack C/B and Carbosieve SIII™, and 2-stages traps filled with Tenax GR™ (Gerstel TDU desorption tubes, conditioned).

The bVOCs entrainments were replicate three times per each phenological stage and a blank or control air collections were made each time as described for samples.

Photosynthetic active radiation (PAR) and air temperature were measured outside the enclosure while emission samples were taken: the mean value of temperature during the sample was 28°C ±2°C, natural photoperiod and light intensity level of 1000 PAR (quality of light intensity: “partial”).

The measurements started about on 1 h after enclosing plants, to allow emissions to stabilize. A preliminary experiment was conducted to define the better time of sampling and rate of flow (data not shown): air flow rate variation was ±2 ml/min for a mean flow rate of 10 ml/min during a 2h sample.

The samples analysis were performed by thermal desorption (TD), gaschromatography (GC) and gaschromatography coupled with mass-spectrometry (GC-MS), on a system consisting of an Agilent 7890 gas-chromatograph with a 5975C inert XL MSD quadrupole mass-selective detector (Agilent Technologies), equipped with a thermal desorption unit (Gerstel TDU, EI inert 350°C), a programmed temperature vaporization inlet (Gerstel CIS 4 PTV) and a Gerstel MPS2 XL autosampler. Desorption temperature was set at 300°C, desorbed compounds were cryo-focused in the PTV inlet at -20°C for 6 min.

Chromatographic conditions were: column Agilent 19091N-233 HP-Innowax 30m, 0,25 mm, ID 0.5 µm; injector and transfer line at 300°C, splitless mode; oven programmed at 40°C for 1 min, then 2°C/min up to 60°C, 3°C/min to 150°C, 10°C/min to 200°C, then 25°C increase per minute up to 260°C, isotherm 260°C for 6 min. The mass spectrometer was operated in scan mode using electron-impact ionization. Scan range was set from m/z 31 to 350 and sampling rate of zero.

Entrainments were repeated in three replicates per each cultivar and phenological stage.

After acquisition, the GC-MS chromatogram was processed algorithm to dynamically subtract the background noise along the chromatograms by (CLEAR VIEW™). The use of enhanced-sensitivity techniques for studying volatile compound profiles may add analytical variability to the data: the technique sensitivity may hide the variability of the phenomenon

under investigation. Therefore, the contribution of the background noise and/or its subtraction becomes a critical issue especially in the case of trace amounts. This problem is generally overcome through algorithms of background subtraction followed by chromatogram deconvolution, which are softwares usually employed for qualitative analysis and compound identification.

The acquired chromatogram were then screened by a spectral deconvolution software (Automated Mass Spectral Deconvolution and Identification System - AMDIS), which deconvolutes the spectra of overlapping chromatographic peaks. AMDIS is very successful at isolating a compound's spectrum from column bleed, other analytes and co-extracted interferences. Compounds identification was performed on the analytes' retention time RT, with a minimum matching factor of 70% and a RT window of 0.5; component width was set at 15 scan, with medium resolution and sensitivity. The deconvoluted spectrum of each hit was then searched against the NIST 05 and Wiley 7 mass spectral libraries, as a further check on peaks identity.

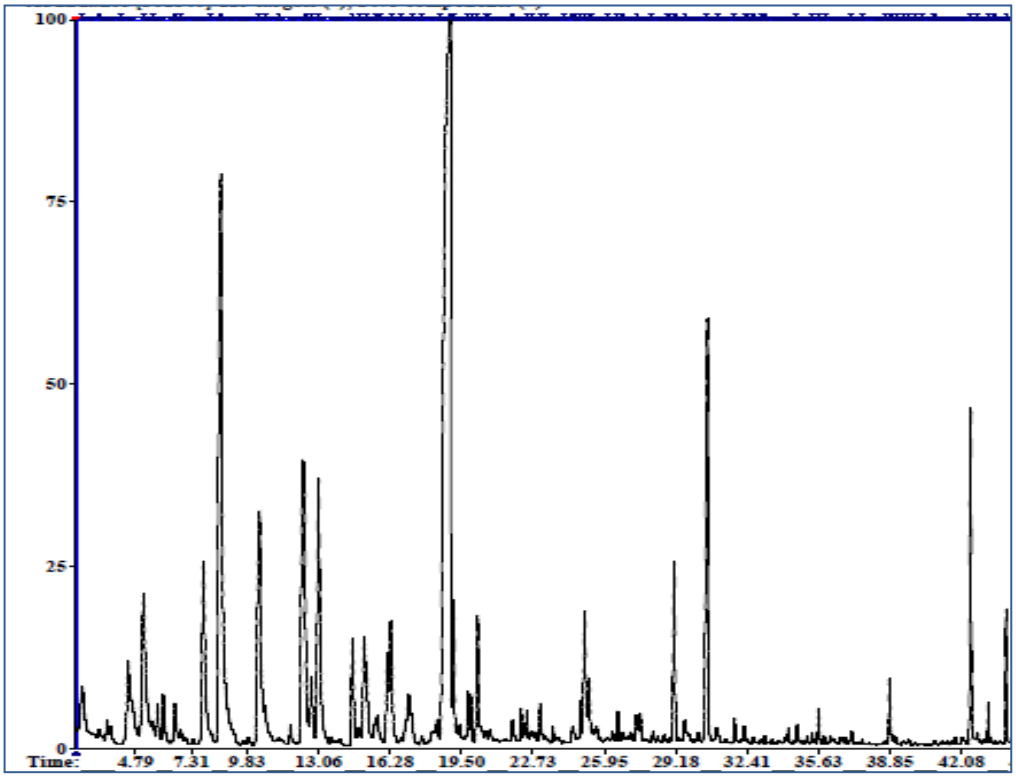

Fig. 25 Chromatogram: VOCs emission from fruiting plants of *Olea europaea* L. (Canino cv) - sample 04/10/2010

4.2.2 Results

Each identified compound is reported with its CAS #, compound name and Base Peak (BP). The compounds reported have a matching quality factor of 80 or more, compounds with a matching factor from 75 to 79 are reported with a leading question mark (?).

The identified compounds were grouped per chemical class (alkanes, alkenes, aldehydes and ketones, alcohols, organic acids, aromatic compounds).

Tab. 14 Volatiles collected from *O. europaea* L. (Leccino cv) in vegetative stage, September, 2010. Each identified compound is reported with its CAS #, compound name and Base Peak (BP). Matching quality factor ≥ 80, matching factors from 75 to 79 are reported with a leading question mark (?) Entrainments performed in three replicates (vg#).

CAS	Name	vg1	vg2	vg3
	alcohols			
100-51-6	benzyl alcohol	x	x	x
67-63-0	2-propanol (cas)			x
104-76-7	1-hexanol, 2-ethyl- (cas)		x	
	alkanes			
111-65-9	? octane (cas)			x
112-40-3	dodecane	x		
544-76-3	hexadecane	x		
629-59-4	tetradecane	x		
930-27-8	furan, 3-methyl-		x	
	aromatic			
100-42-5	styrene	x	x	x
108-38-3	? m-xylene		x	
108-88-3	toluene	x	x	x
95-47-6	? o-xylene	x		
95-47-6	o-xylene	x	x	x
	carbonyls			
100-52-7	benzaldehyde (cas)	x	x	x
112-31-2	decanal (cas)		x	
124-13-0	? octanal	x		
124-19-6	nonanal (cas)		x	x
116-09-6	? 2-propanone, 1-hydroxy- (cas)		x	
67-64-1	acetone	x	x	x
98-86-2	acetophenone	x	x	x

Tab. 15 Volatiles collected from *O. europaea* L. (Leccino cv) in fruiting stage, September, 2010. Each identified compound is reported with its CAS #, compound name and Base Peak (BP). Matching quality factor ≥ 80, matching factors from 75 to 79 are reported with a leading question mark (?) Entrainments performed in three replicates (ft#).

CAS	Name	ft1	ft2	ft3
	alcohols			
100-51-6	benzyl alcohol	x	x	x
104-76-7	? 1-hexanol, 2-ethyl- (cas)	x	x	x
67-56-1	methanol (cas)			x
78-83-1	? 1-propanol, 2-methyl- (cas)	x		
67-63-0	2-propanol (cas)	x		x
	alkanes			
544-76-3	? hexadecane		x	
930-27-8	furan, 3-methyl-			x
	aromatic			
100-42-5	styrene	x	x	x
108-38-3	m-xilene		x	x
108-88-3	? toluene	x		
108-88-3	toluene	x	x	x
	carbonyls			
100-52-7	benzaldehyde (cas)	x	x	x
498-60-2	? 3-furaldehyde		x	
112-31-2	decanal (cas)	x	x	x
124-19-6	nonanal (cas)			x
124-19-6	? nonanal (cas)	x	x	
116-09-6	? 2-propanone, 1-hydroxy- (cas)		x	x
67-64-1	acetone	x	x	x
98-86-2	acetophenone	x	x	x
98-86-2	? acetophenone	x		x

Examining the compounds detected, it's worth to note the presence of toluene and octane, reported in Scarpati (1993) as olfactory-attractive compounds.

Comparing vegetative and fruiting phenological stages, scarce differences could be detected: emissions appear to be very similar. Basing on the lists of identified compounds, the qualitative influence of growth stages is almost not detectable, consistently with preliminary results.

However, dodecane, octanal e tetradecane only occur in the vegetative stage, whereas 3-furaldehydes, methanol e 1-propanol, 2-methyl- only occur in the fruiting stage.

Further samplings of volatile emissions from different growth stages of *O. europaea*, Canino cv, were performed in three replicates per phenological stage. An internal standard solution (ethyl-hexanoate) was added to samples, to be used for area normalization of the analytes.

Each identified compound is reported with its CAS #, compound name and Base Peak (BP). The compounds reported have a matching quality factor of 80 or more, compounds with a matching factor from 75 to 79 are reported with a leading question mark (?).

The peak area of the inner standard was then used for normalizing the peak areas of the identified compounds. This allowed a rough quantitative comparison of the individual compounds among different samples and sampling times, even if no comparison of the analytes concentration ratios in the same chromatogram can be done. An absolute quantization through calibration curves for each identified analytes was not done, because the main purpose of the study was the characterization of the broadest possible range of volatile compounds and to investigate the possible changing trends of biological volatile emissions during development stages. The identified compounds were first grouped per chemical class (alkanes, alkenes, aldehydes and ketones, esters, alcohols, acids, phenolic compounds) and then listed from the most abundant to the least.

Tab. 16 Volatiles collected from *O. europaea* L. (Canino cv) in vegetative stage, October 2010 (3 replicates). Each identified compound is reported with its CAS #, compound name and Normalized Base Peak (BP). Matching quality factor ≥ 80, matching factors from 75 to 79 are reported with a leading question mark (?)

CAS	Name	Normalized Base Peaks
	alcohols	
98-00-0	2-furanmethanol (cas)	0,06
100-51-6	benzyl alcohol	0,30
104-76-7	1-hexanol, 2-ethyl- (cas)	1,19
111-27-3	1-hexanol	0,04
111-87-5	? 1-octanol (cas)	0,05
111-87-5	1-octanol (cas)	0,04
617-94-7	1-hydroxycumene	0,03
67-63-0	2-propanol (cas)	5,40
78-83-1	1-propanol, 2-methyl- (cas)	0,09
	alkanes	
111-65-9	octane (cas)	0,13
124-18-5	decane (cas)	0,26
590-73-8	hexane, 2,2-dimethyl-	4,18
930-27-8	furan, 3-methyl-	0,03
	alkenes	
3779-61-1	? 1,3,6-octatriene, 3,7-dimethyl-, (e)- (cas) (ocimene, trans-β-)	0,01
673-84-7	cis-allo-ocimene	0,01
764-13-6	2,4-hexadiene, 2,5-dimethyl-	0,12
502-61-4	.alpha.-farnesene	0,01
	aromatic	
100-42-5	styrene	12,97
103-65-1	benzene, propyl-	0,82
108-38-3	m-xilene	0,28
108-88-3	toluene	16,31
622-96-8	benzene, 1-ethyl-4-methyl- (toluene, p-ethyl-)	0,24
95-47-6	o-xylene	1,30
98-82-8	cumene	0,56
	carbonyls_aldehydes	
100-52-7	benzaldehyde (cas)	2,23
110-62-3	pentanal (cas)	0,11
111-71-7	heptanal (cas)	0,19
112-31-2	decanal (cas)	0,57
112-44-7	undecanal	0,03
112-44-7	? undecanal	0,01
124-13-0	octanal	0,22
124-19-6	nonanal (cas)	1,03
2277-19-2	nonenal	0,23
2277-19-2	? nonenal	0,05
2363-89-5	2 octenal	0,03
3913-81-3	? 2-decenal, (e)-	0,01
57266-86-1	2-heptenal, (z)-	0,01
66-25-1	hexanal	0,62

6789-80-6	? cis-3-hexenal	0,01
78-85-3	2-propenal, 2-methyl- (cas)	0,10
	carbonyl_ketones	
110-93-0	6-methyl-5-hepten-2-one	0,16
116-09-6	2-propanone, 1-hydroxy- (cas)	1,29
67-64-1	acetone	4,38
67-64-1	? acetone	0,03
98-86-2	acetophenone	0,30
	cycloalkenes	
138-86-3	limonene	0,20
	organic acids	
107-92-6	butanoic acid (cas)	0,11
109-52-4	pentanoic acid (cas)	0,11
140-11-4	acetic acid, phenylmethyl ester	0,19
142-62-1	hexanoic acid (cas)	0,82
142-62-1	? hexanoic acid (cas)	0,03
3891-98-3	dodecane, 2,6,10-trimethyl-	0,09
64-19-7	acetic acid (cas)	1,48
75-98-9	propanoic acid, 2,2-dimethyl-	0,25
79-09-4	propanoic acid (cas)	0,07
79-31-2	propanoic acid, 2-methyl-	0,04
79-31-2	? propanoic acid, 2-methyl-	0,01
VOCSISTD.isl-n1004	***etil esanoato d11***	***1,00***

Tab. 17 Volatiles collected from *O. europaea* L. (Canino cv) in fruiting stage, October 2010 (3 replicates). Each identified compound is reported with its CAS #, compound name and Normalized Base Peak (BP). Matching quality factor ≥ 80, matching factors from 75 to 79 are reported with a leading question mark (?)

CAS	Name	Normalized Base Peaks
	alcohols	
617-94-7	1-hydroxycumene	0,10
98-00-0	2-furanmethanol (cas)	0,05
100-51-6	benzyl alcohol	1,34
104-76-7	1-hexanol, 2-ethyl- (cas)	1,55
111-27-3	1-hexanol	0,06
111-87-5	1-octanol (cas)	0,12
67-56-1	methanol (cas)	2,82
67-56-1	? methanol (cas)	1,33
78-83-1	1-propanol, 2-methyl- (cas)	0,05
67-63-0	2-propanol (cas)	0,66
67-63-0	? 2-propanol (cas)	0,28
	alkanes	
111-65-9	octane (cas)	0,15
124-18-5	decane (cas)	0,14
590-73-8	hexane, 2,2-dimethyl-	3,55
930-27-8	furan, 3-methyl-	0,05
	alkenes	
3779-61-1	? 1,3,6-octatriene, 3,7-dimethyl-, (e)- (cas) *(ocimene, trans-β-)*	0,24
673-84-7	cis-allo-ocimene	0,01
502-61-4	.alpha.-farnesene	0,11
764-13-6	2,4-hexadiene, 2,5-dimethyl-	0,08
2492-22-0	cis-2,6-dimethyl-2,6-octadiene	0,13
	aromatic	
100-42-5	styrene	8,99
103-65-1	benzene, propyl-	0,57
108-38-3	m-xilene	0,42
108-38-3	? m-xilene	0,01
108-88-3	toluene	16,31
622-96-8	benzene, 1-ethyl-4-methyl- (toluene, p-ethyl-)	0,14
622-96-8	? benzene, 1-ethyl-4-methyl- (toluene, p-ethyl-)	0,04
95-47-6	o-xylene	0,86
98-82-8	cumene	0,51
	carbonyls_aldehydes	
100-52-7	benzaldehyde (cas)	2,89
110-62-3	pentanal (cas)	0,12
111-71-7	heptanal (cas)	0,37
112-31-2	decanal (cas)	0,70
112-44-7	undecanal	0,04
124-13-0	octanal	0,41
124-19-6	nonanal (cas)	1,62
124-19-6	? nonanal (cas)	0,03
2277-19-2	nonenal	0,23

2277-19-2	? nonenal	0,06
2363-89-5	2 octenal	0,07
3913-81-3	2-decenal, (e)-	0,03
57266-86-1	2-heptenal, (z)-	0,08
66-25-1	hexanal	0,64
6789-80-6	cis-3-hexenal	0,02
79-09-4	? propanoic acid (cas)	0,03
78-85-3	2-propenal, 2-methyl- (cas)	0,09
	carbonyls_ketones	
110-93-0	6-methyl-5-hepten-2-one	0,36
116-09-6	2-propanone, 1-hydroxy- (cas)	0,51
116-09-6	? 2-propanone, 1-hydroxy- (cas)	0,23
67-64-1	acetone	3,26
98-86-2	acetophenone	0,60
	cycloalkenes	
138-86-3	limonene	0,10
	organic acids	
107-92-6	butanoic acid (cas)	0,29
107-92-6	? butanoic acid (cas)	0,02
109-52-4	pentanoic acid (cas)	0,34
140-11-4	acetic acid, phenylmethyl ester	0,36
142-62-1	hexanoic acid (cas)	1,97
64-19-7	acetic acid (cas)	2,72
79-09-4	propanoic acid (cas)	0,11
75-98-9	propanoic acid, 2,2-dimethyl-	0,20
75-98-9	? propanoic acid, 2,2-dimethyl-	0,03
79-31-2	propanoic acid, 2-methyl-	0,07
vocsistd.isl-n1004	***etil esanoato d11***	***1,00***

Tab. 18 Comparative outline, emission from *O. europaea* in vegetative and fruiting phenological stages.

CAS	Name - vg samplings	Norm. BP	CAS	Name - ft samplings	Norm. BP
108-88-3	toluene	16,31	108-88-3	toluene	16,31
100-42-5	styrene	12,97	100-42-5	styrene	8,99
67-63-0	2-propanol (cas)	5,40	590-73-8	hexane, 2,2-dimethyl-	3,55
67-64-1	acetone	4,38	67-64-1	acetone	3,26
590-73-8	hexane, 2,2-dimethyl-	4,18	100-52-7	benzaldehyde (cas)	2,89
100-52-7	benzaldehyde (cas)	2,23	67-56-1	methanol (cas)	2,82
64-19-7	acetic acid (cas)	1,48	64-19-7	acetic acid (cas)	2,72
95-47-6	o-xylene	1,30	142-62-1	hexanoic acid (cas)	1,97
116-09-6	2-propanone, 1-hydroxy-	1,29	124-19-6	nonanal (cas)	1,62
104-76-7	1-hexanol, 2-ethyl- (cas)	1,19	104-76-7	1-hexanol, 2-ethyl- (cas)	1,55
124-19-6	nonanal (cas)	1,03	100-51-6	benzyl alcohol	1,34
			67-56-1	? methanol (cas)	1,33
VOCS ISTD.isl-n1004	***etil esanoato d11***	***1,00***	***VOCS ISTD.isl-n1004***	***etil esanoato d11***	***1,00***
142-62-1	hexanoic acid (cas)	0,82			
103-65-1	benzene, propyl-	0,82	95-47-6	o-xylene	0,86
66-25-1	hexanal	0,62	112-31-2	decanal (cas)	0,70
112-31-2	decanal (cas)	0,57	67-63-0	2-propanol (cas)	0,66
98-82-8	cumene	0,57	66-25-1	hexanal	0,64
629-59-4	tetradecane	0,33	98-86-2	acetophenone	0,60
98-86-2	acetophenone	0,30	103-65-1	benzene, propyl-	0,57
100-51-6	benzyl alcohol	0,30	98-82-8	cumene	0,51
108-38-3	m-xilene	0,28	116-09-6	2-propanone, 1-hydroxy-	0,51
124-18-5	decane (cas)	0,26	108-38-3	m-xilene	0,42
75-98-9	propanoic acid, 2,2-dimethyl-	0,25	124-13-0	octanal	0,41
622-96-8	toluene, p-ethyl-	0,24	111-71-7	heptanal (cas)	0,37
2277-19-2	nonenal	0,23	110-93-0	6-methyl-5-hepten-2-one	0,36
124-13-0	octanal	0,22	140-11-4	acetic acid, phenylmethyl ester	0,36
138-86-3	limonene	0,20	109-52-4	pentanoic acid (cas)	0,34
111-71-7	heptanal (cas)	0,19	629-59-4	tetradecane	0,29
140-11-4	acetic acid, phenylmethyl ester	0,19	107-92-6	butanoic acid (cas)	0,29
110-93-0	6-methyl-5-hepten-2-one	0,16	67-63-0	? 2-propanol (cas)	0,28

Searching for qualitative differences between the phenological stages, once more it could be detectable the emission of methanol from fruiting plants, which doesn't occur in the vegetative stage, and cis-2,6-dimethyl-2,6-octadiene. The compounds normalized peak areas allows a rough quantitative comparison of the individual compounds among different samples and phenological stages, even if no comparison of the analytes concentration ratios in the same chromatogram can be done. Because of the emission variability and the high sensitivity of the method, the data collected can't be spent to rate the actual quantitative differences between the growth stages. To appreciably cover the wide statistical variability of the phenomenon further samplings are needed.

The most abundant compounds emitted were toluene and styrene for both the phenological stages, as well reported as allelochemicals in Scarpati *et al.* (1993) together with xylene and octanal. Among the numerous, identified compounds it's worth to highlight the presence of nonanal and farnesene, evaluated as allelochemicals in previous studies (Beroza&Green, 1963; Mazomenos&Hamotakis, 1985; Rotundo, 2001; De Cristofaro *et al.* 2005).

During olfactory bioassay carried out in Scarpati (1993), toluene was found to be attractive on the olive fruit fly *Bactrocera oleae* (Rossi). Besides, the Authors reported the highly attractive and oviposition-stimulant features of styrene, even more than α-pinene. Octane and xylene were weaker oviposition activants than styrene.

Nonanal, which constitute the sexual pheromone molecule of *B. oleae* (Mazomenos&Hamotakis, 1985; Rotundo, 2001) is reported among the Tephritid olfactory attractants, particularly active on *Ceratitis capitata* (Wiedemann) (Beroza&Green, 1963). The olfactory sensivity of *B. oleae* to α-farnesene and limonene was confirmed via EAG in previous studies (De Cristofaro *et al.*, 2005; Anfora *et al.*, 2010).

4.2.3 Discussion and Conclusions

There is no clear consensus on the purpose of hydrocarbon production and emission by vegetation, various hypotheses suggest that different plant species may produce biogenic hydrocarbons for different reasons, depending upon their environment and the particular compound produced. The plant loss of energy and assimilated carbon due to hydrocarbon emissions ranges between few thousandths and some percent. A small release could represent an “optimized loss” , but quite high losses should be discussed as physiological and ecophysiological reactions with advantages for the plant species, because biological systems act economically.

Plant development stages could strongly affects quality and quantity of the plant emissions: there are clear evidences that phenological events, such as budding, flowering and fruiting, induce typical variations of the VOC pool, indirectly related to the growing seasons (Kesselmeier and Staudt, 1999). From bud break to a fully developed leaf, plants change their habitat as well as their physiology. The consequent changes both in emission amount and in emission composition could be additionally coupled with variations of the short-term response to light. The development stage appears to have significant influence on alcohols emission behaviour, e.g. linalool emissions by orange (Arey 1991, Seufert 1997).

Jasmonic and salicylic acids and their derivatives, coumarine, some terpenes and ethine are reported to be plant signals. The emission of ethene appears to be stimulated after injury or stress like arid conditions, extreme temperatures or air pollution. Ripening fruits and injuried tissue release ethylene. A stress-induced release of acetaldehydes is also known.

Although there remains a lack of comprehensive knowledge concerning the exact function of biogenic VOCs, their distribution in plant tissues and their emission pathways, in many cases the fundamental role of monoterpenes in plant defence reactions has been well clarified.

No comparable functions is known for isoprene so far, the functional explanation for isoprene emission is still under debate but it has been hypothesized that isoprene within chloroplasts could protect the photosynthetic apparatus of leaves against abiotic stresses or that isoprene production could work as membrane protection against heat stress, like monoterpenes appear to do: one hypotheses is that production of isoprene occurs in order to increase the thermal tolerance in leaves (Sharkey, 1996).

Unlike isoprene, the ecological role of monoterpenes as feeding deterrents against generalist herbivores and toxins against fungal pathogens, is well known. Damage by herbivores can also activate monoterpenes cyclases, enzymes responsible for monoterpenes production from GPP. Also, monoterpenes appear acting as the solvent for higher molecular weight terpenoid compounds, such as diterpenes and resin acids, that are solids at ambient temperature.

There are large number of interrelations between plants and insects based on volatile hydrocarbons: monoterpene species such as linalool are typical components of flowering fragrances attracting pollinators. Despite the ecological significance of the release from flowers and fruits, the great part of the plant emissions originates from green leaves.

Many monoterpenes and other VOCs are released during long-term defence reactions of plants against herbivore or pathogens attacks. For instance, damaging the bark of a pine there will be an instantaneous release of resin al the plant surface, then the induction of neo-

synthesis of terpenes around the damaged tissues will last over days to weeks. Infected pine species appear to emit high amounts of alcohols, especially of ethanol, that are primary attractants for pine beetles. Ethylene and methyl-jasmonate are compounds that induce defensive reactions in the plants where they are product, acting as signals for other plants (Lerdau *et al.*, 1997).

Some of the monoterpenes released from plants have an allelopathic function, for instance against seeds germination and growth of other species nearby; other monoterpenes act directly as defence compounds against pathogens and herbivores: β-pinene might limit fungal and microbial growth within the leaf tissues flooding the intercellular space to inhibit the respiration. Resin monoterpenes play a fundamental role in antimicrobial defence mechanisms of woundered coniferous trees, preventing pathogens colonization. The intense odour and taste of monoterpenes stop herbivorous from feeding and protect against insects feeding and oviposition. Turlings (1990) found out that corn seedlings, not releasing terpenes under normal conditions, react against caterpillars attacks by emitting monoterpenes. This emissions act as attractants for a species of endoparassitic wasp. Similar, complex interrelations could be found between several species of plants and insects.

VOCs emission consequently to phytophagous attack may have a role in plant indirect defences, attracting natural enemies of phytophagous (induced synomones) (Walling, 2000), as recorded for more than 15 different tritrophic systems.

The return of feral olive fly populations to the olive groves after the first abundant summer rains it must certainly be due to volatile compounds emitted by the plants and active on the olive fly (Girolami et al, 1982; Scarpati *et al.*, 1993; Scarpati *et al.*, 1996). Olfactory-attractive compounds were found to be ethylbenzene (from leaves), toluene and α-pinene (leaves and fruits), n-octane (fruits). During oviposition test carried out by the Authors, the most active compound on the olive fruit fly *Bactrocera oleae* (Rossi) was found to be α-pinene, which is the most abundant components of leaves emission, also released by fruits.

In a few days after the adults return to the orchards, ovipositions follow: the reproductive behaviour seems to be regulated by chemical stimuli (attractants and oogenesis-stimulating substances) emitted from the host plant at different phenological stage (ripening of fruits) (Girolami, 1982). The Author suggests that just before the pit-hardening stage, the volatile compounds emitted by olive plants could attract adult flies to the groves. The flies return after the so-called "wjite period" may be anticipated by irrigation or rainfall, but nevertheless occur even in dry conditions. Likewise, the production of mature eggs in the ovaries could be due to olive volatiles compounds, and egg-laying may in addition undergo different *stimuli*, i.e. tactile *stimuli* (Girolami, 1982). Consistently, Scarpati suggests that the most active compounds stimuling the oviposition may be leaf volatiles more than fruit volatiles (Scarpati, 1993), being the great part of the plant emissions originates from green leaves.

The present study reports toluene and styrene among the most abundant compounds emitted by olive plants, as well reported in Scarpati *et al.* (1993) together with xylene and octanal as host allelochemicals. Among the numerous, different compounds identified, it's worth to highlight the presence of nonanal, farnesene and limonene, evaluated as allelochemicals in previous studies (Beroza&Green, 1963; Mazomenos&Hamotakis, 1985; Rotundo, 2001; De Cristofaro *et al.* 2005; Anfora *et al.*, 2010). During olfactory bioassay carried out in Scarpati (1993), toluene was found to be attractive on the olive fruit fly *Bactrocera oleae* (Rossi). Besides, the Authors reported the highly attractive and oviposition-

stimulant features of styrene, even more than α-pinene. Octane and xylene were weaker oviposition activants than styrene. Nonanal, which constitute the sexual pheromone molecule of *B. oleae* (Mazomenos&Hamotakis, 1985; Rotundo, 2001) is reported among the Tephritid olfactory attractants, particularly active on *Ceratitis capitata* (Wiedemann) (Beroza&Green, 1963). The olfactory sensivity of *B. oleae* to α-farnesene and limonene was confirmed via EAG in previous studies (De Cristofaro *et al.*, 2005; Anfora *et al.*, 2010).

These results involve that host-plant odours play the fundamental ecological role of olfactory attractants and oviposition stimulants interacting with *B. oleae,* consistently with Girolami *et al.* (1981).

During the last few decades, much has been done in order to understand chemical communications in insect-insect interactions and about the insect responses to specific volatiles compounds. Steam distillation and extraction methods have generated most useful information in the past decades, headspace techniques and the use of GC and GC/MS is routine by far. It's a matter of fact that considerable are the differences between volatiles from picked material and essential oil obtained from the same plant by distillation or extraction. Besides, certain differences could be highlighted between emissions of picked material and emissions from living plants.

Research emphasis is now directed towards insect-plants interactions: it needs to properly investigate and recognize the volatiles released by living plants first of all, testing bioactive aspects of such compounds and correlating insect responses in the second place.

In line with IPM and biological management purposes, ending the use of non-selective long-lasting poisons and beginning the use of insects attractants, not just pheromones, could potentially become a powerful management tool, interfering with plant and insects at vulnerable steps.

At this regard, the main purpose of the study was the characterization of the broadest possible range of volatile compounds and to investigate the possible changing trends of biological volatile emissions during development stages.

The method developed appears to be functional for a true detection of biogenic compounds from whole olive trees, monitoring the changing trend of volatile profile characteristics during time and obtaining the corresponding volatile profile characteristic for the different phases. For the first time, an early comparison of the volatile organic compounds emitted from plants in different phenological stages (vegetative and fruiting stage) was performed. Despite the usual low emission rates of the specie, the variations of VOCs emission between different phenological stages of *Olea europaea* L. were clearly displayed.

The information accumulated forms an excellent base of knowledge for studying plant-insect interactions and are likely to improve the current strategies against this pest.

The present study, reporting on the original results of VOCs detection from whole, living olive trees (*Olea europaea* L.), could be effective to better clarify natural occurrence and the ecological role of host allelochemicals, i.e. as attractants of the olive fruit fly *Bactrocera oleae* Rossi, the major olive pest.

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"In collocando beneficio et in refenda gratia, si cetera paria sunt, hoc maxime officii est, ut quisque maxime opis indigeat, ita ei potissimum opitulari;

quod contra fit a plerisque."

Cicerone, *De off., 1, 49.*