INTEGRAÇÃO DE DIFERENTES MEIOS DE PROTECÇÃO CONTRA A MOSCA DA AZEITONA, *BACTROCERA OLEAE* (ROSSI) EM AGRICULTURA SUSTENTÁVEL

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Integração de diferentes meios na protecção contra a mosca da azeitona, *Bactrocera oleae* (Rossi) em agricultura sustentável

Integration of different control means against the olive fly, *Bactrocera oleae* (Rossi) in sustainable olive growing

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Abstract

The olive fly, *Bactrocera oleae* (Rossi) is a key-pest of olive tree in the Mediterranean region, traditionally controlled using insecticides. This thesis aimed to study the integration of different control methods against olive fly in sustainable olive growing. The results obtained show that the maintenance of spontaneous vegetation in the soil of the olive grove promoted a positive effect in the biodiversity of arthropodofauna in the olive groves studied either by the abundance and richness of carabid, edaphic predators of olive fly pupae either by the diversity of arthropods present in *Chondrilla juncea* L., especially Diptera immature, particularly abundant, and which may act as alternative hosts for parasitoids of the pest. In the laboratory it was found that the longevity of the parasitoid *Psyttalia concolor* (Szépligeti) increased when sugar is used as a food source. The strain of *Beauveria bassiana* (Balsamo) Vuillemin, Bb2T/08, showed high mortality (93.9%) in bioassays with *B. oleae* pupae showing potential in biological control against the pest. The hole size is a determining factor in the Olipe trap effectiveness. Smaller diameters have high infestation rates when compared with larger diameters, however the larger diameters were more harmful for beneficial insects.

Key words: *Bactrocera oleae* (Rossi), control methods, spontaneous vegetation, *Beauveria bassiana* (Balsamo) Vuillemin, Olipe traps.

Resumo

A mosca-da-azeitona, *Bactrocera oleae* (Rossi), é praga-chave da oliveira na região mediterrânica, e tradicionalmente as suas população têm sido combatidas com luta química. A presente tese teve como objetivo estudar a integração de diferentes meios de luta na proteção contra a mosca-da-azeitona em olivicultura sustentável. Os resultados obtidos mostram que a manutenção da vegetação espontânea no coberto do olival teve um efeito positivo na biodiversidade da artropodofauna nos olivais em estudo, pelo incremento da abundância e riqueza de carabídeos, predadores edáficos de pupas de mosca-da-azeitona, e diversidade de artrópodes presentes em *Chondrilla juncea* L., especialmente estados imaturos de dípteros que podem atuar como hospedeiros alternativos para parasitóides da praga. Em laboratório, a utilização de açúcares como fonte alimentar, incrementou a longevidade do parasitóide *Psyttalia concolor* (Szépligeti). A estirpe de *Beauveria bassiana* (Balsamo) Vuillemin, Bb2T/08, evidenciou alta mortalidade (93,9%) em bioensaios com pupas de *B. oleae* mostrando potencial na luta biológica contra a praga. O tamanho do orifício das armadilhas Olipe mostrou ter influência na eficácia da armadilha. Diâmetros menores apresentam maior infestação da praga quando comparados com diâmetros maiores, contudo os últimos foram mais nefastos para a fauna auxiliar.

Palavras-chave: *Bactrocera oleae* (Rossi), meios de luta, vegetação espontânea, *Beauveria bassiana* (Balsamo) Vuillemin, armadilhas Olipe.

Resumo alargado

A oliveira encontra-se distribuída por toda a região do Mediterrâneo, onde tem grande importância económica, ecológica e social e é um elemento característico da paisagem. Esta cultura é atacada por diversas pragas e doenças que diminuem o seu rendimento. De entre as pragas, a mosca-da-azeitona, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), é praga-chave da oliveira na maioria dos países mediterrânicos. Esta praga causa sérios prejuízos quantitativos, resultantes da queda prematura dos frutos e da destruição da polpa pelas larvas e qualitativos decorrentes da perda de qualidade do azeite proveniente dos frutos atacados. Tradicionalmente, o combate a esta praga tem sido feito com recurso à luta química o que pode por em causa a qualidade e segurança alimentar dos produtos do olival e ter implicações negativas para o ambiente. Os prejuízos causados pela praga e as limitações de uso de pesticidas em modos de produção sustentável, como o modo de produção biológico, justificam a procura e o desenvolvimento de meios alternativos de proteção. A presente tese teve como objetivo estudar a integração de diferentes meios de luta na proteção contra a mosca-da-azeitona em olivicultura sustentável, nomeadamente o estudo: (1) de estratégias de fomento da ação dos inimigos naturais pelo maneio da flora autóctone; (2) da virulência de diferentes isolados do fungo entomopatogénico *Beauveria bassiana* (Balsamo) Vuillemin sobre pupas da praga; e (3) da optimização da utilização de armadilhas tipo Olipe na captura em massa do inseto. Pretende-se que os resultados obtidos contribuam para a adequada proteção contra a mosca-da-azeitona, aspecto considerado limitante do aumento do olival em modos de produção sustentável. Os resultados obtidos mostram que a manutenção da vegetação espontânea no coberto do olival teve um efeito positivo na biodiversidade da artropodofauna nos olivais em estudo. Neste estudo foi encontrada uma grande diversidade de artrópodes presentes em *Chondrilla juncea* L., sendo Aphididae, Diptera e Thysanoptera os grupos mais abundantes. Alguns dos artrópodes encontrados nesta planta, como por exemplo afídios e tripes, têm sido referidos como presas alternativas e/ou hospedeiros para alguns predadores e parasitóides presentes nos olivais. O grande número de larvas e pupas de dípteros encontrados nesta planta é particularmente importantes pois os estados imaturos de dípteros podem também atuar como hospedeiros alternativos para os parasitóides da praga. O conhecimento dos artrópodes associados a esta planta é uma importante ferramenta no sentido de desenvolver táticas de proteção biológica de conservação. Neste trabalho, observou-se uma grande abundância de carabídeos nos olivais estudados. Em ambos os olivais, a subfamília Platyninae esteve presente durante todos os meses de estudo, sendo a subfamília mais

abundante com cinco espécies identificadas. Entre as espécies, *Calathus granatensis* Vuillefroy foi a mais abundante e o pico de abundância dessa espécie ocorreu entre o final do Verão e o meio de Outono, período que coincide com um aumento gradual de pupas da mosca-da-azeitona no chão, especialmente as gerações de Outono. A ocorrência de *C. granatensis* entre o final do Verão e Outono pode contribuir para a luta biológica da mosca-da-azeitona através de predação de pupas encontradas no solo. A informação recolhida neste estudo pode conduzir ao desenvolvimento de estratégias de modo a aumentar as espécies mais abundantes, a fim de promover a conservação biológica contra esta praga. Em laboratório, a utilização de açúcares (frutose e sucrose) como fonte alimentar, incrementou a longevidade do parasitóide *Psyttalia concolor* (Szépligeti) e em geral o tempo médio de vida foi significativamente maior para as fêmeas de *P. concolor* do que para os machos, tendo as fêmeas vivido o dobro de o tempo dos machos. Relativamente à produção da descendência, verificou-se que esta foi maior quando a glucose ou a frutose foram usadas como fonte alimentar. Estes resultados sugerem também que a utilização de açúcares como fonte alimentar pode aumentar a proporção de fêmeas obtidas na descendência. Estas fontes alimentares podem ser utilizadas para a criação em massa deste parasitóides em laboratório. O conhecimento dos requisitos energéticos do parasitóide *P. concolor* é uma importante ferramenta no sentido de melhorar a criação e manutenção desse parasitóide em laboratório e na manipulação de habitat para garantir o sucesso na introdução de parasitóides em programas de luta biológica. Nos bioensaios com pupas de *B. oleae*, realizados em laboratório, todos os isolados do fungo entomopatogénico *B. bassiana* testados contra a mosca-da-azeitona foram capazes de causar micoses nas pupas deste insecto e a percentagem de pupas com presença de micose variou entre18% e 94%. Uma correlação positiva foi encontrada entre a concentração e a mortalidade. A concentração mais alta (10^8 conídeos/mL) evidenciou maior patogenicidade contra pupas de *B. oleae*. Entre os isolados a estirpe de *B. bassiana*, Bb 2T/08, foi a que evidenciou a mais alta mortalidade nos bioensaios (94% de pupas mortas). A concentração letal (LC_{50}) dos isolados testados variou de $1{,}6\times10^6$ conídeos/mL para o isolado mais patogénico até $1{,}8\times10^8$ conídeos/mL para o isolado menos patogénico. Os resultados deste ensaio mostram que os isolados de *B. bassiana* testados têm potencial na luta biológica contra a praga. Pela análise das curvas de voo nos diferentes anos pode-se verificar que a praga esteve presente nos três anos de estudo, atingindo um pico populacional em Outubro. Os dados fenológicos da mosca-da-azeitona mostram um gradual aumento das populações imaturas desde meados do Verão até meados de Outubro, registando-se também um aumento progressivo da percentagem de frutos atacados. Neste trabalho observou-se que o tamanho do

orifício das armadilhas Olipe pode ter influência na protecção contra a mosca-da-azeitona. As armadilhas Olipe com tamanho de orifício menor parecem ser menos eficientes, que as armadilhas com diâmetros maiores, em reduzir níveis populacionais desta praga. As armadilhas com tamanhos de orifício maiores (10 e 8 mm de diâmetro) podem reduzir níveis de infestação para níveis abaixo do nível económico de ataque. Este estudo demonstrou também que o tamanho do orifício das armadilhas Olipe pode ter um impacto nefasto sobre a fauna auxiliar. Embora as armadilhas com tamanhos de orifício maiores aumentem as capturas de mosca-da-azeitona elas também aumentam a captura da fauna auxiliar do olival, tendo-se registado um aumento de capturas de insectos benéficos, principalmente de adultos de crisopídeos, um importante predador associado ao olival. As armadilhas Olipe com menor tamanho de orifício (4 mm de diâmetro) mostraram menor impacto na fauna auxiliar. Independentemente do tamanho de orifício usado verificou-se que as formigas foram o grupo mais capturado nas armadilhas Olipe, o que está em linha com estudos prévios realizados em Espanha e Portugal, provavelmente devido a atração destes insectos pelo fosfato biamónio. O uso de armadilhas Olipe selectivas para a fauna auxiliar é uma questão importante no olival, devido à grande diversidade de insectos que são importantes na luta biológica contra algumas pragas. Neste sentido as armadilhas Olipe com tamanho de orifício de 8 mm mostraram ser o melhor compromisso por ser aquelas que menor impacto tiveram na fauna auxiliar. A captura em massa com armadilhas Olipe pode ser uma alternativa aos tratamentos convencionais em modo de produção sustentável, devido ao seu baixo custo e eficácia, as quais podem reduzir populações para níveis considerados aceitáveis. O interesse da utilização deste tipo de armadilhas é reforçado pelo facto de não existirem alternativas a luta química no combate à mosca-da-azeitona em olivais conduzidos sob o modo de produção biológico, e quer pelo baixo custo das armadilhas e eficácia demonstrada, este meio de luta será uma alternativa a ser considerada.

Palavras-chave: *Bactrocera oleae* (Rossi), meios de luta, vegetação espontânea, *Beauveria bassiana* (Balsamo) Vuillemin, armadilhas Olipe.

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CHAPTER 1

Literature review

"Numa manhã, ao despertar de sonhos inquietantes, G. Samsa deu por si na cama transformado num gigantesco insecto. Estava deitado sobre o dorso, tão duro que parecia revestido de metal, e, ao levantar um pouco a cabeça, divisou o arredondado ventre castanho dividido em duros segmentos arqueados, sobre o qual a colcha dificilmente mantinha a posição e estava a ponto de escorregar. Comparadas com o resto do corpo, as inúmeras pernas, que eram miseravelmente finas, agitavam-se desesperadamente diante de seus olhos."

Kafka, Metamorphosis (1915).

1.1. Introduction

The olive tree, *Olea europaea* L., is distributed in all regions of the world with Mediterranean climate. In this region, olive growing is an activity with great economic, ecological and social importance.

World olive growing is estimated of around 1,000 million of olive trees, occupying an area of 10.2 million hectares and more than 90% of the total area is located in the Mediterranean basin. Spain (with 61% of production) is the world's largest olive oil producer country and together with Italy and Greece account for about 96% of EU olive oil production (IOC, 2014).

In Portugal the olive tree is distributed throughout the country, currently occupying a great area of around 347,000 hectares (FAOSTAT, 2015). The production of olive oil in Portugal round the 56,500 ton representing 3.2% of the EU olive oil production (IOC, 2014) highlighting the Alentejo, Trás-os-Montes and Beira Interior regions as the main producing regions of olive oil. In Trás-os-Montes region the olive grove occupies an area of 76,031 ha, producing an average of 59,114 ton of olive fruits and providing about 95,096 hl of olive oil (INE, 2013). Concerning the production of table olives, this region represents an area of 3,886 ha with the production of about 3,208 ton of olive fruits (INE, 2013). In this region, the olive groves constitutes a system of production that contributes greatly to generate income and employment in the region, not only directly through their cultivation, but also due to oil processing units and associated services to the olive growing and olive oil sector (Duarte et al., 2006).

Olive crop is attacked by a great number of pests and diseases that reduce their yield. Among pests, the olive fly, *Bactrocera oleae* (Rossi) (Diptera, Tephritidae), is considered the most important pest attacking olives being the key pest in Mediterranean countries (Daane & Johnson, 2010). Yield losses changing according the region, the year and the destination of the fruits. When fruits are for olive oil extraction the losses can reach 80-90%, which can reach 100% when fruits are for table olives preparation (Broumas et al., 2002). The control of the olive fly traditionally is based in insecticide application that can affect the food quality and safety of olive products and have negative impacts on environment. And, this control measures is not compatible with the production of olives in organic agriculture. Sustainable and effective protection against this pest should be based on integrating different control methods which is clearly in accordance with the principles and guidelines of the International

Organization for Biological and Integrated Control (Boller, 1998; IOBC/WPRS, 2012). The need to ensure effective control against olive fly in sustainable olive production systems, as integrated protection and organic farming, requires the development of control strategy that should be based on the improvement of crop protection level based in knowledge of the *B. oleae* bioecology, their factors of susceptibility, correct fly population monitoring systems, establishment of economic threshold levels, and the use of selected control means.

1.2. Olive fly, *Bactrocera oleae* (Rossi)

1.2.1. Taxonomy

Olive fly belongs to Tephritidae family (Table 1.1). This family of Diptera is the most diverse, with more than 4,500 described species, where are including some of the world's most significant agricultural pests (Duyck et al., 2004). The olive fly is in the large subfamily Dacinae and tribe Dacini, which contains primarily Afrotropical, Australasian, and Oriental species (Daane & Johnson, 2010). Within Dacinae are some of the more damaging fruit fly pests of fruits and vegetables, including species in the genera *Bactrocera*, *Dacus* and *Ceratitis* (Daane & Johnson, 2010).

Table 1.1. Systematic of *Bactrocera* (Daculus) *oleae* (Gmelin) (Adapted from Fauna Europaea, 2016 and CABI, 2016).

Class	Insecta
Order	Diptera
Suborder	Brachycera
Family	Tephritidae
Subfamily	Dacinae
Tribe	Dacini
Genus	*Bactrocera*
Subgenus	*Daculus*
Species	*oleae*

B. oleae is known in several countries and has several designations. "Mosca-da-azeitona" in Portugal, "mosca del olivo" in Spain, "mouche de l'olive" in France, "mosca delle olive" in Italy, "olive fly" in English-speaking countries and "olivenfliege" in Germany (Cantero, 1997).

1.2.2. Geographic distribution

Bactrocera oleae is associated with plants of genus *Olea* and its distribution is generally limited to the regions where cultivated and wild olive trees are found (Table 1.2).

Table 1.2. Countries or regions where the olive fly, *Bactrocera oleae* (Rossi) is present and last reference cited in the distribution. (Adapted from CABI, 2016).

Countries or regions	
Albania	Libya
Algeria	Jordan
Angola	Kenya
Arabian Peninsula	Malta
Azores	Mexico
Canary Islands	Montenegro
Corsica	Morocco
Croatia	Pakistan
Cyprus	Portugal
Egypt	Sardinia
Eritrea	Seychelles
Ethiopia	South Africa
France	Spain
Greece	Sudan
Georgia	Switzerland
Italy	Syria
India	Tunisia
Iran	Turkey
Israel	United States
Lebanon	

Bactrocera oleae was known primarily from the Mediterranean area of southern Europe, and is found in North Africa, Caucasian region and from Middle East to India (Fauna Europaea, 2016). It is also present in countries of sub-Saharan Africa, South Africa, Sudan and Kenya (Copeland et al., 2004). In 1998 it was found in United States of America and Mexico (Rice, 2000). Reports of *Bactrocera* species collected on wild olives in China bring in

to question the olive fruit fly's presence in Asia (Daane & Johnson, 2010). The wide dispersion of this species has been favored by high flight capacity of adults, which also explains the ease of reinfection in areas in which it has been controlled. There have been reported movements of this species from 200 m in the presence of olive hosts to much as 4 km in search of host, as well as movements of up to 10 km over open water in Mediterranean (Rice, 2000).

The origin of olive fly and ancient history of olive tree is still matter of debate (Nardi et al., 2010). Actually, it has been proposed that *B. oleae* is not native to southern Europe, but rather Africa (Nardi et al., 2005, 2010). Recent molecular analysis of *B. oleae* population suggests it may have evolved in Africa and followed the expansion of olive cultivation into the Mediterranean area, south-central Asia, and California (Nardi et al., 2005).

1.2.3. Morphology

Adults: The fly (Figure 1.1) is normally 4-5 mm long and 10-12 mm in wingspan (Cantero, 1997). The thorax is dark brown with 2-4 gray or black longitudinal bands. The scutellum is almost entirely yellow-ivory (Neuenschwander et al., 1986; Civantos, 1999). The abdomen is reddish-brown with darker areas on the sides of each segment (Neuenschwander et al., 1986). Wings containing dark veins and a small dark spot at each wing tip (Daane et al., 2004). Females can be distinguished from males by the ovipositor, a pointed structure at the end of female's abdomen (Cantero, 1997). Males are generally smaller than females.

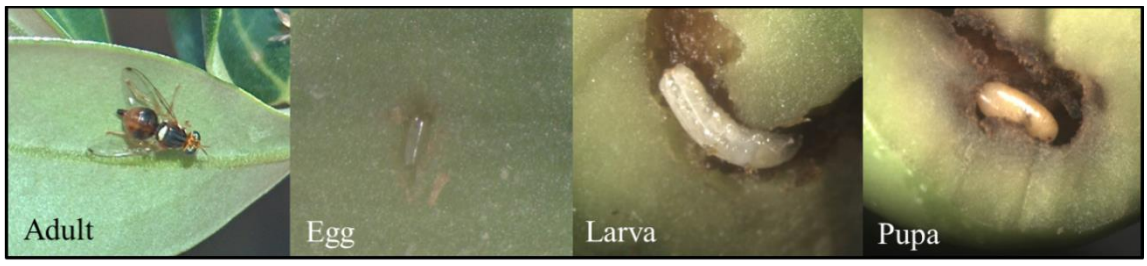

Figure 1.1. Adult and immature stages (egg, larva and pupa) of *Bactrocera oleae* (Rossi).

Eggs: Eggs are elongated and cylindrical, white and very small and difficult to see. It dimensions are about 0.7 mm long and 0.2 mm wide (Neuenschwander et al., 1986).

Larva: The larvae are apodous, cylindrical white-yellow and with pointed and dark head. Larvae pass through three instars during development. Newly larvae measure about 1 mm long and at the end of development can reach about 7-8 mm (Civantos, 1999).

Pupa: Pupae have elliptical shape with pointed head. Their colour varies from pale white to light yellow, with the colour intensity depending on the colour of the olive pulp that they infest (Daane et al., 2004). It dimensions are about 4-4.5 mm long and 2 mm wide (Civantos, 1999). Unlike most other Tephritidae species, mature olive fly larvae pupate in fruit during the summer, but leave fruit in the fall and winter to pupate in the soil under the tree (Daane et al., 2004).

1.2.4. Life cycle

Female fruit flies are oligogamous and mate 1-3 times during their life (Tzanakakis et al., 1968). On the other hand, male flies are polygamous and they can mate daily if receptive females are available (Zervas, 1982). Olive fly female have been reported to lay from 10 to 40 eggs per day (Tzanakakis, 1989), generally one egg in each fruit, and from 200 to 500 eggs during their lifetime (Tzanakakis, 1989; Daane et al., 2004).

Adults feed on a variety of organic sources including insect honeydews, plant nectar, plant pollen, fruit exudates (Daane & Johnson, 2010), and other opportunistic sources of liquid or semi-liquid food (Weems & Nation, 2003). The availability of adult food for reproduction and/or survival may be critical to olive fly populations' existence during periods when fruit flies are unsuitable for oviposition. For that reason, adult flies can be found on many different plants where adult food sources are found. The larvae, however, are monophagous on olives in the genus *Olea*, including *O. europea* (cultivated and wild), *O. verrucosa*, and *O. chrysophylla* (Daane & Johnson, 2010). As such, the olive flies' survival is dependent of olive fruits condition and availability.

The life cycle of the olive fly is closely linked to the seasonal development of the olive tree, and the local climate (Zalom et al., 2003, 2009). Generally olive fly develops two to five generations per year in Mediterranean region (Civantos, 1999). *B. oleae* overwinters either as an adult or as pupa in the soil, a few deep, 1 to 3 cm (Neuenschwander et al., 1986). Some immature stages can stay inside infested fallen fruits which have not been collected (Conti, 2007). New adults from overwintered pupae or first generation begin to emerge in spring, generally in April and May (Civantos, 1999), and immediately begin their activity looking for

food. Olive fly females are particularly attracted by materials with volatile nitrogen compounds (Conti, 2007) that could be related with the great importance of nitrogen compounds, which contain protein, in the maturation of the ovary in females and consequently with egg production and development (Conti, 2007).

During the preoviposition period the female is maturing the ovary and the first set of eggs. From late June to July as new olives develop, females actively seek and oviposit in early maturing fruits (Zalom et al., 2003), beginning the second generation. Fruit susceptibility begins at the time of stone hardening (the olive phonological stage considered receptive to oviposition) usually in July in Mediterranean region (Rice, 2000). Eggs are laid in olives in a chamber under the epidermis created by the ovipositor, such that the neonate larva has access to food. Although eggs may be laid in small fruit, the larvae do not successfully develop until the fruit grows to sufficient size (Zalom et al., 2009). In nature, females usually lay one egg per fruit, and they avoid laying in an olive fruit in which a female has already oviposited (Tzanakakis, 1989). The egg hatching occurs over a variable period depending on weather conditions, usually in 2 to 4 days in summer (Katsoyannos, 1992; Daane et al., 2004) (Table 1.3).

Table 1.3. Duration of individual life stages of olive fly (Adapted from Katsoyannos, 1992).

Stage	**Summer**	**Autumn-Winter**
Egg	2-4 days	4-10 days (autumn); 12-19 days (winter)
Larva	9-14 days	18 days or more
Pupa	16 days	To 3 months (pupae wintering)
Adult	2-3 months	Several months

The newly hatched larva feeds and grows as a fruit borer in the mesocarp of olives developing a gallery inside. Olive fly larva initially tunneling a superficial gallery which becomes deeper with larval development, reaching endocarp. In this gallery the larva passes through different instars until the end of its development. Then, when close to third instar larva move to the fruit surface and then pupates, lasting its development between 9 to 14 days, depending on temperature (Katsoyannos, 1992). After pupate the adults emerge and fly away leaving an emerging hole (Civantos, 1999) beginning a new generation. Larval stages develop from mid-summer to late autumn when there are fruits available.

Additional generations of flies are produced through the late summer and autumn into December depending upon fruit availability and climatic conditions (Vossen et al., 2004). Unlike other tephritid species, olive flies pupate within the host fruit during warmer months, but in late autumn and winter, its behaviour changes, and larva leaves the fruits to pupate in the ground or in any protected niche during winter (Daane et al., 2004). The pupation site selection was considered by Kapatos & Fletcher (1983) as a survival mechanism: thus, in the summer generations olive fly pupates in the fruit to avoid high temperatures that can be reached in the soil surface, while in the autumn generations the insect pupates in the soil to avoid the predation by birds or death due to early harvest. Although the olive fly does not have a true diapause, development is sufficiently slowed during the winter, so pupae produced in late fall do not emerge until the following spring (March to April) (Conti, 2007).

In field, the duration of development of the insect is highly dependent on environmental conditions, particularly temperature and food (Burrack & Zalom, 2008). In summer olive fly can complete a generation in 30 to 35 days at optimum temperature to 130-160 days in winter (Katsoyannos, 1992).

1.2.5. Factors affecting insect populations dynamics

Olive fly populations are subject to natural mortality factors such as **climatic factors**, **agronomic factors** and the **action of parasitoids and predators**. The main natural factors that limit olive fly populations are temperature and humidity (climatic factors). The optimal temperature for *B. oleae* development range between 20ºC to 30ºC (Civantos, 1999). Nevertheless, adults can survive at temperatures between 6ºC (lower temperature threshold) to 35ºC (upper limit). The optimal temperature for eggs development has been reported ranging from 30ºC to 32ºC (Tsitsipis, 1977). Although immature development could be completed at 30ºC, up of 35ºC no larvae are able to reach adult (Tsitsipis, 1977; Wang et al., 2009). The action of relative humidity acts in combination with temperature. High summer temperatures associated with low relative humidity reduces the probability of immature stages and increase mortality (Pucci et al., 1985) and impeding female maturation (Katsoyannos, 1992). In opposite, during winter, the combined action of low temperatures and high soil moisture can cause high mortality in pupae buried in the ground (Neuenschwander et al., 1986).

Relatively to agronomic factors, *B. oleae* has olive cultivar preference (Gonçalves et al., 2012). Fruit size and weight, colour, fruit epicarp hardness, surface covering, phenological stage of the crop and chemical factors play a role in host susceptibility to attacks of *B. oleae* (Neuenschwander et al., 1985; Iannotta et al., 2007). It is known that in some cultivars, there is a reaction of the olive fruit to female oviposition, occurring a tissue suberization of mesocarp crushing and destroying the egg (Neuenschwander et al., 1986). Olive hardness is considered an important factor in determining the choice of drupes for *B. oleae* oviposition. In the early developing and ripening period of olives, when all drupes are completely green, hardness of olives plays an important role in oviposition (Rizzo & Caleca, 2006). Also first instar larvae may suffer high mortality when the olive fruits are still very green because they are unable to obtain adequate food or become encysted, as a result of a reaction of the gallery suberization as well as in fully matured olives, with high oil content, larvae can die from suffocation (Neuenschwander et al., 1986). The size of drupes is considered by several authors one of the most important factors in the choice of olives by *B. oleae* females (Jimenez, 1988). Larval development is influenced by fruit size and pulp consistency, as well as the water content, thus, cultivars of large size fruits are preferred in earlier infestations because offer better protection against high summer temperatures and high water content reduces risk of desiccation (Neuenschwander et al., 1985; Burrack & Zalom, 2008). Also olive coloration seems to play a role in females choice and it was shown that green olives resulted more infested than brown ones (Katsoyannos, 1989).

The complex of parasitoids associated with the olive fly in the Mediterranean basin is relatively poor and does not provide effective biological control of the olive fly (Bigler et al., 1986). Except for *Eurytoma martelli* Domenichini (Hymenoptera: Eurytomidae), the chalcidoid species are not specific to *B. oleae* and are somewhat to highly polyphagous, attacking unrelated hosts in several different insect orders (Hoelmer et al., 2011). *Eupelmus urozonus* Dalman (Hymenoptera: Eupelmidae) is also a facultative hyperparasitoid of other parasitoids including *Pnigalio mediterraneus* Ferrière & Delucchi (Hymenoptera: Eulophidae) (Noyes, 2011). Among predators, is attributed some importance to birds including the blackbird (*Turdus merula*), the spanish sparrow (*Passer hispaniolensis*) the mistle thrush (*Turdus viscivorus*) the robin (*Turdus migratorius*), ravens (*Corvus* sp.), etc, (Cavalloro, 1984) playing an important role in consuming attacked fruits, and to some soil predators, mainly by its action on the pupae such as carabids, staphylinids, centipedes and Dermaptera (Neuenschwander et al., 1983).

1.2.6. Damage and loses

The olive fly larvae feed exclusively on olives or it is the only life stage that causes significant damages. In regions of the world where the olive fly is endemic and uncontrolled, its feeding can result in total loss of production when olives are destined to table olives (Broumas et al., 2002) and 80% loss when the production is destined for olive oil extraction (Tzanakakis, 2006). Economic damages results from the olive fly ovipositions in fruits and feeding larvae into the drupe. Direct damages results from premature fall of fruit to soil that depending on the year could reach 90% of the production (Bento et al., 1999, 2003, 2009) and pulp destruction by larvae feeding (Neuenschwander & Michelakis, 1978), ranging from 3 to 20% depending on the olive size (Kapatos & Fletcher, 1983). Larval consumption of fruit pulp has been estimated to range from 50 to 150 mg per larva depending on cultivar (Neuenschwander & Michelakis, 1978). Indirect damages results from the emergence holes of adults that favour the attack of bacteria and fungi that decompose the pulp (Vossen et al., 2004) and causes deterioration on olive oil quality, increasing hydrolysis and oxidation, and decreasing the antioxidant compounds of oil resulting in total trade devaluation in case of table olives (Civantos, 1999; Pereira et al., 2004a). This relationship is influenced by the presence of microorganisms such as bacteria (*Xanthomonas*), yeasts (mostly *Torulopsis* and *Candida*), and molds (mainly *Fusarium* and *Penicillium*), with a positive logarithmic relationship between microflora populations and oil acidity (Torres-Villa et al., 2003). The quality of the olive oil can also be affected particularly when the fruits are stored for long periods of time. The emergence holes of the olive fly adults facilitate installation of fungi and bacteria, which find here conditions of temperature and humidity, favourable to their development (Pereira, 2000).

Traditionally, the losses caused by the olive fly in Trás-os-Montes region were considered of reduced economic significance and this region was considered area of low incidence of *B. oleae* or an adacic region till few decades ago (Azevedo, 1965).

1.2.7. Risk assessment and economic thresholds

Early detection of olive fly is essential to prevent crop losses in commercial production areas. The **risk estimation** on the olive fly is made by capturing adults in traps and visual observation of the attack in fruit samples. Adult fly population are usually monitored with yellow sticky traps containing a sex pheromone of insect (1,7-dioxaspiro[5.5]undecane).

Yellow sticky traps consist of a flat yellow plastic plate, about 20 cm × 20 cm, of a yellow colour, gummed on both sides. Traps are usually spaced by at least 50 m and placed in south side of the tree (Civantos, 1999). According Kapatos & Fletcher (1983) and Dimou et al. (2003) yellow sticky traps with the sex pheromone provide more consistent data than McPhail traps. The McPhail trap is used too for monitoring, and in some cases for mass trapping (control) as well. They are made of either glass or plastic with a reservoir for liquid bait containing a 4% solution of ammonium salts (ammonium bicarbonate or ammonium phosphate) as bait attractants (Vossen et al., 2004). However they are difficult to manage and keep filled with the ammonium bait attractant (Varela & Vossen, 2003) because they dry out quickly in hot weather. For visual observation on the attacked fruits, it is recommended to collect randomly 10 fruits per tree on 20 olive trees in olive grove, weekly from stone hardening (Gomes & Cavaco, 2003).

The economic thresholds level recommended for Portugal corresponds in the case of table olives, 1 female per day in McPhail traps, 1% of attacked fruits with living forms and over 50% of fertile females. In the case of olive oil, 5 females per day in McPhail trap and over 60% of fertile females or 3 adults per day in yellow sticky traps and more of 60% fertile females and 8 to 12% of attacked fruits with live forms (DGADR, 2010).

1.3. Indirect control measures

The need to reduce pesticide applications in sustainable production such as organic agriculture and integrated pest management, justify the development of alternative means to control olive fly (Andrea et al., 2005) and this control should be based on the integration of different control methods which are clearly in accordance with the principles and guidelines of the International Organization for Biological Control and Integrated Pest Management (IOBC/WPRS, 1995; Malavolta & Perdikis, 2012). Moreover, with the publication of Directive 2009/128/CE, from 1 of January 2014, all operators have to adopt agricultural protection systems compatible with the general principles of integrated pest management provided in Annex III, in the same Directive (Directive 2009/128/CE). In accordance with these guidelines, this strategy should be based primarily on the use of indirect measures of protection or preventive and only when they are not effective should be utilized direct measures.

1.3.1. Conservation biological control

As alternative measures for olive pest control, the integrated production rules of the International Organization for Biological Control and Integrated Pest Management (Malavolta and Perdikis, 2012) recommend the promotion of biodiversity, to be considered an important element of agricultural sustainability. Promoting the natural control of crop enemies constitutes a tactic of conservation biological control (Amaro, 2003). Conservation biological control involves manipulation of the environment to enhance the survival, fecundity, longevity, and behaviour of natural enemies to increase their effectiveness. Habitat management is a strategy of conservation biological control which seeks enhancing natural enemies in agricultural systems (Landis et al., 2000). In this strategy, the abundance and diversity of natural enemies can be enhanced by favouring ecological infrastructures in agroecosystems that providing, in space and time, the resources necessary for its effective activity, in particularly: 1) **food sources**, such as honeydew, pollen, nectar; 2) **habitats for hosts/alternative prey**; 3) **shelters**, such as habitats for wintering, nesting and mating, protection from natural enemies, favourable microclimates (Landis et al., 2000; Franco, 2010).

1.3.1.1. Natural control of olive fly

With the objective of enhancing natural enemies in the olive groves has been dedicated of particular attention both to increase plant diversity associated with ecosystem, or to implement on the crop, artificial foods in order to enhance the beneficial fauna (Torres, 2007). In olive groves, the use of ecological infrastructures can have an important role in improving and conservation of biodiversity. The implementation of vegetation covers in the olive groves is a kind of ecological infrastructures and consists in leaving the soil covered by herbaceous plants during part of the year, with cultivated species or spontaneous vegetation (Saavedra & Pastor, 2002).

Tillage or herbicide sprays are traditionally used for the control of spontaneous vegetation in olive groves with negative consequences for soil erosion, destruction of olive roots, decrease of organic matter and also the negative effects on biodiversity (Campos et al., 2000). The use of spontaneous vegetation in olive groves is considered particularly interesting, because it can provide shelter for many entomophagous insects and can be a reservoir of alternative preys for predators and parasitoids (Campos & Civantos, 2000). Some

studies show a positive effect of some species of spontaneous vegetation on beneficial arthropods. For example, fennel (*Foeniculum vulgare* Mill.) is attractant to many beneficial arthropods including lacewings, syrphids, coccinellids and hymenoptera parasitoids and can act as an arthropod reservoir (Coelho et al., 2011a) or *Coleostephus myconis* (L.) Rchb.f., that also may constitute a relevant arthropod reservoir of alternative prey and hosts for predators and parasitoids of olive pests (Villa et al., 2012). The spontaneous vegetation exert a positive effect on the increase of biodiversity and maintenance of several species of Hymenoptera parasitoids belonging to different families such as *Braconidae, Ichnemonidae, Pteromalidae, Eulophidae, Chalcididae, Eurytomidae* and *Elasmidae* (Escudero et al., 2002), soil predators (Pereira et al., 2004b; Paredes et al., 2013), alternative hosts and also could serve as shelter.

Efforts to incorporate biological control in management of *B. oleae* were initially made using a braconid wasp, *P. concolor*, which was introduced into Italy from Tunisia in 1914 and later in others Mediterranean countries. This parasitoid was repeatedly introduced but it did not establish widely in Europe, which was attributed to unsuitable climatic conditions (Miranda et al., 2008), resulting in low rates of parasitism in the olive fly (Jiménez et al., 1990) and not providing adequately a control of *B. oleae* population (Delrio et al., 2003).

With the aim of controlling the olive fly, were tested in Italy, inundative releases of the *E. urozonus*, considered one of the most important parasitoids of the olive fly in the north of the Mediterranean basin (Civantos, 1999). However, the results suggest that, in the olive grove, *E. urozonus* behaves primarily as hiperparasitoid, especially of *P. nigalioagraules* and only occasionally as a parasitoid of the olive fly, so their interest, according to Delrio et al. (2005) is questionable.

These parasitoid species are present in the olive groves from July to October and their discontinuity is related to the absence of other insects or alternative host, which can be present in the spontaneous vegetation being necessary for reproduction of parasitoids during the spring period and wintering (Arambourg, 1986). As an example refers to the case of *E. urozonus*, a polyphagous species, which action on olive fly is exerted mainly in August/September, decreased in October, probably because find more attractant alternative hosts (Jiménez, 1985). In Mediterranean region some plants are sources of alternative hosts for beneficial fauna that attack the olive fly, as in the case of *Dittrichia viscosa* (L.) which flowers are attacked by *Myopites stylata* Fabricius being the larvae alternative hosts for *E. urozonus* (Warlop, 2006). *E. urozonus* parasite *M. stylata* larvae in autumn and spring, and from spring to autumn behaves as a parasitoid on the olive fly (Katsoyannos, 1992). Under

the presented conditions the development of *D. viscosa* in olive groves can be of interest and should be enhanced in order to enhance the action of *E. urozonus* (Warlop, 2001).

The recent invasion of olive fly in California resulted in renewed interest in classical biological control of this pest (Daane & Johnson, 2010; Hoelmer et al., 2011) and recent surveys have been made intending to introduce new parasitoids into infested regions. *Fopius arisanus* (Sonan) originally collected from puparia of the oriental fruit fly, *B. dorsalis* (Hendel) (Diptera: Tephritidae) was tested in Italy (Calvitti et al., 2002) and a small number of braconids, *Psyttalia lounsburyi* (Silvestri), *Psyttalia humilis* (Silvestri), *Psyttalia ponerophaga* (Silvestri) and *Bracon celer* Szépligeti native of wild olives of Sub-Saharan Africa were tested in California since 2000 (Daane et al., 2008; Sime et al., 2006; 2007; Yokoyama et al., 2006). On wild olives parasitism levels range from 57% in Kenya by *P. lounsburyi* and *U. africanus*; 37% in Pakistan by *P. ponerophaga*; and 28% in South of Africa by *P. lounsburyi*, *B. celer*, *U. africanus* (Daane & Johnson, 2010). Among the various parasitoids considered for olive fly control in California, *P. lounsburyi* was especially attractant because in quarantine studies it appeared to be more of a specialist on olive fly than other parasitoid species tested (Daane et al., 2008).

Although efforts have been made to introduce these parasitoids in new habitats, the field trials showed some difficult in parasitoids establishment on *B. oleae*. Hoelmer et al. (2011), refers that detailed biological studies are needed to clarify the relationship between parasitoid ecology, behaviour and efficacy, including the influence of the olive host on the parasitoids.

An important predator of *B. oleae* is the olive cecidomid, *Lasioptera berlesiana* Paoli (Diptera: Cecidomiidae). The olive cecidomid is widely distributed in the Mediterranean region and exerts olive fly egg predation, reducing its populations to 30% in some conditions (Civantos, 1999). The development of olive cecidomid occurs essentially in the mastic tree (*Pistacia lentiscus*) in association with leaf galls produced by *Aceria stefani* (Nalepa), where reproduces from late May through September. From this month it begins reproducing in olives in the lesions made by the olive fly laying, and the emerged larva preys on the olive fly eggs or young larvae (Sasso & Viggiani, 2005).

In the olive groves, the spontaneous vegetation is important for some soil predators able of acting on the olive fly pupa like carabids, rove beetles and ants (Neuenschwander et al., 1983; Warlop, 2001). As olive fly larvae feed deep inside the fruit (Tzanakakis, 2006), the immature stages are protected from most generalist predator. From the end of September till harvesting, the third instar larvae begin to exit the fruit to pupate on the soil (Tzanakakis,

2003), staying exposed to soil predators, such as ants and predaceous beetles, mainly carabids and staphylinids.

Ants have an important role in the olive agroecosystem, participating actively in natural control exercising predatory action on *B. oleae* larvae and pupae in canopy and soil (Arambourg, 1986; Katsoyannos, 1992) and other phytophagous species (Varela & González, 1999). According to Neuenschwander et al. (1983), many species of ants could attack *B. oleae* larvae as well as pupae inside the fruit and in the soil, including some species as *Aphaenogaster simonelli* Emery, *Crematogaster sordidula* (Nylander) and *Tetramorium caespitum* (L.). Orsini et al. (2007) found that native ant *Formica aerata* (Francoear) could be responsible by high mortality of olive fly pupae in California. Carabids and staphylinids also have potential in controlling *B. oleae* pupae. Neuenschwander et al. (1983) referred the existence of more than twenty species in Crete (Greece) that exerted predation on larvae and pupae of the olive fly. *Pterostichus creticus* Frivaldszky and *Carabus banoni* Dejean were the most common species of carabids, followed by other predatory species such as *Poecilus cupreus* Linnaeus, *Platyderus minutus* Reiche, *Calathus fuscipes graecus* Dejean & Boisduval, *Chlaenius festivus* Panzer and *C. vestitus* Paykull, and *Ocypus olens* Mueller and *O. fulvipennis* Er. were two species of staphylinids observed exerting predation on larvae and pupae of *B. oleae*. Also, in laboratory trials conducted in Italy it was found that the species *Pterostichus* sp. *Calathus fuscipes* (Goeze), *Pseudoophonus rufipes* (De Geer) and *Laemostenus cimmerius* (Fischer von Waldheim) fed regularly on olive fly pupae (Odoguardi et al., 2008).

1.3.1.2. Selection of resistant varieties

Olive varieties vary in terms of *B. oleae* preference and studies about host preference found several factors influencing the preference for oviposition such as fruit size, colour, and epicarp hardness and chemical stimuli, mainly aliphatic waxes, have been suggested to play a role in ovipositional preference (Neuenschwander et al., 1985). In Portugal, laboratory studies (Gonçalves et al., 2008) confirmed that Cobrançosa presents lower susceptibility to olive fly when compared with either Madural or Verdeal Transmontana, a trend also observed in field studies (Cardoso et al., 2006; Bento et al., 2009).

The knowledge of the existence of differences in sensitivities of olive varieties to olive fly attacks, have great interest at the time of installation of the olive grove, especially in areas

of greatest risk of attack. As recommended protection against olive fly on integrated protection strategy should be used varieties of epicarp with great hardness (Gomes & Cavaco, 2003).

1.3.1.3. Early harvest

The harvest timing is considered an indirect mean to control olive fly damage It is known that most of fruit drop occurs between early November and early December (Patanita et al., 1997), falling down in this period about 73% of all fallen infested fruits. Thus, an earlier harvest time, relative to the traditional ones, eliminates pest control applications late in the growing season and reduces the damage caused by olive fly attacks. According Topuz & Durmusoglu (2008) the suitable harvest time, it was found when fruit maturation index is between 2.5 and 3.5. This index point coincides with the ripening stage when the purple speckles form on the fruits until a complete darkness. After that time the rise in olive oil formation and fruit drops caused by natural factors and *B. oleae* increase. Anticipation of harvesting is indicated in Italy (Petacchi et al., 2003) as preventive measure to reduce impact of olive fly. Studies in Trás-os-Montes region also showed interest in anticipation of harvest time (Cardoso et al., 2006).

1.4. Direct control measures

The aim of direct control measures is to control and if possible to destroy the enemy of crop and to prevent eminent losses (Amaro, 2003). Among the direct means of protection against olive fly compatible in organic production, the application of copper and kaolin-based particle film as physical barrier or repellent against adults of olive fly have been tested. On the other side biological control using entomopathogenic fungi and nematodes as well the use of *Bacillus thuringiensis* Berliner were used. For biotechnical control various types of traps, including mass trapping with OLIPE trap, was been used.

1.4.1. Biological control using entomopathogenic fungi

The use of entomopathogenic fungi as biological control agents has shown great potential against agricultural pests. The entomopathogenic fungi act, usually by contact,

penetrating through the cuticle of insects (Federici, 1999). The spore or conidia in contact with the cuticle of the insect, under favourable conditions of humidity, germinate and germ tube penetrates through the cuticle. When reached the hemolymph, the hyphae develop and colonize the entire body of the insect, causing their death within 7 to 10 days. Some fungi produce protein toxins and these strains may shorten the death of the insect within 48 hours (Federici, 1999). Are known about 800 entomopathogenic species, but only about a dozen of species have demonstrated capability for use as bioinsecticide due to technical and economic difficulties in its production (Fargues, 2001). *Metarhizium anisopilae* (Metchnikoff) Sorokin, *Beauveria bassiana* (Balsamo) Vuillemin and *Paecilomyces fumosoroseus* (Wize) Brown & Smith have been recognized as some of the most important entomopathogenic fungi against dipteran (Steinkraus et al., 1990; Watson et al., 1995). Some preliminary works of laboratory, semi-field and field trials showed that Tephritidae *C. capitata*, *Rhagoletis cerasi* Loew and *B. oleae* are susceptible to infection caused by *B. bassiana* (Konstantopoulou & Mazomenos, 2005; Daniel et al., 2008; Quesada-Moraga et al., 2008; Mahmoud, 2009) and soil applications of entmopathogenic fungi have low impact on beneficial fauna (Garrido-Jurado et al., 2011). Also Youself et al. (2013) showed that *M. brunneum* (Petch) have potential for controlling adults and preimaginals stages of *B. oleae*. As a result of these studies, microbial control with entomopathogenic fungi has been shown to have potential as an alternative approach to olive fly management (Quesada-Moraga et al., 2009). Furthermore, it was also demonstrated that soil applications of entomopathogenic fungi beneath the tree canopy for the control of *B. oleae* pupae could have an impact on soil-borne plant pathogens (Lozano-Tovar, et al., 2013). However, according to Maurer et al. (1997) the ability of entomopathogenic fungi on insect populations regulate depends on the specific association of host-pathogen thus, the use of local isolates and knowledge of their diversity, in bioinsecticide formulation is essential for their future use in a given region.

1.4.2. Biological control using entomopathogenic nematodes

The entomopathogenic nematodes have been used against many insects, including Diptera (Grewal et al., 2005; Karagoz et al., 2009). Nematodes, especially of the genera *Steinernema* and *Heterorhabditis* have reduced size (1 to 3 mm) and are parasitic in the soil. Nematodes penetrate their host by anal orifice and the mouthparts, through the cuticle and spiracles (Figueiredo, 1997). Inside the insect, the nematode reaches hemolymph and feed, releasing a symbiotic bacterium that colonizes the insect and by action of toxins causes their

death in less than 24 hours (Figueiredo, 1997; Federici, 1999). Nematodes of the Steinernematidae and Heterorhabditidae families are the only ones considered entomopathogenic for causing insect death within 24 hours being the remainder considered parasites (Figueiredo, 1997). Tests carried out by Sirjani et al. (2009) show that a commercial product based on the nematode *Steinernema feltiae* is effective in some applications to the soil against larval instar of *B. oleae*.

1.4.3. Other microorganisms and their products

Bacillus thuringiensis – *B. thuringiensis* (Bt) is a biopesticide based on *B. thuringiensis* spp. kurstaki and Bt spp. conjugate kurstaki X aizawai. Many crystalline proteins characterized by their entomopathogenic activity are highly specific for several insect orders as Lepidoptera, Diptera, Coleoptera and other invertebrates (Ilias et al., 2013). *B. thuringiensis* is a common member of the microbiota in the olive tree environments, and in the last years there has been a great interest in search, analyze and triage isolates of that bacteria in different geographical olive growing regions (Alberola et al., 1999; Cinar et al., 2007), to verify the existence of strains of *B. thuringiensis* toxic to larvae and adults of olive fly (Alberola et al., 1999; Navrozidis et al., 2000). The biological activity of Bt strains against larvae and in new emerged adults was demonstrated (Alberola et al., 1999; Ilias et al., 2013) opening the possibility of using this bacterium against this pest.

Spinosad – The Spinosad, a mixture of spinosyns A and D derived from the naturally occurring soil bacteria *Saccharopolyspora spinosa* Mertz & Yao (Sparks et al., 1998), is a relatively new insecticide with efficacy against a wide range of insects, including the olive fly (Poullot & Warlop, 2002). It was commercially introduced in 1997 and is formulated as a bait (GF-120 Naturalyte Insecticide; Dow Agrosciences, Indianapolis, IN) for control of *Ceratitis* spp. and *Bactrocera* spp. (Tomlin, 2004), and has shown good efficacy on the olive fly under laboratory tests (Poullot & Warlop, 2002) and later it was used in commercial olives, mainly in California (Nadel et al., 2007). According to Zalom et al. (2003) GF-120 applications should start when olive fly adults are captured on the monitoring traps or at least 2 to 3 weeks before pit hardening. In Portugal is recommended by DGADR (DGADR, 2011) a commercial product (Spintor Isco) based in Spinosade, for *B. oleae* control.

Others bacteria – Recently, a study performed by Mostakim et al. (2012) shown that a biological compound produced by *Pseudomonas aeruginosa*, which is an antifungal product

constituted by antimicrobial metabolites such as siderophore pyoverdine and salicylic acid, have larvicidal activity against third instar larva of *B. oleae*.

1.4.4. Mass trapping with Olipe traps

The bottle trap known as Olipe trap was developed in Spain for olive groves in organic production in 1997, by the cooperative Olivarera de los Pedroches (Olipe) (Caballero, 2001). This trap consists of a polyethylene terephthalate (PET) translucent bottle with 1.5-2 liters of capacity (Figure 1.2) used in the drink marketing, perforated and which is placed inside of the bottle the food attractant, generally ammonium salts or hydrolysed proteins, and sometimes a pheromone. The addition of a spiroketal pheromone (1,7-dioxaspiro-[5.5]undecane) to improve the attractantness to male flies had been used in some countries when there is high population of *B. oleae*, which are placed in trees from September (Luque & Pereda, 2003). The holes are drilled with a metal template, with the desired diameter, heated in flame, melting several holes in the plastic bottle on the upper part to allow the release of volatiles and the entry of the flies. Traps are hanged in a branch south side of the tree.

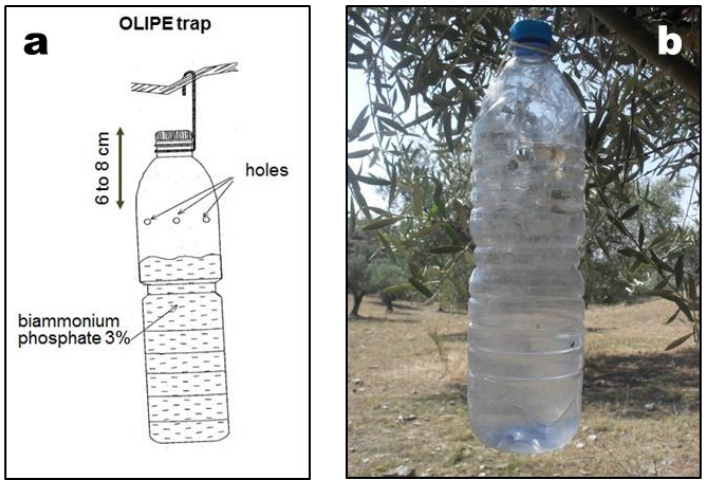

Figure 1.2. Representative scheme of Olipe trap (adapted from Vossen, 2006) (a), and its hanging in the tree (b).

The use of these traps may be an adaptation to the traps used by Zervas (1982) in Greece, which consisted of plastic bottles of 1.5 liters, with a solution attractant placed inside of the bottle and coated outside with an adhesive to capture insects. In the case of Olipe traps

developed in Spain, flies are attracted by the solution, usually ammonia, entering through holes made in the bottles with sufficient size to allow entry and eventually drowned in the solution.

For the mass trapping results effective, traps should be placed at a high density on extensive surface of olive groves and before starting stings in fruits (June) until harvest (Broumas et al., 2002). According to Caballero (2001) and Luque & Pereda (2003) the recommended period of use for Olipe traps begins in late spring/early summer and ends in late autumn.

According to Vossen (2006), the success of the Olipe traps in Spain may due in part to the high temperatures and scarcity of water. Besides the use in Spain, Olipe traps for controlling olive fly population, were also tested in recent years in some countries of Mediterranean region as Greece (Eliopoulos, 2007), Italy (Caleca & Maltese, 2007), Portugal (Pavão et al., 2007), Middle East (Sorosh et al. 2011; Tabic et al., 2011) and also in the United States of America (Vossen, 2006). Mass trapping with Olipe traps was tested in Portugal for the first time in organic olive groves in Trás-os-Montes region (Bento et al., 2003) and later in Beira Interior region (Coutinho et al., 2011).

In organic olive growing, mass trapping with Olipe traps is a promising option due to its low cost and effectiveness (Altolaguirre-Obrero et al., 2003), being in some cases sufficient to control pests and keep the damage below 10% (Vossen et al., 2004). However, when there is high population density of olive fly, mass trapping with Olipe traps alone cannot control *B. oleae* and this technique should be complemented with others preventive measures such as anticipation of harvesting.

Although this control method is able to reduce the olive fly population levels it also showed limited efficacy (Zervas, 1982; Ros et al., 2009; Coutinho et al., 2011) and adverse effects on beneficial fauna (Pereira et al., 2007; Seris et al., 2007; Porcel et al., 2009a) which emphasizes the need to improve the effectiveness of these traps and reduce the side effects on beneficial fauna. Despite the effectiveness of the attractant has been studied in greater detail, other features used in relation to bottles, which can play a role in the efficacy and selectivity of this type of trap, as well as colour of bottle, number of holes, the hole size and position of holes in relation to the bottle top. A better understanding of these characteristics can help achieving more effective traps to capture olive fly adults and reduce their impact on beneficial fauna.

1.4.4.1. Food attractants used in Olipe traps

The ammonia salts and attractants based on hydrolysed proteins have been widely used by farmers in all olive growing regions with Olipe traps for the control of adult flies in olive growing under organic production. Ammonia salts (aqueous solutions of sulphate, carbonate or ammonium phosphate) were the first attractant sources used to control olive fly and remain the most used attractant in Olipe traps in the regions where that kind of traps are used by farmers due its low price. The use of ammonium phosphate as attractant for the olive fly is referenced to the year 1933 in Spain (Garcia-Rojas et al., 2002), being considered the most effective attractant for controlling *B. oleae* being actually the most used attractant in capture assays for this pest. In these first studies on the attractant power of ammonium phosphate, conducted using McPhail traps was demonstrated that low concentrations (2-3%) in phosphate would be the best to attract olive fly adults (Garcia-Rojas et al., 2002). Actually, ammonium salts concentration 3-4% has been the most used in trials with Olipe traps), being the Olipe traps renewed weekly (Garcia-Rojas et al., 2002; Luque & Pereda, 2003; Coutinho et al., 2011) or fortnightly (Ros et al., 2009; Coelho et al., 2010; Muñoz & Marí, 2012).

The ammonium salts, when used in combined with Olipe traps, have demonstrated lower attractantness than most of the hydrolysed protein used (Zervas, 1982; Ros et al., 2009; Despite the lower power of attraction of the phosphate, the choice for solutions with sulphate and ammonium phosphate as attractant for Olipe traps is mainly due to its low price compared to other attractants with higher attractantness (Zervas, 1982; Ros et al., 2005). Also ease of preparation of the solution and easier handling and count the insects is one aspect to consider. Relatively to the use of ammonium salts, the duration of this appeal may be a limitation of Olipe traps because as the aqueous solution evaporates, the concentration of ammonia increases, losing the power of attraction of the solution. Due to loss of attraction by solution evaporation, Luque & Pereda (2003) recommends a maximum time of 15 to 20 days without renewal of attractant. However, Olipe traps require less maintenance than, for example, McPhail traps, because it does not evaporate so quickly in plastic bottles, although the solution has to be changed regularly (Devarenne & Vossen, 2007).

The attractant food based in hydrolysed proteins, used very often for Mediterranean fruit fly, *C. capitata*, monitoring, attracting and capturing in association with McPhail traps (Leblanc et al., 2010a) and it was subject of study in recent years in Olipe traps to control olive fly. This kind of attraction is based on the need for protein by the Tephritidae females during egg formation (Piñero et al., 2002). Hydrolysed corn protein also known as Nulure

(Vargas & Prokopy, 2006) was used as food attractant which generally is added 3% sodium borate (borax) as a preservative in order to retard the decomposition. Hydrolysed protein Tephri Lure® (Sorygar Co, Madrid), another attractant food has already incorporated glycol and sodium borate (Seris et al., 2007; Ros et al., 2009) attractant, Entomela 50® (Garcia-Rojas et al., 2002), a nutritional liquid based on animal proteins and plant, used at a concentration of 33%, have been tested in recent years in Spain in Olipe traps. The food attractant Endomosyl, a concentrated solution of hydrolysed protein was used in Olipe traps, especially in tests carried out in Greece (Zervas, 1982) and Portugal (Pereira et al., 2007; Pavão et al., 2007). In Greece, the attractant food Dacus bait®, a solution based on hydrolysed vegetable protein, rich in amino acids containing 3-4% ammonium salts and hydrolysed protein Buminal® were also tested for the control of olive fly in organic olive groves. In the United States, the use of Torula in controlling several species of Tephritidae has been very common. This attractant contains yeasts of the genus *Cryptococcus* (before Torula) proceeding from production of *Cryptococcus luteolus* (Saito) Skinner, using as substrate sulfite solution which originates as waste from the production of wood pulp in papermaking (Frágenas et al., 1996). Torula yeast tablets (ERA International, Freeport, NY) dissolved in water was used by Vossen (2006) in Olipe traps in trials conducted in California (USA) for controlling olive fly.

In Spain, the use of hydrolysed proteins in Olipe traps to capture *B. oleae* has demonstrated better efficacy in the capture of olive fly adults when compared to aqueous solutions of ammonium salts (Ros et al., 2009). Also in California (USA) Vossen (2006) showed that the attractant based on Torula yeast captured four times the olive fly than the ammonia-based solution. In olive groves under organic production of Trás-os-Montes region (Portugal), the use of hydrolysed protein in Olipe traps resulted in a reduction of *B. oleae* infestation in the plots tested with traps compared to the control (Bento et al., 2003).

One disadvantage in using these proteins as attractant in Olipe traps is its high price and economic studies will be needed to see if the cost of mass trapping with these proteins is compatible with sustainable olive growing.

Besides the attractant ammonium base and hydrolysed proteins other compounds have been studied as attractant in Olipe traps for controlling the olive fly in organic olive groves. In Trás-os-Montes (Portugal) urea was tested (Pavão et al., 2007; Pereira et al., 2007) as food attractant in Olipe traps. Although efficacy was similar to hydrolysed protein in olive fly catches, this compound revealed a negative impact on beneficial fauna. Caleca et al. (2007) based on use by Italian farmers of baits based on sardines in plastic bottles to control olive fly

in organic groves, tested the use of salt as sardine as food attractant for traps Olipe trap, having this kind of attraction shown to be effective in capturing of olive fly adults.

It is known that climatic factors influence both the duration of the attractant or the effectiveness of traps. Thus, high temperatures and wind facilitate evaporation which can be a disadvantage because the traps lose effectiveness (Caballero, 2002) due to loss of water in solution increasing the concentration of ammonia, losing its power of attraction. Also frequent rainfall, which often is recorded in October, decreases the effectiveness of these traps (Altolaguirre-Obrero et al., 2003; Caballero, 2002).

1.4.4.2. Characteristics of bottles used as Olipe traps

Olive fly is very mobile and has the ability to seek out cooler areas within the olive grove (Vossen et al., 2004). In the case of mass trapping with Olipe traps is recommended to hang the traps, usually between 1.5 and 2.0 meters high, in the shade of the south or southeast side of the tree, since this orientation is preferred for oviposition by *B. oleae* (Montiel-Bueno & Vásquez, 1984). It is preferable to choose it placement within the canopy to prevent evaporation of the attractant (Caballero, 2002). For mass trapping, Olipe traps can be placed at a high density (up to one per tree) (Vossen et al., 2004). The high density of traps used in this method requires a high number of traps and manpower either for transport of traps inside the olive grove and hanging traps in each tree either to periodically renew the attractant (Caballero, 2002), which can be a disadvantage of this method. According Caballero (2002) decreasing trap density to 1 trap every 2-3 trees the economic cost would be lower.

According to Luque & Pereda (2003) some variables concerning to bottle used as Olipe traps such as colour, number of holes, the hole size and position of holes in relation to the bottle top may influence the effectiveness of these traps in catching *B. oleae* adults.

In order to evaluate the effect of different number and hole sizes in catching olive fly adults some studies have been conducted in recent years in olive groves under organic farming. The hole size is of great importance, because its size may interfere with the number of catches of olive fly adults either the amount of volatile released from solution used as attractant. Also, according to Luque & Pereda (2003) increasing the number of holes we can increase the effectiveness of these traps to capture more flies, without changing its selectivity due to increased ventilation, facilitating the evaporation of ammonia compounds. In

perspective to improve these characteristics different holes sizes were tested ranging the diameter between 3 and 20 millimeters.

In Spain, the study of the different hole sizes of Olipe traps revealed that traps with larger sizes diameters are those that capture more adults of olive flies (Altolaguirre-Obrero et al., 2003; Duatis et al., 2006). Altolaguirre-Obrero et al. (2003) found in Olipe traps with 8 mm of hole size that catches were higher than other hole sizes, obtaining even higher catches than McPhail traps. According to Luque & Pereda (2003) the Olipe traps with best performing are those that have 7 mm of hole size in catches of *B. oleae* adults, although its selectivity is lower compared to smaller diameters. Also in Trás-os-Montes region (Portugal) where different hole sizes were tested (4, 6, 8 and 10 mm of diameter), has been found that the percentage of infested fruits was low in blocks tested with Olipe traps with greater hole size (8 and 10 mm of hole size) (Coelho et al., 2011b). Relatively to position of holes in bottle, the few existent studies did not allow to retain conclusions about the influence of position of holes in the efficacy of traps.

Tests concerning colour of the bottles used as Olipe traps were performed by Zervas (1982) in Greece. In his assays he demonstrated that regardless of attractant used, catches of olive fly adults were always higher in bottles that were painted with yellow colour. Also García-Rojas et al. (2002) refer that if the bottles were painted with yellow colour it could increase the effectiveness of these traps. The use of yellow Olipe traps could be an alternative to consider since the olive fly, as well as many other species of Tephritidae respond strongly to visual stimuli in nature, being very attracted by the yellow colour (Prokopy et al., 1994; Economopoulos, 2002), especially fluorescent yellow, which reflects between 500 and 580nm like bright leaves (Economopoulos, 2002). According to Duatis et al. (2006) the use of yellow colour in the trap did not increase the capture efficiency of the trap. In other hand, painting the traps with the yellow colour has the disadvantage of attracting other insects and when used in high densities can cause adverse effects on beneficial fauna. Beyond the side effects this practice may not be economically viable from the point of view of organic olive growing.

1.4.4.3. Impact of Olipe traps on beneficial fauna

Although the environmental impact of Olipe traps is considered low in relation to chemical control, mass trapping can change the arthropod community due to the capture of beneficial fauna (non-target) (Zervas, 1982; García-Rojas et al., 2002; Luque & Pereda, 2003;

Pereira et al., 2007; Seris et al., 2007; Porcel et al., 2009a; Coelho et al., 2010). The degree of selectivity of the mass trapping is conditioned by the kind of trap and attractant used. Due to the low selectivity of this trap and attractant used that can be harmful for beneficial fauna, various studies have been performed involving either the diameter of the hole size and the different attractant used in these traps in order to analyse the secondary effects of mass trapping with Olipe traps.

The food attractants are known to attract together with the target pest many non-target arthropods (Leblanc et al., 2010b). For a better understanding about the impact of different attractants on the beneficial fauna, the combination of Olipe traps with different attractant was tested in several field trials carried out in Iberian Peninsula. The attractants most commonly used in Olipe traps, ammonia salts and hydrolysed proteins, had a negative impact in crysopidae population in the various tests carried out (Pereira et al., 2007; Coelho et al., 2010; Seris, 2011). Chrisopids are generalist predators and important natural enemy of several olive pests as olive moth, (*Prays oleae*) black olive scale (*Saissetia oleae*) and olive psyllid (*Euphyllura olivina*) (Campos & Ramos, 1983). Some studies also showed that Chrysopidae adults are found among the predators more captured in traps used for *B. oleae* control (Zervas, 1982; Campos & Ramos, 1983). Also urea, an attractant tested in Trás-os-Montes region besides negative impact on the capture of non-target arthropods showed to be harmful for Chrysopidae adults (Pereira et al., 2007). Relatively to Hymenoptera parasitoids, which represents an important beneficial group in olive agroecosystem and have a principal role in the biological control of some olive key pests (Bento et al., 1998), Porcel et al. (2009b) found that ammonia salts used in Olipe traps, in Trás-os-Montes region, were the most harmful for parasitoids among the attractants tested. This author, in another study in Spain, also verified that Entomela attractant had a negative impact in parasitoid population when compared with ammonia salts (Porcel et al., 2010). Ants were another important group abundant in catches by Olipe traps. Regardless of the characteristics of the bottle used as a trap or the kind of attractant used into the trap has been found that Olipe traps have an impact on ants (Caballero, 2001; Pereira et al., 2007; Porcel et al., 2009a; Coelho et al., 2010; Seris, 2011). Ants have an important role in the olive agroecosystem, participating actively in natural control exercising predatory action on *B. oleae* larvae and pupae in canopy and soil (Arambourg, 1986; Katsoyannos, 1992) and other phytophagous species (Varela & González, 1999).

In field studies where it was tested the effect of different hole size on beneficial fauna it was found that traps with bigger hole sizes have lower selectivity for beneficial fauna,

exerting a negative impact especially on populations of parasitoids (Luque & Pereda, 2003) and crisopids (Luque & Pereda, 2003; Coelho et al., 2010). Although there is greater efficiency in capturing olive fly adults in Olipe traps with bigger hole size, also the selectivity of the traps decreases when increases the hole size. Luque & Pereda (2003) points out that ideal trap would be if it is used hole size with a diameter between 3 and 5 mm. The adaptation of a net in this kind of traps, when high hole sizes are used, to prevent the passage of non-target insects may be useful to help preserve the beneficial fauna. It is known that the adaptation of the nets in other kind of traps has been provided with good results (Seris, 2011).

The use of selective Olipe traps for beneficial fauna becomes important because in the olive groves there is a great diversity of insects that are important in biological control of many pests.

1.5. Other methods

The use of repellents and anti-oviposition in control of Tephritidae has great interest in organic farming (Caleca et al., 2010), being verified the effectiveness of some clays and copper products in *B. oleae* in the past. Both products have been widely used as physical barriers or repellents against adults of olive fly and are listed in the European Council Regulation (Reg. CEE 2092/91).

The application of copper, universally known as a fungicide and bacteriostatic, makes the fruit less attractant for the females to lay their eggs due to loss on the surface of bacterial compounds (Belcari et al., 2003). The application of kaolin to olive tree forms a thin film which gives a brilliant white colour and may compromise the possibility for the location of the fruits/plants by the insects. On the other hand the surface texture of the fruit will repel gravid females that contact them (Saour & Makee, 2004).

Recently, the use of kaolin and copper based products in controlling *B. oleae* has awakened great interest in Italy (Caleca et al., 2010) in Spain (Pascual et al., 2009) and in Syria (Saour & Makee, 2004), with its use in preliminary studies revealing interesting results. Although this control measures has no negative effect on olive oil (Perri et al., 2005), it was found that the application of kaolin (Surround WP) (Iannotta et al., 2007; Pascual et al., 2010; Bengochea et al., 2013; Bengochea et al., 2014) and copper (Iannotta et al., 2007; Gonçalves & Torres, 2012; Bengochea et al., 2014) have an impact on the community of beneficial

arthropods. However it should be noted the restrictions on the use of these products by Regulation CE nº 473/2002, due to the risk of accumulation on soil.

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CHAPTER 2

2. Objectives

This doctoral thesis had the overall aim to study the integration of different control methods to manage the olive fly, in sustainable olive growing, particularly: (1) strategies to increase the role of natural enemies by native flora management; (2) the virulence of different isolates of the entomopathogenic fungus, *B. bassiana,* on *B. oleae* pupae; and (3) optimizing the use of Olipe traps in olive fly mass trapping. To achieve this overall objective, the following specific objectives were established as indicated below:

Objective 1 – effect of natural vegetation in the biodiversity and natural control of olive fly:

1.1) to investigate the abundance of arthropods on a representative herbaceous plant, *Chondrilla juncea,* in an olive grove from Trás-os-Montes (Portugal) and their role in provide alternative hosts for parasitoids of olive fly as a measure of conservation biological control against the pest (*Chapter 3*).

1 2) to study the abundance and diversity of carabids in olive groves with soil covered by spontaneous vegetation in Trás-os-Montes region (Portugal) (*Chapter 4*).

1 3) the effect of different food sources on the life parameters, longevity and reproduction of *P. concolor*, with the objective to improve the rearing and maintenance of this parasitoid in the laboratory and in the manipulation of habitat to ensure success in the parasitoid introduction in biological control programs (*Chapter 5*).

Objective 2 – Evaluation of the use of *Beauveria bassiana* isolates in controlling olive fly in laboratory tests:

2.1) compare the pathogenicity of four *B. bassiana* isolates on *B. oleae*, *C. capitata*, and *R. cerasi* pupae using sand-conidial suspension incorporation bioassay and determine the most virulent fungal isolates against these fruit flies (*Chapter 6*).

Objective 3 – Mass trapping using Olipe traps in the control against olive fly:

3.1) to report and discuss the progress registered in the knowledge of the *B. oleae* bio-ecology in the northeast of Portugal, the losses caused by the insect, the monitoring of the pest and evaluating mass trapping effectiveness using Olipe traps as control method against olive fly in sustainable olive production system, testing different bottle hole size (10, 8, 6 and 4 mm) and observing the difference between different hole sizes in reducing fruit infestation levels (*Chapter 7*).

3.2) study the effect of different bottle hole sizes in a mass-trapping experiment with Olipe traps, observing the difference between four different access hole sizes (4, 6 8 and 10 mm) in captures of non-target arthropods and their impact in beneficial groups of olive grove (*Chapter 8*).

CHAPTER 3

Arthropodofauna associated to *Chondrilla juncea* L. and their possible role in conservation biological control in an organic olive grove from Trás-os-Montes (Portugal)



E abaixava-se a agarrar uma louva-a-deus. Tirava um frasco do bolso, pegava na infeliz com mil cuidados, não lhe fosse quebrar um braço, e bojo do vidro com ela.

A princípio, todos arregalaram os olhos, num justo e desconfiado espanto. No que dera o filho do Sr. Adriano Gomes! Mas apenas lhes arrendou, por umas cascas de alho, os bens de que passara a ser dono, e o viram contente com a transacção, mudaram de ideias e puseram-se a vender-lhe quantos insectos havia nas redondezas. Bastava chegar ao pé dele e mostrar-lhe uma joaninha, para que a comprasse logo por um tostão. De modo que semelhante maluqueira era uma mina, vista por qualquer lado.

Miguel Torga – Os bichos (1940)

Abstract

Currently, the management of agricultural habitats to optimize the action of natural enemies is a form of conservation biological control. Spontaneous vegetation, as components of agroecosystems, can positively affect the dynamics of beneficial insects. In this work, the action of *Chondrilla juncea* L., an abundant plant in the olive groves of the Northeast of Portugal, was evaluated in order to understand their role as host of alternative preys to the beneficial fauna fauna of olive groves, which may contribute to the increase of natural enemies of olive pests and consequently to increase natural biocontrol. In an olive grove from Trás-os-Montes (Portugal), the abundance of arthropods in *C. juncea* were evaluated weekly in 50 plants randomly collected during its flowering period in three consecutive years (2009, 2010 and 2011). In laboratory the arthropods found were separated, sorted and identified to order, family or species. A total of 11,098 arthropods were observed in three years of the study belonging to 20 taxa. The most abundant taxa were Aphididae, Diptera and Thysanoptera. The Diptera are particularly important either by number of individuals or by being attacked by different species of parasitoids, which may act as natural enemies and/or prey/host of predators of the olive fly, *Bactrocera oleae* (Rossi). *C. juncea* may also provide alternative hosts to parasitoids, may be important in establishing and maintaining populations of parasitoids that may contribute for the control of *B. oleae*.

Key-words: conservation biological control, aphids, Diptera immatures, parasitoids

3.1. Introduction

The olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is a key-pest of olive tree in the Mediterranean region, and is also considered one of the most important enemies of crop in the Trás-os-Montes region. Its control has been traditionally carried out by insecticide application (Broumas and Haniotakis, 1994; Haniotakis *et al*., 1991) having negative effect on beneficial fauna of olive groves (Ruano *et al*., 2001) in losses of biodiversity and development of pest resistance (Hawkes *et al*., 2005; Marc *et al*., 1999). The need to reduce pesticide applications in sustainable production such as organic agriculture and integrated pest management, justify the development of alternative measures to control olive fly. As alternative measures for olive pests control, the integrated production rules of the International Organization for Biological Control and Integrated Pest Management (Boller *et al*., 2004; Malavolta and Perdikis, 2012) recommend the promotion of biodiversity, to be considered an important element of agricultural sustainability. Once, the increase of diversity may be a requisite for improving the natural control of arthropods pests (Landis *et al*., 2000). Habitat management is a strategy of conservation biological control which seeks enhancing natural enemies. In this strategy, favoring ecological infrastructures in agroecosystems may provide food, alternative hosts and shelter to natural enemies (Landis *et al*., 2000). Weeds as components of agroecosystems could positively affect the dynamics of beneficial insects. Weeds offer many important requisites for natural enemies such as alternative prey/host, pollen, or nectar as well as microhabitats (van Emden, 1965). Some studies have shown that non-crop vegetation provide habitat for natural enemies (Maudsley *et al*., 2002; Schmidt and Tscharntke, 2005) and may support alternative hosts and prey for predators and parasitoids (Clark *et al*., 1997; Boller *et al*., 2004). The presence of alternative hosts and prey may increase parasitoid and predator populations which can result in an improvement of pest control (Altieri, 1994; Landis *et al*., 2000; Östman, 2004; Tscharntke *et al*., 2005; Pontin *et al*., 2006).

In traditional non-irrigated olive groves from Trás-os-Montes (Northeast of Portugal), the long dry season leads to soil tillage or herbicides application in order to eliminate non crop plants that compete for water with olive trees. However, herbicide applications have negative impacts for arthropod biodiversity (Campos *et al*., 2000) and tillage promotes soil erosion, destroying olive roots, and decreasing soil organic matter. The use of spontaneous vegetation in olive groves is considered particularly interesting to protect the soil from erosion, helps to increase water infiltration, provide organic matter, and improves microbial

activity (Saavedra and Pastor, 2002). On the other side, favors the development of beneficial fauna and may contribute to the biological control of pests as they are often source of nectar, pollen or honeydew of other insects, which are vital to maintaining high populations of beneficial insects within an ecosystem (Escudero, 2004). Some studies have reported also that native plants can be as valuable as the exotic, with the advantage of being locally adapted and its use could contribute to increase the local biodiversity (Fielder and Landis, 2007). In recent years, a systematic work was developed to evaluate the action of some plants as sources of host/alternative prey to the beneficial fauna of olive groves (Campos and Civantos, 2000; Villa *et al*., 2012; Paredes *et al*., 2013), which may contribute to the increase of natural enemies of olive pests and consequently to increase its action.

Chondrilla juncea L., (Asteraceae), known as skeleton weed, is a biennial to perennial plant distributed from Southern and Central Europe to Northern Africa, Southern Russia, and Southwest Asia. This plant is widespread in north to south of Portugal and it is grows in cultivated and uncultivated fields and it is also abundant in olive groves in Trás-os-Montes region. .

Interactions between weeds and beneficial arthropods form the basis for increasing biodiversity as a mean to enhance arthropod pest management (Norris and Kogan, 2005). In this context, the aim of this study was to investigate the abundance of arthropods on a representative herbaceous plant, *C. juncea*, in an olive grove from Trás-os-Montes (Portugal) and their role in providing alternative hosts for parasitoids of olive fly as a measure of conservation biological control.

3.2. Materials and Methods

3.2.1. Study area

The work was conducted in three consecutive years (2009, 2010 and 2011) in an olive grove located in Cedães (Mirandela - Northeast of Portugal, 41º29’20.76”N, 7º07’36.02”W) following to Integrated Pest Management practices since 2001.The olive trees are medium sized and belong to cv. Cobrançosa. The area of the plot is 4 ha and the planting density is of 7 × 7 m and soil is conducted with natural vegetation. The trees are pruned every two to three years. No phytosanitary treatments were done during the experiments.

3.2.2. Arthropod sampling

Sampling was carried out weekly during the peak of the flowering period of *C. juncea* from July to November during three consecutive years (2009, 2010 and 2011). To study the arthropodofauna existing in *C. juncea* were harvested randomly in the olive grove fifty plants. Plants have been put into plastic bags to prevent the insects from escaping and transported to the laboratory. In the laboratory, harvested plants were observed under the binocular stereomicroscope and all arthropods found in leaves, stems or flowers were collected, including immature stages present in the flowers. They were all, counted and transferred for tubes with 70% ethanol to proceed to the subsequent identification. Arthropods were sorted and identified until orders, family or species taxa and the total number of each taxon was recorded. Formicidae, Coccinellidae and Aphididae families were identified to species level. Formicidae family was identified according to Collingwood and Price (1998). Coccinellidae species were identified according Raimundo and Alves (1986). Spiders were identified according to Nentwig *et al*. (2013) and Roberts (1985). Aphididae was identified according to Nieto Nafría and Mier Durante (1998) and Nieto Nafría *et al*. (2002, 2005). The abundance and average number of arthropods per plant during the test was calculated. Results are presented as percentage or mean and standard error (SE). The number of species present - richness (S), their relative abundance - evenness (E) and diversity (H', D and 1-D) were used in this study as biodiversity descriptors. The value of E ranges between 0 and 1 with 1 representing a situation in which all species are equally abundant. Shannon's index (H') accounts for both abundance and evenness of the species present. The proportion of species (i) relative to the total number of species (pi) is calculated, and then multiplied by the natural logarithm of this proportion (*lnpi*). S is the numbers of species encountered.

$$H' = -\sum_{i=1}^{s} piLnpi$$

Simpson's index (D) is based on the probability of any two individuals drawn at random from an infinitely large community belonging to the same species, where pi is the proportion of individuals found in species i. The value of D ranges between 0 and 1. With this index, 1 represents infinite diversity and 0, no diversity.

$$D = \sum p^2 i$$

From Simpson's index (D), Simpson diversity index (1-D) was found. For these analyses, larvae and pupae of diptera were considered together.

3.2.3. Survey of **Chondrilla juncea L.** *as parasitoid reservoir*

In laboratory, Diptera immature stages (larvae and pupae) found in flowers of *C. juncea* were recorded and placed in Petri dishes at room temperature until the emerge of the dipterans or parasitoids. Larvae were reared with flowers of *C. juncea* and in each Petri dish it was placed a small filter paper with humidity. The occurrence of parasitoids and fly adults was checked daily, and the parasitoids were removed when they emerged. The emerged dipterans and parasitoids in Petri dishes were transferred to Eppendorf tubes without ethanol for later identification. Parasitism was estimated as percent parasitoids emergence, which was calculated by dividing the total number of emerged parasitoids by the sum of the number Diptera immature. In 2011 the counting of the number of flowers it was made in each plant and the number of flowers attacked by Diptera (larvae and pupae). Percentage of attacked flowers was calculated.

3.3. Results

3.3.1. Arthropod abundance

The arthropods collected in *C. juncea* were classified into two classes, Insecta and Arachnida, and 11 orders: Acari, Araneae, Coleoptera (larvae and adults of Coccinelidae and other Coleoptera), Diptera (larvae, pupae and adults), Hemiptera (Aphididae, Heteroptera, and others Hemiptera), Hymenoptera (Formicidae and Hymenoptera parasitoids), Lepidoptera, Neuroptera (larvae and adults of Chrysopidae), Orthoptera and Thysanoptera. The other eggs, larvae and pupae were classified separately.

A total of 11,100 arthropods were collected from *C. juncea* during the three years of sampling. In 2009, 4,486 arthropods (eggs included) were observed in *C. juncea*, 1,607 in 2010 and 5,007 in 2011 (Table 3.1). The class Insecta represented 96.7% of the total arthropods recovered in 2009, 94.3% in 2010 and 96.6% in 2011. The class Arachnida represented 2.7% of total arthropods recovered in 2009, 1.7% in 2010 and 3.4% in 2011.

In 2009, *C. juncea* arthropod community was numerically dominated by Aphididae (order Hemiptera), which represented 84.8% (4.2±0.3) (mean±SE) of total specimens

followed by Diptera immature (7.8%) (0.4±0.0) and Thysanopetra (3.0%) (0.2±0.0). In 2010, the arthropod community present in *C. juncea* was numerically dominated by Diptera immature (70.8%) (1.3±0.1) followed by Thysanoptera (11.3%) (0.2±0.0) and Aphididae (9.0%) (0.2±0.1).

Table 3.1. Number of arthropods captured in *Chondrilla juncea* L., in 2009 (*n=900*), 2010 (*n=850*) and 2011 (*n=1000*).

Taxa		2009	2010	2011	Total
Insecta					
Hymenopera					
	Parasitoids	6	9	1	16
	Formicidae	7	6	5	18
Coleoptera					
	Coccinellidae (L)	9	0	1	10
	Coccinellidae (A)	7	0	3	10
	Other Coleoptera	2	0	0	2
Neuroptera					
	Chrysopidae (L)	5	0	1	6
	Chrysopidae (eggs)	1	20	1	22
Diptera					
	(Diptera) Adults	1	5	3	9
	larvae	187	403	921	1,511
	pupae	161	734	1,774	2,669
Hemiptera					
	Aphididae	3,806	144	2,155	6,105
	Other Hemiptera	2	1	0	3
	Heteroptera	0	11	0	11
Thysanoptera		134	181	121	436
Lepidoptera		9	0	0	9
Orthoptera		0	1	0	1
Arachnida					
Araneae		8	15	5	28
Acari		115	13	13	141
Non-identified eggs		19	47	0	66
Non-identified larvae		5	1	3	9
Non-identified pupae		2	16	0	18
Total		4,486	1,607	5,007	11,100

L – larvae, A – adult, *n* – number of plants observed

In 2011, arthropod community present in *C. juncea* was numerically dominated by Diptera immature with 53.9% (2.7±0.1) of total specimens, being Diptera pupae represented 35.4% and Diptera larvae represented 18.4%, followed by Aphididae (43.0%) (2.4±0.2) and Thysanoptera (2.4%) (0.1±0.0).

The higher value of richness of groups recovered in *C. juncea* was found in 2009 with 18 groups of arthropods identified (Table 3.2), however, the diversity of groups was greater in 2010 than 2009 or 2011. In 2011, it was observed a dominance of two groups (Aphididae and Diptera immature). The groups found in different years were not evenly distributed, highlighting for each year a high dominance of a few groups of arthropods

Table 3.2. Richness (S), evenness (E) and diversity (H', D and 1-D) of arthropods captured on *Chondrilla juncea* L. in different years (2009, 2010 and 2011).

Year	S	E	H	D	1-D
2009	18	0.23	0.65	0.72	0.28
2010	15	0.41	1.11	0.52	0.48
2011	13	0.32	0.84	0.48	0.52

A total of 6,105 aphids were recovered in three years of the study. Population of aphids was higher in 2009 and 2011 than 2010. In 2009, 3,806 aphids were counted, representing 84.9% of all arthropods. Aphids were always present from 27th July to 2nd November, although the number of aphids per plant was very variable during this year, with maximum aphids per plant of 18.0±2.0 in middle October and minimum aphids per plant of 0.1±0.1 in begin of September (Figure 3.1).

In 2010 the aphids population was lower, representing aphids 9.0% of all arthropods and in some periods of the year aphis were not detected on *C. juncea*. The maximum aphids per plant of 2.7±1.2 occurred at 19th October. In 2011, aphids were always present from 29th June to 9th November, with maximum aphids per plant of 9.3±1.8 at 26th October, and minimum was less than 0.01 aphids per plant in the end of August. In all years there was an increase of aphids populations in *C. juncea* in middle/end of October. After identification of aphids specimens it was found one single species associated with *C. juncea* in this olive grove, *Uroleucon chondrillae* (Nevsky) (Aphididae: Dactynotinae). The presence of *U.*

chondrillae in *C. juncea* has also been described by other authors (Wapshere, 1970; Cullen, 2012).

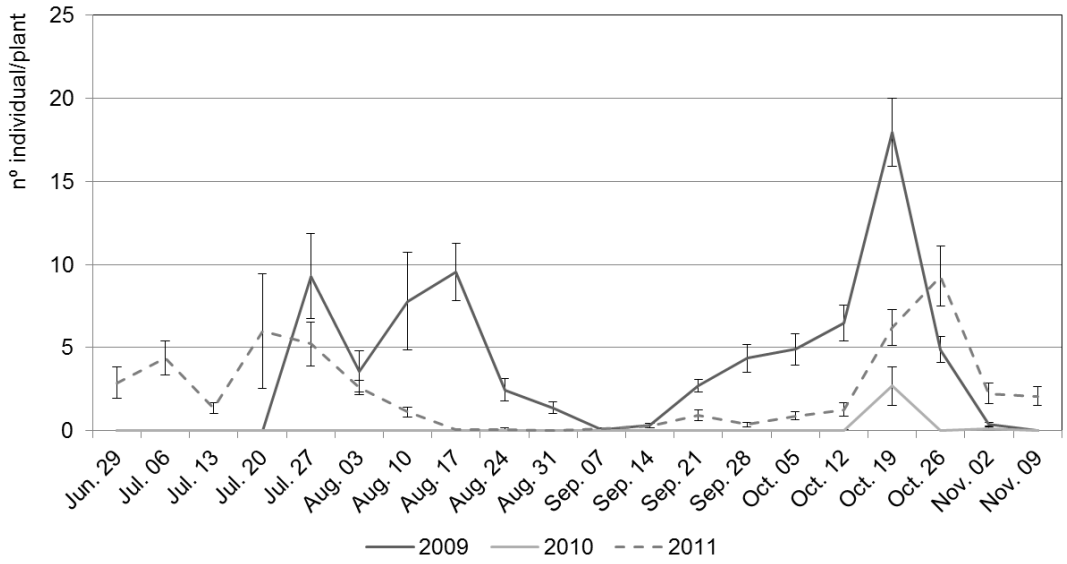

Figure 3.1. Mean number of aphids (Aphididae: Hemiptera) per plant present in *Chondrilla juncea* L. during the time of study (2009, 2010 and 2011) (vertical lines mean standard error).

The groups Araneae, Formicidae, larvae of Chrysopidae, larvae and adults of Coccinellidae are important predators in olive orchards. These groups of predators and Hymenoptera parasitoids are groups regarded as beneficial fauna in olive groves by their important role in biological control through depredation and parasitism (Neuenshwander, 1982; Varela and Gonzáles, 1999; Ruano *et al.*, 2001). The beneficial fauna represented 1.9% of total arthropods in 2010. In 2009 and 2011, their number was lower than 1.0%.

A total of 18 specimens of Formicidae belonging to three subfamilies and seven species were collected in all three years in *C. juncea*. The identified species in decreasing order of abundance were: *Crematogaster auberti* (Emery, 1869) with seven individuals, *Tapinoma nigerrimum* (Nylander, 1856) with three individuals, *Messor barbarus* (Linnaeus, 1767), *Leptotorax* sp., and *Tetramorium forte* (Forel, 1904) with two individuals each, *Cataglyphis hispanicus* (Emery, 1900) and *Plagiolepis pygmaea* (Latreille, 1798) with one individuals each.

On the Coleoptera order 20 individuals of Coccinellidade family were observed, 10 Coccinellidae adults and 10 Coccinellidae larvae and two individuals of other Coleoptera, one

belonging to family Phalacridae and other belonging to family Chrysomelidae. The different species of Coccinellidae captured in *C. juncea* were *Scymnus apetzi* Muls. with six individuals, *Exochomus nigromaculatus* (Goeze) with three individuals and *Hippodamia* (*Adonia*) *variegata* (Goeze) with one individual. Thysanoptera was one of the groups most representative in this study. A total of 436 individuals of Thysanoptera were recollected in *C. juncea* in three years of this study. The maximum Thysanoptera per plant occurred at 4th August (1.0±0.4) in 2009, at 27th July (0.8±0.3) in 2010 and both 4th and 19th July (0.4±0.1) in 2011.

Hymenoptera parasitoids collected in aerial part of the *C. juncea* include two superfamilies, Chalcidoidea with 15 individuals and Platygastroidea with one individual. In superfamily Chalcidoidea five individuals belong to Eurytomidae family, four individuals belonging to Eulophidae family, three individuals belonging to Pteromalidae family, two individuals belonging to Aphelinidae family and one individual belonging to Tetracampidae family. The superfamily Platygastroidea was represented by a single family, Scelionidae.

A total of 28 Araneae were collected during the study, one adult and 23 juvenile. Four individuals were not possible to identify. Araneae were composed by six families, Thomisidae with 11 individuals, Araneidae with five individuals, Miturgidae with four individuals, Linyphiidae with two individuals, Dictynidae and Philodromidae with one individual respectively.

In 2010 a total of 20 Chrysopidae eggs were found in *C. juncea* representing 1.2% of total arthropods recovered in this plant. In 2009 and 2011 their number didn't reach 1.0%.

Relatively to other groups with less importance, Acari represented 2.6% in 2009 and less of 1.0% in 2010 and 2011 of total arthropods recovered. The non-identified eggs represented 2.9% of total arthropods recovered in 2010, being represented in 2009 less of 1.0%. In 2011 it wasn't found non-identified eggs on *C. juncea*. The other groups represented in all years of this study, less of 1.0% of total arthropods recovered.

3.3.2. Parasitoids abundance on Diptera present in flowers

Diptera immature were very abundant into the rosette of flowers. It was observed a high number of immature Diptera, 2,669 pupae and 1,511 larvae in whole years in a total of 4,180 individuals. A total of 187 larvae and 161 pupae were collected from flowers of *C. juncea* in

2009. The maximum number of Diptera immature per plant it was observed at 6th October (1.6±0.2) (Figure 3.2).

In 2010, it was collected 403 larvae and 734 pupae. At September 29 it was reached the maximum number of Diptera immature (5.5±0.7) per plant. And in 2011 it was collected 921 larvae and 1,774 pupae. The maximum number of Diptera immature per plant it was observed at 22nd September 22 (8.2±0.8).

From Diptera immature 1,118 parasitoids and 884 dipterans were emerged in whole three years of the study. Fifty three parasitoids and 263 dipterans were recovered in 2009, 287 parasitoids and 136 dipterans were recovered in 2010 and 778 parasitoids and 485 dipterans were recovered in 2011. By parasitoids emerging from Diptera immature, parasitism rates were of 15.2%, 25.3% and 28.9% in 2009, 2010 and 2011 respectively (Table 3.3).

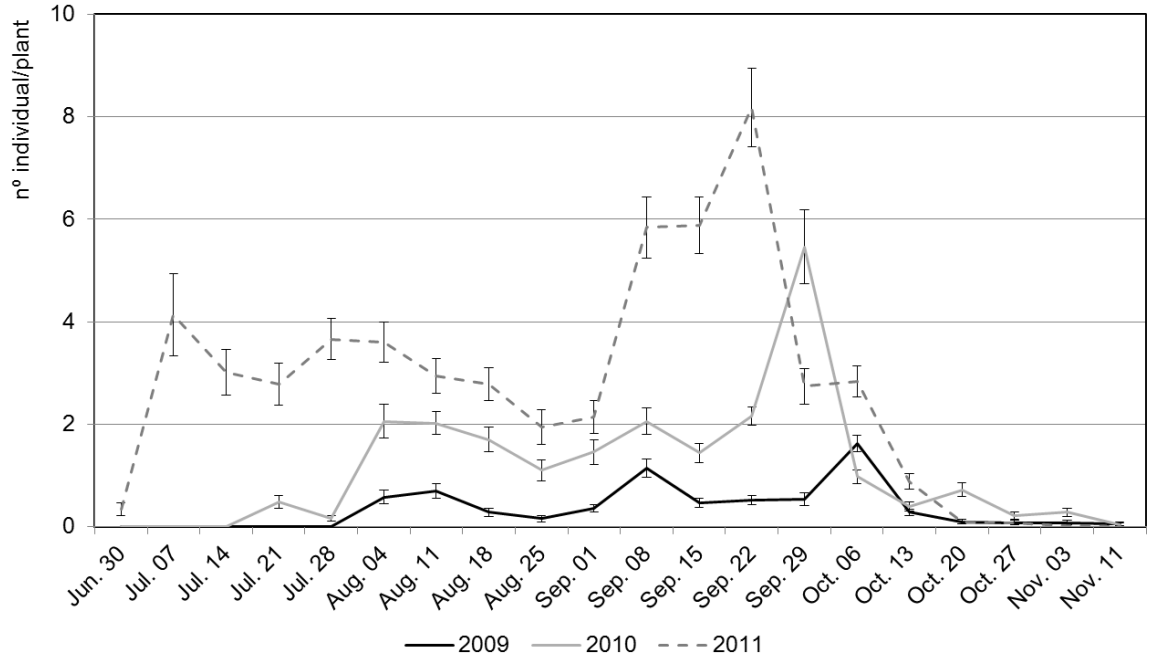

Figure 3.2. Average number of Diptera immature (larvae and pupae) per plant present in *Chondrilla juncea* L. in 2009, 2010 and 2011 (vertical lines mean standard error).

Concerning the distribution of parasitoids emergence along the time, two periods of great abundance of emergences can see observed, mainly in 2011, corresponding the first period to the month of July and first week of August and the second period to medium/late September (Figure 3.3). In 2010 one peak of abundance can be observed in late September.

Due to the lower abundance of parasitoids verified in 2009 it was unable to distinguish any period of abundance.

Table 3.3. Emergences and parasitism rates (%) found in Diptera immature in 2009, 2010 and 2011.

	Immature	Emergences		Percentage of parasitism
		Parasitoids	Diptera	
2009	348	53	263	15.23
2010	1,137	287	136	25.25
2011	2,695	778	485	28.87
Total	**4,180**	**1,118**	**884**	

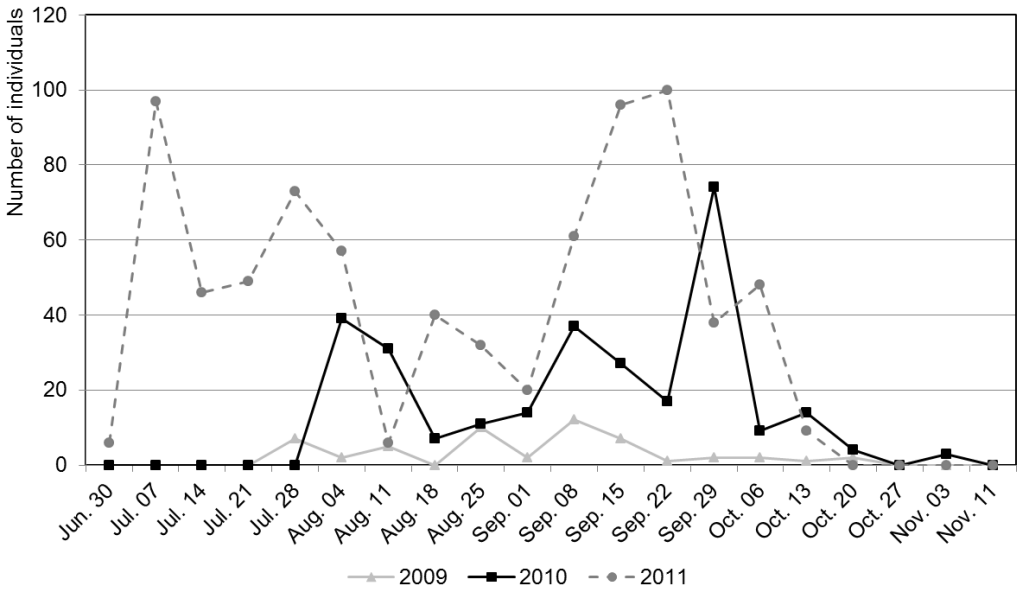

Figure 3.3. Period of emergence of total parasitoids from *Chondrilla juncea* L. flowers in 2009, 2010 and 2011.

From 1,118 individuals of parasitoids emerged from dipteran immature it was found three superfamilies, Chalcidoidea with 1,109 individuals, Platygastroidea with three individuals and Ichneumonoidea with five individuals. In superfamily Chalcidoidea 962

individuals belong to Pteromalidae family (Table 3.4), 93 individuals belong to Eurytomidae family, 20 individuals belonging to Torymidae family, 18 individuals belonging to Ormyridae family, 15 individuals belonging to Eupelmidae family and one individual belonging to Encyrtidae family. Superfamily Platygastroidea and Ichneumonoidea were represented by a single family, Scelionidae with three individuals and Braconidae with five individuals respectively. The family Pteromalidae was the dominant family, representing 75.5% of total arthropods recovered in 2009, about 80.0% of total arthropods recovered in 2010 and about 90.0% of total arthropods recovered in 2011.

Table 3.4. Total abundance of parasitoids by families recovered from *Chondrilla juncea* L. in 2009, 2010 and 2011.

	Number of individuals			
Superfamily and Families	**2009**	**2010**	**2011**	**Total**
Superfamily Chalcidoidea				
Pteromalidae	40	229	693	962
Eurytomidae	4	37	52	93
Torymidae	2	8	10	20
Ormyridae	0	8	10	18
Eupelmidae	1	4	10	15
Encyrtidae	1	0	0	1
Superfamily Platygastroidea				
Sceliniodae	0	1	2	3
Superfamily Ichneumonoidea				
Braconidae	5	0	0	5
Others	0	0	1	1
Total parasitoids	**53**	**287**	**778**	**1,118**

Diptera immature were present during all time of this study in 2011 on flowers of *C. juncea* and the percentage of attacked flowers varied between 0.8% at 3rd November and 28.1% at 22nd September. The higher percentage of attacked flowers coincides with the highest value of Diptera immature recovered (Figure 3.4). The percentage of parasitoids recovered from Diptera immature in 2011 varied from 4.1% at 11th August to 46.9% at 7th July.

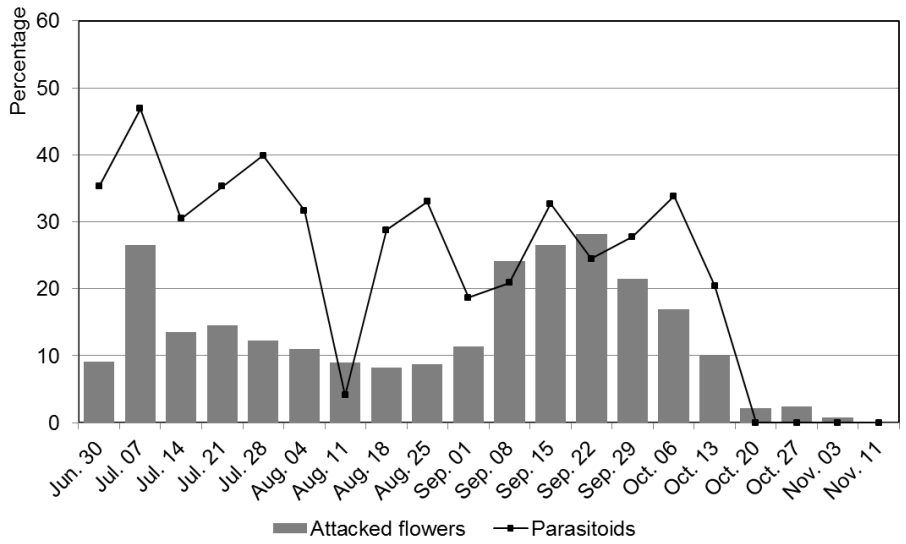

Figure 3.4. Percentage (%) of attacked flowers and percentage of parasitoids found on *Chondrilla juncea* L. during the time of study in 2011.

3.4. Discussion

The maintenance of spontaneous vegetation into the orchards is a kind of ecologic infrastructure designed to improve the action of natural enemies (Franco, 2010). In Trás-os-Montes region, *C. juncea* is naturally spread in olive groves, and in accordance with the Rules of Integrated Protection of Olive of OILB/SROP, the beneficial fauna conservation should go through the provision of infra-structures presenting great ecological diversity in their structure and composition and, if possible, using or encouraging the development of native species (Malavolta *et al*., 2002).

In this study, a great number of specimens belonging to different taxa were collected in *C. juncea*. If excluding immature stage of Diptera, the most abundant taxa in all years were Aphididae and Thysanoptera. Phytophagous arthropods feeding on weeds can serve as a food source for beneficial arthropods (first-order carnivores), and thus weeds can indirectly serve as a resource for such beneficial arthropods (Norris and Kogan, 2005). Different species of Aphididae and Thysanoptera are used as prey and hosts by many predators and parasitoids, respectively (Gordh *et al*., 1999; Hagen *et al*., 1999). In the olive grove, they could constitute alternative prey for generalist predators as some species of Chrysopidae or Coccinellidae. Also honeydew from aphids could be used by *Chrysoperla* spp. adults since, together with pollen, for egg production (Hagen *et al*., 1999). It is important to note that many species of natural enemies of pests in different olive groves, despite their preferences for parasitism or

predation, are polyphagous and can attack a wide range of phytophagous species in olive groves that are present in natural vegetation (Varela and González, 1999).

Relatively to groups considered as beneficial in olive groves (spiders, ants, green lacewings, coccinellids and parasitoids), 112 individuals (immature and adults) were collected from the aerial part of this plant in three years of the study, representing 1.0% of total arthropods observed. Spiders, being in olive groves one of the groups most abundant (Santos *et al*., 2002; Rodrigues *et al*., 2003; Gonçalves and Pereira, 2012) are present in lower number on *C. juncea*, being identified one adult and 23 juveniles. Their presence in this plant can be considered important as olive fly adults feed on plant nectar or pollen (Daane *et al*., 2010), spiders can play an important role in predation of adults of olive fly. Ants have an important role in the olive agroecosystem, participating actively in natural control exercising predatory action on *B. oleae* larvae and pupae in canopy and soil (Arambourg, 1986; Katsoyannos, 1992) and other phytophagous species (Varela and González, 1999). The number of Formicidae collected in *C. juncea* was low, counting 18 individuals belonging to seven species. Coccinellids, either due to their abundance or to their diversity, are a major component of the generalist predator community within the olive grove being considered to feed mostly on *Saissetia oleae* (Olivier) (Argyriou and Katsoyannos, 1977; Santos *et al*., 2009). In this plant it was found 20 individuals (10 adults and 10 larvae) of Coccinellidae, being identified *E. nigromaculatus* and *H. variegata* described as predators of aphids (Raimundo and Alves, 1986) and *S. apetzi* predator of *S. oleae* (Argyriou and Katsoyannos, 1977). It was observed that green lacewing lay eggs in *C. juncea*. It was observed 22 eggs and six larvae on *C. juncea*. It is known that beneficial arthropods can use plants that are not a food source as oviposition sites (Norris and Kogan, 2004), thus, egg laying on this plant may be occurred in the absence of prey for the larvae on the olive tree, becoming this plant important for larvae survival by found here high population of aphids. Parasitoids represent an important beneficial group in olive agroecosystem and have a principal role in the biological control of some olive key pests in Trás-os-Montes region (Bento *et al*., 1998; Pereira *et al*., 1998). On this plant were collected 16 parasitoids from the aerial part in all years of this study founding in this plant food and shelter. It's known that some parasitoids feed directly on plant material such nectar or pollen (Jervis *et al*., 1993; Wäckers, 2005) and as it was observed that a high variety of Hymenoptera parasitoids use spontaneous vegetation as shelter (Escudero *et al*., 2002). According to Murdoch *et al*. (1985) alternative host or preys can serve as reservoir of polyphagous natural enemies, maintaining them during periods of pest scarcity or absence,

until the pest becomes available. Thus, this plant can constitute a ready source of natural enemies, favoring their early establishment on crop at the initial phase of pest colonization.

Relatively to other groups with less importance their presence in this plant was very low, though, is to emphasize the number of non-identified eggs. Non identified eggs found in this plant could also play a role not only as alternative host for parasitoids but also as alternative food for *Chrysoperla carnea* (Stephens) which has been reported to be an important oophagous predator on *P. oleae* eggs (Bento *et al*., 1999).

The Diptera immature found in *C. juncea* are particularly important either by number of individuals or by being attacked by different species of Hymenoptera parasitoids. The larval stage of many Diptera are described in literature as living in and feeding on living tissues of plants in specific ways: surface leaf feeders, leaf-miners, gall-formers, or feeding in stems, roots, flowers, seeds and fruits, where are included some Diptera regarded as pest (Smith, 1989). It was observed in this plant, that dipteran females oviposite into the rosette of flowers where immature stages develop. Larvae feed on flowers of *C. juncea* leading to destruction of the flowers. It was observed in our study that such larvae serve as alternative host for many generalist parasitoids, belonging mainly to superfamily Chalcidoidea. Also Caresche and Wapshere (1975) reported the presence of alternative hosts on *Chondrilla* spp. in the eastern Mediterranean region for two species of Eulophidae. The authors reported that this plant is attacked by a Cecidomyiidae (Diptera) known as cecidomyiid gall midge. Caresche and Wapshere (1975), Littlefield (1980) and Mendes (1982) provided details about biology and ecology of this insect in Europe and Pacific Northwest. They describe that the insect is active from April until late October, completing four or five generations. The adult female oviposit into the lower epidermis of the plant where small circular to ovoid raised galls are produced on the rosette, stem leaves and the stem. The galls are formed in 10 to 12 days approximately and larvae feeding on leaf mesophyll or stem parenchyma. Larvae normally pupate within the galls.

3.5. Conclusions

The present work presents a list of arthropods captured in *C. juncea* between 2009 and 2011. Summarizing, some of the arthropods found in this plant have been referred as alternative prey and hosts for predators and parasitoids present on olive groves. *C. juncea* is a source of preys for many natural enemies by the number of aphids and thrips found on plant.

The Diptera immature found on *C. juncea* can serve as alternative host to parasitoids which may act as natural enemies of olive fly. Thus, the presence of preys and alternative hosts can contribute to maintaining or enhancing parasitoid and predator populations on olive grove, resulting in improved pest control. The knowledge of arthropods associated with this plant is an important tool in order to develop schemes of conservation biological control.

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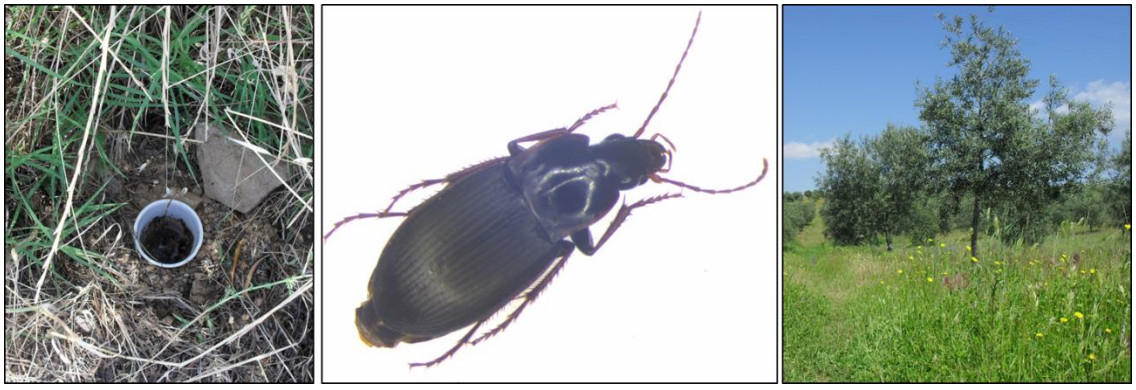
CHAPTER 4

Biodiversity of carabids in olive groves with spontaneous vegetation in Trás-os-Montes region (northeastern Portugal)



Observou, a névoa cor-de-rosa aparecendo de novo à sua frente, como algumas das criaturas se voltavam e dirigiam para ele, como se sentissem que ainda estava vivo. Pestanejou com força e tentou focá-los. Sim, conseguia distinguir alguns deles agora. Lá estava o escaravelho-hércules, abrindo e fechando em patético desespero os grotescos cornos pretos da sua fronte, enquanto tentava arrastar o seu pesado corpo de cinco polegadas de comprimentos sobre o metal. Enquanto olhava, parou repentinamente, fez mais uma tentativa para mover as duas patas anteriores e ficou quieto. Mais atrás dele, o escaravelho-veado, mais pequeno (…), movia-se mais depressa, tal como o seu parente mais distante, a cabra-loura. Masters não podia contar quantos havia de cada, à medida que cerca de uma dúzia de diferentes escaravelhos se moviam para ele.

Richard Lewis, Devil's coach-horse (1979).

Coelho, V.; Santos, S.A.P.; Mexia, A; Bento, A. & Pereira, J.A. Biodiversity of carabids in olive groves with spontaneous vegetation in Trás-os-Montes region (northeastern Portugal).

Abstract

Edaphic carabid beetles (Coleoptera: Carabidae) are components of different agroecosystems and can act as generalist predators of a variety of pests that spend part of their life cycle in soil. In this work, the abundance and diversity of carabids were studied in two organic no-tilled olive groves from Trás-os-Montes region, and their possible action on the olive fly was discussed. From April to November, on a two-weekly basis, five pitfall traps were placed in each grove and in the laboratory carabids were identified to species. In both olive groves, a total of 543 carabids were collected belonging to 19 species and eight subfamilies. Significant differences on carabids catches were observed between groves. *Calathus granatensis* Vuillefroy, with 70.9%, was the most abundant species followed by *Brachinus variventris* Schaufuss (with 7.9%) and *Pterostichus globosus* (Fabricius) with 7.6%. The peak of abundance of *C. granatensis* occurred between the end of August and the middle of October, which coincides with the gradual increase of pupae of the olive fly, *Bactrocera oleae* (Rossi), on the ground. Thus, the presence of this species may be important for biological control of the pest, through predation of pupae found in soil.

Key words: generalist predators, pitfall traps, natural enemy abundance, *Calathus granatensis*, olive fly.

4.1. Introduction

Carabids (Coleoptera: Carabidae) occur in a large number of terrestrial ecosystems and are one of the most abundant and diverse families of beetles (Dillon & Dillon, 1972). Agricultural practices such as pesticide sprays, tillage operations and the maintenance of spontaneous vegetation may have impacts on beetle communities (Andersen, 1999; Clark, 1999; Holland & Luff, 2000; Marasas et al., 2001; Shah et al., 2003; Clark et al., 2006). In general, less disturbed areas are the most favorable for high abundance and diversity of carabids (Kromp, 1999).

In agricultural areas, carabid beetles are important polyphagous predators of insect pests that attack different crops (Torres & Bueno, 2000; Symondson et al., 2002). Their action against immature stages of some fruits flies of economic importance has been documented. In laboratorial experiments, *Pseudophonus rufipes* De Geer was an efficient predator of the third instar larvae and pupae of the Mediterranean fruit fly, *Ceratitis capitata* (Weidemann) (Monzó et al., 2011). And in field experiments, *Pterostichus melanarius* (Illiger) was considered a potential predator of immature blueberry maggot (*Rhagoletis mendax* Curran) (Renkema et al., 2011).

In olive groves, the olive fly, *Bactrocera oleae* (Rossi), is a key pest throughout the Mediterranean region (Haniotakis, 2005). This fly is able to develop two to five generations per year (López-Villalta, 1999). Eggs are laid in olives and larvae develop inside the fruit where they are protected from generalist predators. As olive fruit matures, third larval stage leaves the fruit to pupate in soil. In this period, pupae can be exposed to the predatory action of natural enemies, among which carabids can be found (Bigler et al., 1986).

Around the Mediterranean region, different studies showed the existence of a high number of edaphic carabids in olive groves (Morris & Campos, 1999; Cotes et al., 2009; Coelho et al., 2012). In southern Spain, the most abundant genera were *Amara*, *Carabus* and *Calathus* (Torres & Bueno, 2000). And, in Crete (Greece), Neuenschwander et al. (1986) referred the existence of more than twenty species that exerted predation on pupae and larvae of the olive fly being *Pterostichus creticus* Frivaldszky and *Carabus banoni* Dejean the most common species, followed by other predatory species such as *Poecilus cupreus* Linnaeus, *Platyderus minutus* Reiche, *Calathus fuscipes graecus* Dejean & Boisduval, *Chlaenius festivus* Panzer and *Chlaenius vestitus* Paykull. However, the information about the community of carabids occurring in the northeast of Portugal is limited.

In this work, we studied the abundance and diversity of carabid beetles in two organic olive groves along the crop season in the northeast of Portugal. The synchrony between carabid abundance and the olive fly life cycle was also discussed.

4.2. Material and methods

4.2.1. Olive groves

The study areas were located in two groves near Mirandela (Northeast of Portugal), Cedães and Valbom-dos-Figos groves, both conducted according to organic growing guidelines. Cedães (41º29'18.84''N, 7º07'36.02''W) grove covers an area of four hectares, with trees of approximately 20 years old belonging to cv. Cobrançosa and spaced 7 x 7 m apart. Valbom-dos-Figos (41º33'04.00''N, 7º80'43.00''W) grove has been conducted according to organic growing guidelines since 1991. The grove covers an area of 3 ha and was planted with trees between 50 and 80 years old, spaced 9 x 9 m apart. The predominant cultivars are Cobrançosa and Verdeal Transmontana. Both olive groves were covered with spontaneous vegetation and were not irrigated. The trees have been pruned every two or three years and, during the survey, no phytosanitary treatments were done.

4.2.2. Carabid sampling

The sampling period occurred between April and November 2010. Five pitfall traps were randomly placed in the field, facing the south of the canopy at 20 cm apart from the tree trunk and spaced by 50 m and were emptied every two weeks. Pitfall traps consisted in plastic cups with 9 cm height and 7 cm of diameter. Each trap was filled with 100 ml of ethanol (70%) used as killing/preservative agent, water (29%) and detergent (1%) to reduce the liquid surface tension and ensure that the arthropods sank. In the laboratory, the collected carabids were separated and identified to species under binocular microscope and stored in 70% ethanol. Carabid species were identified according to Aguiar & Serrano (2012).

4.2.3. Statistical analysis

Data were evaluated for normality and homogeneity of variances with Kolmogorov–Smirnov test and Bartlett's test, respectively, and the transformation log10(x + 1) was used to normalize the data. The abundance of carabids captured in both olive groves over different

times was compared by repeated measures analysis of variance using IBM-SPSS Statistics, version 19 (SPSS Inc, 2010).

The catches of carabid are presented as mean ± standard deviation. Species richness (number of species), abundance and Simpson diversity index (1/D) were used in this study to describe the carabid communities.

4.3. Results and discussion

During the period of the study, 543 specimens of Carabidae were collected in both groves, belonging to 19 species and eight subfamilies. From them, 323 carabids were collected in Cedães and 220 in Valbom-dos-Figos (Table 4.1). Significant differences ($F_{1, 8}$ = 24.2, $P < 0.05$) were found between the abundance of carabid observed in Cedães (2.2 ± 3.3) (mean ± SD) and Valbom-dos-Figos (1.6 ± 3.0). In both olive groves, the subfamily Platyninae was present during all months of the study being the most abundant subfamily, with five species identified, *Anchomenus dorsalis* (Pontoppidan), *Calathus granatensis* Vuillefroy, *C. melanocephalus* (Linnaeus), *C. mollis* (Marsham) and *Laemostenus terricola* (Herbst), and this subfamily represented 83.3% of the total carabids captured in Cedães and 64.5% of total carabids captured in Valbom-dos-Figos. Within this subfamily, the genus *Calathus* was the most abundant in both olive groves, with 1.8 ± 3.0 individuals captured per trap in Cedães and 1.0 ± 0.2 in Valbom-dos-Figos.

The subfamily Brachininae was the second most abundant in both olive groves with 26 individuals (8.1%) and two species identified (*Brachinus bodemeyeri* Apfelbeck and *B. variventris* Schaufuss) in Cedães and 29 individuals (13.2%) and three species identified (*B. bodemeyeri*, *B. explodens* Duftschmid, *B. variventris*) in Valbom-dos-Figos.

The subfamily Pterostichinae was the third most abundant with 18 individuals (5.6%) in Cedães and with 29 individuals (13.2%) in Valbom-dos-Figos grove. Within this subfamily, the species *Pterostichus globosus* (Fabricius), with 13 individuals collected in Cedães and 28 individuals in Valbom-dos-Figos, was the most representative. It was also observed that the subfamilies Harpalinae, Lebiinae, Nebriinae and Trechiinae were less abundant in Cedães, with 2 individuals each, and subfamily Patrobinae was the least abundant in Valbom-dos-Figos represented by one species with one individual.

Table 4.1. Total abundance, mean ± standard error, richness and diversity of carabids captured in pitfall traps in the olive groves of Cedães (n = 145) and Valbom-dos-Figos (n = 135) in 2010.

	Cedães		Valbom-dos-Figos	
Carabidae	N	Mean ± SE	N	Mean ± SE
Brachininae				
Brachinus bodemeyeri Apfelbeck, 1904	3	0.02 ± 0.19	8	0.06 ± 0.40
Brachinus explodens Duftschmid, 1812	0	0.00 ± 0.00	1	0.007 ± 0.009
Brachinus variventris Schaufuss, 1862	23	0.16 ± 0.57	20	0.15 ± 0.77
Harpalinae				
Harpalus rubripes Duftschmid, 1812	1	0.007 ± 0.08	2	0.01 ± 0.12
Harpalus anxius Duftschmid, 1912	1	0.007 ± 0.08	0	0.00 ± 0.00
Lebiinae				
Microlestes corticalis (Dufour, 1820)	0	0.00 ± 0.00	1	0.007 ± 0.009
Paradromius linearis (Olivier, 1795)	1	0.007 ± 0.08	0	0.00 ± 0.00
Syntomus foveolatus (Dejean, 1831)	1	0.007 ± 0.08	1	0.007 ± 0.009
Nebriinae				
Nebria salina Fairmaire & Laboulbénè, 1854	2	0.01 ± 0.12	0	0.00 ± 0.00
Patrobinae				
Penetretus rufipennis (Dejean, 1828)	0	0.00 ± 0.00	1	0.007 ± 0.009
Platyninae				
Anchomenus dorsalis (Pontoppidan, 1763)	0	0.00 ± 0.00	1	0.007 ± 0.009
Calathus granatensis Vuillefroy, 1866	246	1.70 ± 2.95	139	1.03 ± 2.49
Calathus melanocephalus (Linnaeus, 1758)	4	0.03 ± 0.16	0	0.00 ± 0.00
Calathus mollis (Marsham, 1802)	12	0.08 ± 0.30	1	0.007 ± 0.009
Laemostenus terricola (Herbst, 1783)	7	0.05 ± 0.43	1	0.007 ± 0.009
Pterostichinae				
Amara aenea (De Geer, 1774)	5	0.03 ± 0.22	1	0.007 ± 0.009
Pterostichus globosus (Fabricius, 1793)	13	0.09 ± 0.31	28	0.21 ± 0.64
Trechinae				
Bembidion lampros (Herbst, 1794)	1	0.007 ± 0.08	3	0.02 ± 0.26
Trechus obtusus Erichson, 1837	1	0.007 ± 0.08	10	0.07 ± 0.36
Others	2	0.01 ± 0.12	2	0.01 ± 0.12
Total of carabids	323		220	
Richness	15		15	
Simpson diversity index	1.70		2.34	

N = total number of captured individuals, n = total number of samples

In both olive groves, *C. granatensis* was the most abundant species. In Cedães, this species represented 45.3% of total species followed by *B. variventris* with 4.2% and *P. globosus* with 2.4%. In Valbom-dos-Figos, C. *granatensis* represented 25.6% of total followed by *P. globosus* with 5.2% and *B. variventris* with 3.7%. For both olive groves, the curve of abundance of *C. granatensis* (Figure 4.1) was similar to the observations made by Cardenas (1994) in the southwest of the Iberian Peninsula where this author observed two peaks of activity for this species, the first in spring, corresponding to the emergence of a new generation and the second, which started in late summer and continues till the early winter with corresponds to the period of reproductive activity.

The peak of abundance of *C. granatensis* occurred between late summer and middle autumn, which coincides with a gradual increase of olive fly pupae on the ground, especially the autumn generations. At this time, most of *B. oleae* larvae leave the fruits (Figure 4.2) to pupate and spend the winter in the soil, usually at a depth between 1 and 3 cm (Neuenschwander et al., 1986).

According to Riddick (2004), the diet of many species of the genera *Agonum*, *Calathus*, *Poecilus* or *Pterostichus* is mostly dependent on the season and availability of specific preys. Thus, the occurrence of *C. granatensis* between the end of summer and autumn may contribute to the natural biological control of olive fly through predation of pupae found in soil. The abundance of the genus *Calathus* in olive groves was also observed by Morris (1997) and Morris et al. (2000) in olive groves in southern Spain, where the species *C. ambiguus* (Payk.) represented 40% of the carabids captured. In Greece, the species *C. graecus* was observed exerting predation on larvae and pupae of *B. oleae* (Neuenschwander et al., 1986). Carabids are generally polyphagous, most with carnivorous habits (Lövei & Sunderland, 1996) attacking a large number of agricultural pests. In organic olive groves, where alternatives to chemical control against the olive fly are scarce and/or with limited efficacy, the presence of carabids may contribute to decrease population levels of olive fly and consequently the importance of this pest.

The Simpson diversity index was 1.70 in Cedães and 2.34 in Valbom-dos-Figos. The value of Simpson's diversity index obtained in Cedães is due to the fact that the carabid community had a clearly dominant species, representing more than 83% of total individuals captured.

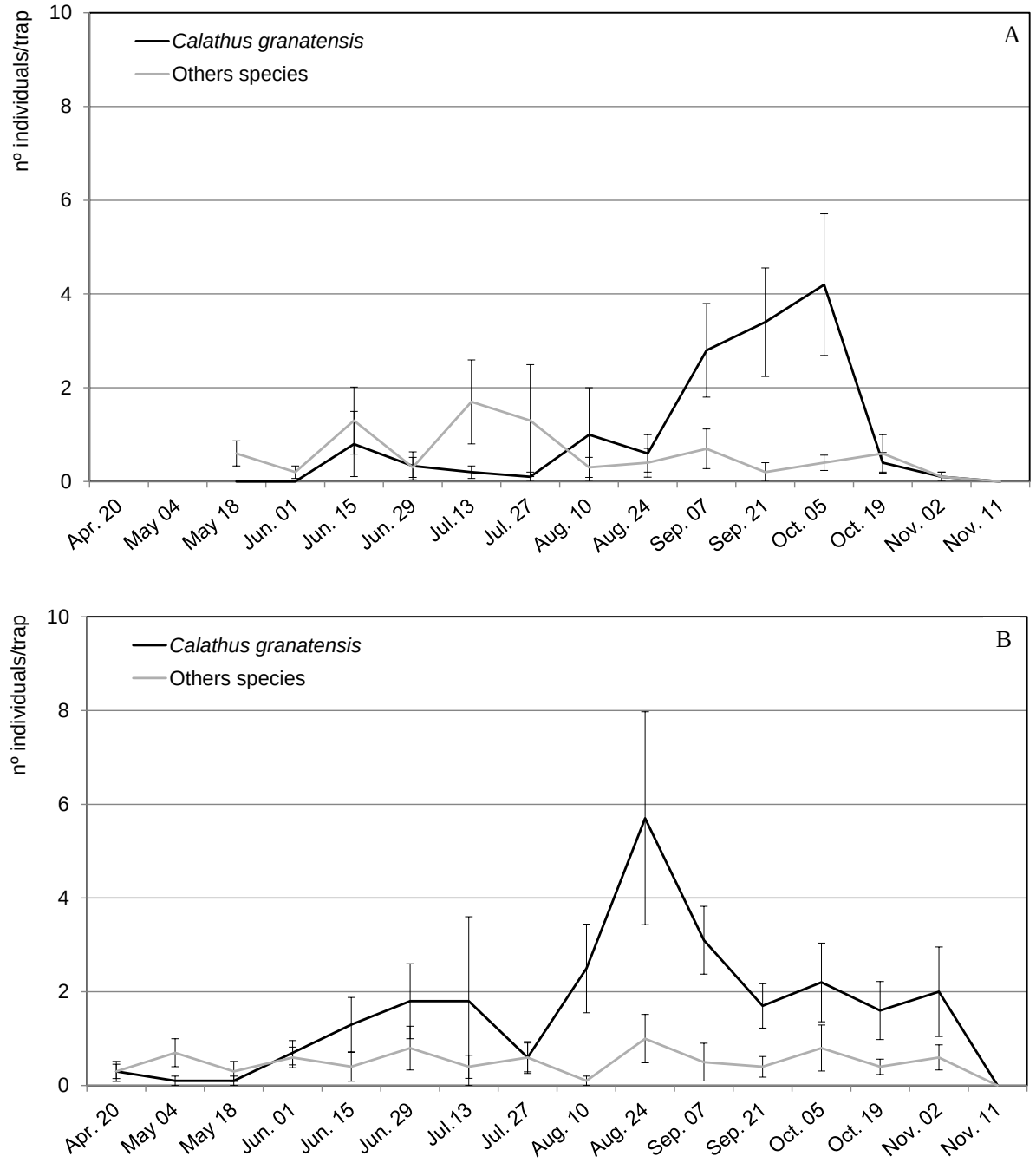
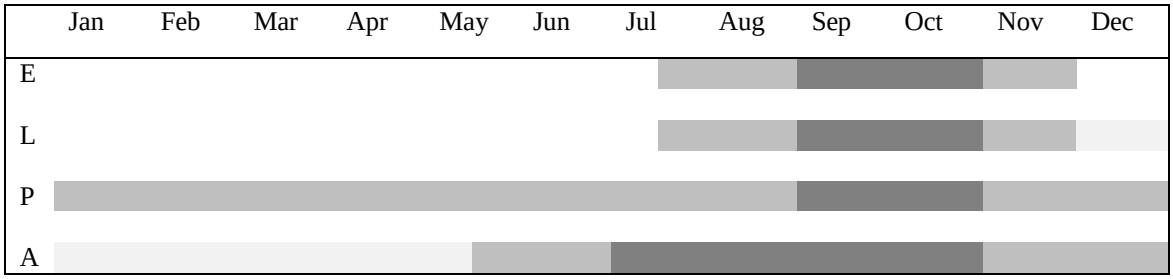


Figure 4.1. Abundance (mean ± standard error) of carabid species captured in pitfall traps in Valbom-dos-Figos (A) and Cedães (B) in 2010.



E – Eggs, L – Larva, P – Pupa, A - Adults

Figure 4.2. Temporal distribution of phenological stages of *Bactrocera oleae* (Rossi) in Mirandela (Trás-os-Montes region) (Adapted from Bento et al., 1999).

Currently, there is a great interest in the management of agricultural ecosystems aiming to optimizing the action of natural enemies. In this context, the management of soil cover is one of the fundamental aspects to promote functional biodiversity in agroecosystems. The studied olive groves have not been tilled in the last five years and the cover vegetation followed its natural phenology. Some studies of ground beetles in annual crops indicated an increase in the abundance and richness of carabids when tillage are reduced or eliminated (Thorbek & Bilde, 2003). Also, Clark et al. (2006) reported that vegetation tends to promote the abundance of carabid by providing conditions of habitat or indirectly by supporting prey populations in organic systems. On the other hand, studies performed by Belaoussoff et al. (2003) showed no favorable effects on the abundance and diversity of species of Carabidae with the reduction of soil tillage. These aspects need to be clarified in the olive grove. However, the control of spontaneous vegetation by tillage or herbicide applications and the negative consequences for soil erosion, destruction of olive roots, decrease of organic matter and also the negative effects on biodiversity (Campos et al., 2000) put forward the maintenance of spontaneous vegetation as generating benefits. In particular, the increase of certain edaphic arthropod predators that act on the olive fly, such as carabids, which find shelter locations here (Neuenschwander et al., 1983; Warlop, 2001).

The information collected may lead to the design of strategies for enhancing the most abundant species in order to promote conservation biological against olive fly.

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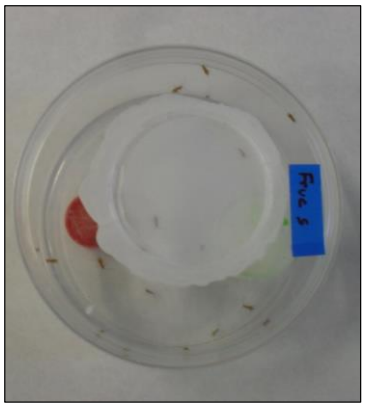
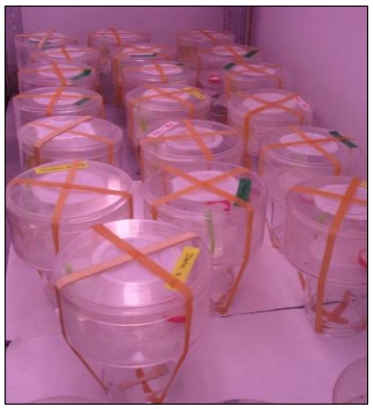
CHAPTER 5

Effect of different food sources on longevity and progeny production of parasitoid *Psyttalia concolor* (Szépligeti, 1910)





Olhou para cima. Havia quatro deles, voando em círculos: enormes insetos dos pântanos, cada um carregando um parasita invisível. Provavelmente, bichos que viviam de carniça, inofensivos — mas a envergadura de suas asas, de mais de três metros, impressionava. Inquieto. Cord lembrou-se do viajante carnívoro e venenoso, que deixara desacordado, perto da Estação. (…) Então, seus pensamentos devanearam novamente; começou a cogitar vagamente sobre a curiosa simbiose em que dois sistemas nervosos de duas criaturas tão diferentes quanto os insetos e seus parasitas podiam estar tão estreitamente ligados, a ponto de funcionarem como um só organismo. (…).

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Coelho, V.; Medina, P.; Bengonchea, P.; Mexia, A.; Bento, A. & Pereira, J.A. Effect of different food sources on longevity and progeny production of parasitoid *Psyttalia concolor* (Szépligeti, 1910). Submitted.

Abstract

Food sources can affect life parameters of adult parasitoids used as biocontrol agents. In this work, the effect of eight different food sources (artificial diet; sucrose; fructose; glucose; honey solution -10% v/v-; pollen; honey solution -10% v/v- and pollen; and *Dittrichia viscosa* flowers) on longevity, parasitism ability and progeny size of *Psyttalia concolor* was studied. At controlled conditions (25±2ºC, 16L:8D and 75±5% RH) five replicates of 20 newly emerged *P. concolor* (10 males and 10 females) were evaluated for each food source. Insect survival was daily checked; parasitism on the alternative host *Ceratitis capitata* larvae ability was measured after 7 days; and progeny size was evaluated on the end of the experiment. Longevity was significantly influenced by food source. *P. concolor* females longevity was significantly highest on sucrose (59[31,66] days) (median[quartiles]) and artificial diet (52[27,61] days), and lowest for *D. viscosa* flowers (2[1,2] days). Glucose showed the highest values of total offspring (20.78±3.78), however, the highest values of sex ratio were recorded when *P. concolor* fed on artificial diet (75.32%). Our results suggest that sugar feeding can increase *P. concolor* longevity and can enhance female-biased progeny.

Key words: Biological control, lifespan, parasitism, sucrose.

5.1. Introduction

The olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is the most serious pest of olives in the main producing countries. Pesticide bait sprays was been usually the control method used again the pest. However, this strategy has high negative impacts on both olive groves and environment and also on safety and quality of olive products. In sustainable olive production the availability of measures to control *B. oleae*, effective and affordable from the economic point of view, is reduced. Biocontrol using parasitoids to suppress olive fly populations can be a useful alternative to the pesticide uses (Daane et al., 2011).

Psyttalia concolor (Szépligeti, 1910) (Hymenoptera: Braconidae) is a koinobiont larval-pupal endoparasitoid of many Tephritidae. This parasitoid is able to attack at least fourteen tephritids on different wild and/or cultivated plants, including pests of great economic importance, such as the medfly, *Ceratitis capitata* (Wiedemann), and also the olive fly (Copeland et al., 2006). In Europe, this species was introduced many times in different regions to control olive fly, but it did not established widely. Unsuitable climatic conditions was been pointed the main reason of this fail (Raspi and Loni, 1994; Miranda et al., 2008), resulting in low rates of parasitism, not sufficient to maintain the olive fly population levels at low density especially in years of severe attacks (Delrio et al., 2003). Besides the weather, other factor could contribute for this reduced action, being the availability of food for *P. concolor* adults a factor which can affect the effectiveness of the parasitoid and their action in controlling the olive fly (Yokoyama et al., 2008).

In conservation biological control, agricultural habitats could be managed to optimize the action of natural enemies (Landis et al., 2000). In this strategy, the diversification of the agricultural ecosystems is a key factor for increase biological control. Vegetation had an important role in the agroecosystem once it can be an important source of foods for natural enemies. Flowering plants can support a broad range of predators and parasitoids that require nectar and pollen sources to survive and reproduce (Winkler et al., 2009). The main energy source for adult parasitoids is sugar which can be obtained in the field from nectar or honeydew (Jervis et al., 1993; Tooker and Hanks, 2000), which provide primarily the disaccharide sucrose and its two monosaccharide components, glucose and fructose (Harborne, 1988).

Adult parasitoids of many species require carbohydrates to achieve maximum longevity and reproduction (Fadamiro and Heimpel, 2001) and their privation can be critical to enhance

biological control of pests (Heimpel and Jervis, 2005). Some species are strongly stimulated by certain sugar sources such as sucrose, glucose and fructose and food rich on them increase parasitoids longevity (Wäckers, 2001). Resources such as pollen provide valuable nutrients for many natural enemies and benefit fitness of several parasitoids (Hickman et al., 1995; Eijs et al., 1998). At laboratorial conditions for mass-rearing of different braconid parasitoids, including *P. concolor*, honey only (Sivinski et al., 2006; Daane et al., 2011) or mixed with pollen (Canale and Beneli, 2012; Zhang et al., 2004) are used as food for adults with good results. Though, in field conditions the lack of suitable food sources has been long suspected to be an important constraint to the success of biological control programs.

Therefore, the objective of this study was to evaluate the effect of eight different food sources, an artificial diet composed by sucrose and yeast extract (4:1), three individual sugars (sucrose, fructose and glucose), honey solution (10% v/v); pollen; a combination of honey solution (10% v/v) and pollen; and *Dittrichia viscosa* (L.) flowers in comparison to water, on the life parameters longevity, parasitism and progeny production of *P. concolor*.

5.2. Materials and Methods

5.2.1. Insects

The laboratorial colony of *P. concolor* and *C. capitata* used in the experiments have been reared in artificial diet for several years in the Laboratory of Crop Protection, Department of Crop Production, Technical University of Madrid, Spain. Both species were reared and all experiments were run at controlled conditions in methacrylate cages (40 x 30 x 30 cm) at 25±2ºC, with relative humidity at 75±5% and a photoperiod of 16:8 (light:dark). The parasitoid *P. concolor* was reared in the laboratory on the alternative host *C. capitata* following the rearing procedure of González-Núñez (1998). For the experiments all *P. concolor* were newly emerged. Fully-grown *C. capitata* larvae were used as host for fecundity tests.

5.2.2. Food sources

Eight different food sources were tested on parasitoids survival: (1) artificial diet (sugar, yeast extract 4:1), (2) sucrose, (3) fructose, (4) glucose, (5) honey solution 10% (v/v), (6) pollen, (7) honey solution 10% (v/v) and pollen combined, (8) flowers of *D. viscosa* in

comparison to the water (treatment 9). Artificial diet, sucrose (Panreac), fructose (D-Fructose, Panreac) and glucose (D-Glucose, Fluka) were offered to parasitoids in solid form. The monosaccharides glucose and fructose and the disaccharide sucrose were selected once honeydew and floral nectar are commonly constitute by these sugars (Wäckers, 2001). Honey solution 10% (v/v) was prepared dissolving 10 ml of honey in 90 ml of water and provided to parasitoids in small vials. Two floral stems and flowers of *D. viscosa*, recently collected in field, were placed in small vials containing water, and changed every two days.

5.2.3. Longevity

For the longevity experiment and for each treatment newly emerged *P. concolor* were placed in groups of 20 individuals (10 females and 10 males) in five cylindrical plastic cages (12 cm of diameter and 5 cm of height) with a total of 100 individuals (50 females and 50 males) were used for each treatment. Parasitoids were removed after emergence from rearing boxes with an aspirator and placed in cylindrical plastic cages. Solid food was placed on small plastic plates of a 2 cm diameter and was replaced weekly. Honey solution was renewing twice a week to avoid contamination by fungi and deterioration. In all treatments it was provided a separated source of water in small vials, except treatment with water. For all treatments food was provided ad libitum. The no-food (water only) treatment served as control. All cages were checked for insect survival on a daily basis and dead parasitoids were promptly removed with an aspirator.

5.2.4. Progeny production

After one week, for each treatment, eight surviving females were isolated for four days in plastic cages and provided with the respective food source. Every day, during four days, 30 fully-grown *C. capitata* larvae were offered to each cage of females for parasitisation following González-Núñez (1998) procedure. One hour later, *C. capitata* larvae exposed were placed into Petri dishes to let them pupate. The number and sex of the emerging offspring were recorded. Parasitism ability was measured as the percentage of attacked host (percentage of puparia without medfly emergence) and progeny size (percentage of parasitoids emerged from parasitized medfly puparia). Pupae without emergency were considered parasitized. Data of first day of parasitisation was not considered since females need to learn how to parasitize.

5.2.5. Data analysis

Average longevity and progeny production were calculated for each different diet and were presented as (median[quartiles]) Statistical analyses were carried out with the program SPSS PASW Statistics 18 for Windows, IBM. Data were evaluated for normality with Kolmogorov-Smirnov test and preceded to the mathematical transformation to normalize the variable using square roots. Survival curves were generated for each treatment and sex. The Kaplan-Meier estimator was calculated using the *Surv* and *survfit* functions from the "*survival"* package and used to asses statistical significant differences between treatments by constructing a diagonal matrix considering all the multiple comparisons between curves. A χ^2 statistic and its associated probability were then calculated using the *survdiff* function for each pair of curves and finally, due to the large number of multiple comparisons (n=36) the Bonferroni correction was applied (0.05/36) in order to avoid an inflated likelihood of error. Since the mean was not used in this analysis, the median accompanied by the first and third quartiles was used as a measure of central tendencies. Statistical differences between sexes were assessed for each treatment using the non-parametric Wilcoxon rank-sum test estimating the probability that a randomly chosen subject from the first group (males) has a higher longevity than a randomly chosen subject from the second group (females). In order to assess the effect of different food sources on the number of *P. concolor* adults (males and females), the sex ratio (measured as the proportion of females), the number of adults of *C. capitata* and the pupae without emergence from parasitation with *P. concolor*, a series of GLMs were developed using the negative binomial distribution except for the proportion of females where the binomial distribution was used. For each model, a post-hoc analysis (Bonferroni corrected Tukey's all-pair comparisons) was carried out in order to identify which treatment differed significantly from the remainder. All analyses were conducted using the software environment for statistical computing R (R Core Team, 2016).

5.3. Results

5.3.1. Longevity

Different food sources had significant effects on *P. concolor* longevity for both females and males. *P. concolor* females survived longer than males, reaching two times more when artificial diet, sucrose and fructose were used as food.

Sugar-based sources had significant effect on female's longevity. Three distinct groups were observed. The highest value was observed on sucrose, either alone or in artificial diet followed by fructose (Table 5.1) and glucose. When pollen honey solution 10% or pollen and honey solution 10% combined were added as food source, the mean value of female longevity was lower than sugar-based sources, but significantly greater than treatment with water. The lowest mean longevity was obtained for flowers of *D. viscosa* (1%).

Table 5.1. Longevity of females and males of *Psyttalia concolor* (Szépligeti, 1910) when reared on different food sources. Different letters in each column means significant differences. An asterisk indicates a significantly higher longevity in females than males.

	Longevity (median[quartiles])		
Food source	Females	Males	Total adults
Artificial diet	52[27,61] b*	20[15,30] b	35.23±22.72
Sucrose	59[31,66] b*	21[15,25] bc	35.22±21.93
Fructose	34[12,65] b*	11[8,18] cd	26.17±22.95
Glucose	20[14,25] c*	10[7,16] de	15.40±8.32
Pollen	15[8,31] c	9[7,16] de	16.07±12.62
Honey	14[12,19] c*	11[10,12] de	14.29±6.94
Honey + Pollen	10[4,17] c*	4[3,10] e	11.80±12.32
Flowers of *D. viscosa*	2[1,2] a	2[1,2] a	2.11±1.25
Water	3[2,4] a	3[2,3] a	2.78±1.15

There was also a significant difference in longevity of males fed in different food sources. The highest longevity was obtained when sucrose was added as food source, in artificial diet followed by sucrose, fructose and honey solution 10%. The lowest mean longevity was obtained for flowers of *D. viscosa*.

When water was only given, *P. concolor* females lived for only 3[2,4] days and males lived for only 3[2,3] days. The floral resource (*D. viscosa*) used in this study did not increase longevity of *P. concolor* females or males, and mean longevity either females (2[1,2]) or males (2[1,2]) was lower than treatment with water.

There were significant differences between sexes in treatments with artificial diet ($W=495$, $P<0.001$), sucrose ($W=387$, $P<0.001$), fructose ($W=629$, $P<0.001$), glucose ($W=497$,

P<0.001), honey (W=519, P<0.001) and honey combined with pollen (W=863, P=0.016). In treatments with flowers of *D. viscosa* (W=1313, P=0.639), pollen (W=917, P=0.086), and water (W=1146, P=0.458) there was not significant differences between sexes.

Survivorship of *P. concolor* females and males on the different food sources is shown in Figure 5.1.

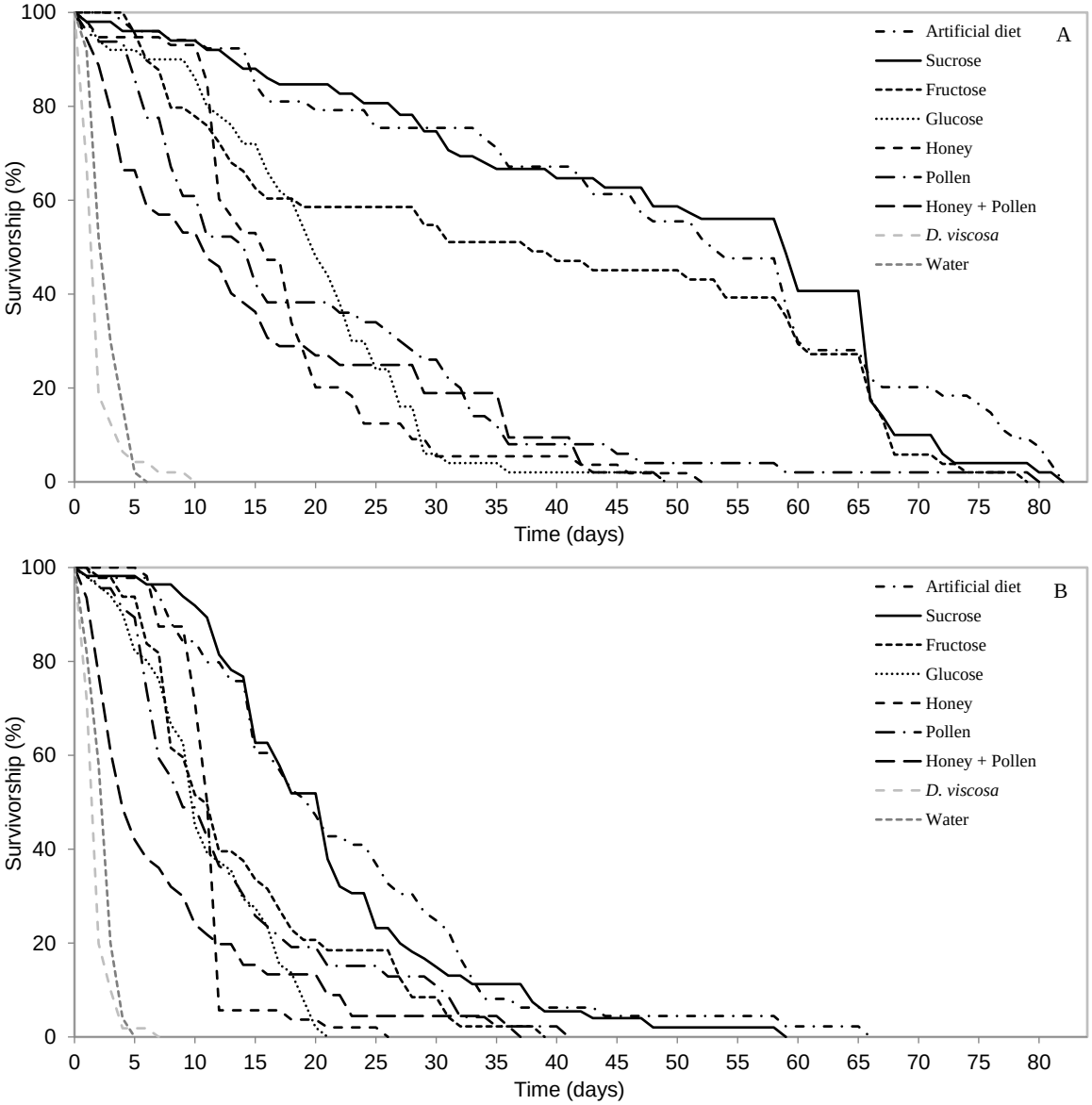

Figure 5.1. Survivorship curves for females (A) and males (B) of *Psyttalia concolor* (Szépligeti) fed with various food sources (artificial diet, sucrose, fructose, glucose, honey solution 10%, pollen, honey solution 10% and pollen combined, flowers of *Dittrichia viscosa* (L.), and water).

Survivorship was different between females and males and generally females lasted longer than males. The values of survivorship ranged from nine days for females feeding on flowers of *D. viscosa* to 81 days for females feeding in sucrose and artificial diet, and ranged from six days for males feeding on flowers of *D. viscosa* (1%) to 65 days for males feeding in artificial diet. The maximum of survivorship for females was registered in experiment with sucrose and artificial diet, where *P. concolor* females survived the maximum of 81 days, followed by treatment with pollen where females survived 79 days and fructose with 78 days. The survival time for *P. concolor* males was shorter, where the maximum of survivorship were recorded in treatment with artificial diet, having males surviving 65 days in this treatment, followed by treatment with sucrose where males survived 58 days, and pollen with 40 days. The minimum of survivorship was recorded in treatment with flowers of *D. viscosa* having males *P. concolor* surviving the maximum of six days and females of *P. concolor* surviving only the maximum of nine days. Neither females nor males survived longer than four days in treatment with water alone.

5.3.2. Progeny production

Mean number of offspring (females and males) produced by *P. concolor* females during parasitisation period when exposed to *C. capitata* is shown in Table 5.2. Offspring produced by *P. concolor* females varies with different source food. Significant differences were found in males ($P \leq 0.001$) and females ($P=0.003$) offspring of *P. concolor* except for treatment with glucose. The highest values of total offspring were obtained by females feed on glucose (20.8±3.8), artificial diet (19.5±2.3) and fructose (18.7±4.3). The lower value of total offspring was obtained by females feed on sucrose (16.5±3.8). The number of female progeny which emerged in parasitasion experiments was greater than males in all sources food tested, and reached its highest mean value in experiments with artificial diet (14.7±2.3) and fructose (13.9±4.4).

The mean number of *C. capitata* adults that emerged ranged between 2.2±2.5 for females feed on artificial diet to 5.8±8.8 for females feed on sucrose and no significant differences ($P=0.092$) were found in different treatments. There were significant differences ($P=0.003$) between pupae without emergence or death pupae in all experiments tested with different source foods.

Table 5.2. Number (mean±SD) of *Psyttalia concolor* (Szépligeti, 1910) males and females, total of adults and percentage (%) of females, adults of *Ceratitis capitata* (Wiedemann) and pupae without emergence from parasitasion with *Psyttalia concolor* females feed on different sources. An asterisk indicates a significantly higher longevity in females than males (*n*=10).

	Psyttalia concolor					
Food source	Females	Males	Total adults	% females	*C. capitata*	Death pupae
Artificial diet	14.65±5.26* a	4.80±2.37 a	19.45±6.13 a	75.32 c	2.20±2.49 a	8.15±4.77 a
Sucrose	10.43±5.47* a	6.05±3.78 a	16.48±7.58 a	63.28 b	5.83±8.80 a	6.95±4.52 a
Fructose	13.88±5.63* a	4.80±2.56 a	18.68±5.93 a	74.30 c	2.45±3.33 a	7.03±3.42 a
Glucose	10.60±5.23 a	10.18±4.97 b	20.78±5.28 a	51.02 a	2.53±3.67 a	5.50±3.25 a
Pollen[1]	11.58±4.79* a	5.69±3.27 a	17.27±5.38 a	67.04 bc	4.14±4.79 a	8.36±3.02 a
Honey	9.95±6.65* a	7.38±5.22 ab	17.33±6.64 a	57.43 ab	4.50±4.79 a	7.38±3.44 a
Honey + Pollen[2]	12.45±6.66* a	5.15±3.21 a	17.60±7.56 a	70.74 c	2.25±2.45 a	5.80±3.24 a

[1] $n = 36$, [2] $n = 20$.

Significant difference in sex ratio (P≤0.001) was recorded in progeny produced. Values of sex ratio of *P. concolor* vary with different source foods. The highest values of sex ratio were recorded when *P. concolor* fed on artificial diet (75.3%), fructose (73.7%) and pollen and honey solution 10% combined (70.7%). The lowest values of sex ratio were obtained when *P. concolor* fed on glucose (51.0%). No significant differences were observed between attacked hosts (P=0.90) after parasitasion experiment with different food sources tested but differences between progeny size were registered (P=0.03) (Figure 2). The highest percentage values of progeny size were obtained in treatment in which *P. concolor* females fed on pollen and honey solution 10% combined (75.5%), glucose (74.8%) and sucrose (72.0%). The lower percentage value was obtained in treatment in which *P. concolor* females fed on pollen (67.0%).

5.4. Discussion

In the present work, the different food source studied had different effects on *P. concolor* longevity. Females and males longevity was enhanced by artificial diet, sucrose and

fructose, since those were the sources of food that gave significantly higher mean longevity when compared with water (no-food treatment). In generally, the survivorship of *P. concolor* adults was higher in experiments where sugar as food source was used, having parasitoids fed on artificial diet, sucrose and fructose lived about 80 days each in laboratory. The artificial diet, a common food for parasitoids mass rearing in laboratory, is based in sugar and yeast extract containing a source of protein (sucrose, yeast extract 4:1). This result suggests that protein seems to be less essential for *P. concolor* females longevity than sugar. Glucose, fructose, and sucrose are the most important sugar in floral nectar and honeydew (Wäckers, 2001). Sugar represents the main energy source for adult parasitoids, and several studies have demonstrated that sugar can increase longevity and fecundity in the laboratory (Olson and Andow, 1998; Olson et al., 2000; Fadamiro and Heimpel, 2001; Wäckers, 2001; Lee et al., 2004). It is known that parasitoids are especially sensitive to sugar deprivation as adult; the laboratory lifespan of many parasitoid species is typically less than 5 days in the absence of sugar but exceeds 2 to 3 weeks when sugar meals are provided (Quicke, 1997; Olson and Andow, 1998; Fadamiro and Heimpel, 2001). In this study, the mean longevity of *P. concolor* females fed on glucose was lower than other sugar tested in this study, fructose and sucrose. It seems to us that *P. concolor* females did not use glucose as effectively as sucrose and fructose.

The mean longevity for *P. concolor* adults fed on honey solution 10% and pollen it was very similar, and no difference was found between *P. concolor* adults fed on honey solution or pollen and *P. concolor* adults fed on glucose. However, when compared with other sugar, the mean longevity was lower, even registering half the average longevity of *P. concolor* adults fed on artificial diet and sucrose. Usually Hymenoptera parasitoids visit plants to feed on nectars as well as pollen. Pollen, an important food for some parasitoids, is frequently consumed when contaminating nectar, honeydew, and water sources, being one of the most nutritious non-prey food sources for parasitoids based on its protein levels (Jervis et al., 2004; Wäckers, 2005). This feeding normally increases both longevity and fecundity as verified for example for parasitoid *Trichogramma* spp. (Zhang et al., 2004). Several works have reported that some food sources rich in proteins are primarily utilized for parasitoid reproduction whereas sugar from plant exudates or aphids secretions as well as honey are primarily utilized for maintenance (Wäckers, 2001; Rivero and West, 2005; Irvin et al., 2007). Honey separately (Sivinski et al., 2006; Yokoyama et al., 2008; Hepdurgun et al., 2009; Daane et al., 2011) and mixed with pollen (Zhang et al., 2004; Loni, 2003; Canale and Beneli, 2012) are typically

used as food source for several species of Opiinae braconid parasitoids, including *P. concolor*, mass-reared in laboratory for augmentative release, and some studies reported high longevity of *P. concolor* in laboratory when honey and water are provided (Sime et al., 2006; Yokoyama et al., 2008), ranging these values from 21.3±15.30 to 77.6 ±15.30 days when performed at 25ºC in laboratory. In our experiments, the mean longevity of *P. concolor* adults fed on honey solution 10% (v/v) (14.29±1.55) was longer than those provided with water alone. However, our results are lower than studies performed by the authors mentioned above. In our case pollen and honey combined did not increase longevity when compared with the treatments where honey solution 10%, pollen or water, were utilized as food sources.

The mean longevity of experiments where *P. concolor* adults fed on flowers of *D. viscosa* were no longer than those provided with water alone and no significantly different were recorded. Although, the survivorship of adults fed on flowers of *D. viscosa* was longer, suggesting that some individuals acquired sufficient floral resources. *D. viscosa* is a perennial aromatic plant associated to agricultural ecosystems, very common in the Mediterranean olive groves margins (Warlop, 2006). It is well known that this plant is attacked by the gall-forming dipteran *Myopites stylatus* (Fabricius) which is further parasitized by several hymenoptera species, some belonging to the parasitoid complex associated with the olive fly (Warlop, 2006).. When flowers of *D. viscosa* were provided as food source, *P. concolor* adults survived up the maximum of nine days.

In general, average lifespan was greater for *P. concolor* females than males. There was significant difference in longevity between males and females in all treatments except in treatments where *P. concolor* were provided with flowers of *D. viscosa* and water. In the present study *P. concolor* females fed on artificial diet, sucrose and fructose survived more than twice the time of males that are in agreement with other works where parasitoids females live longer than males when fed on sugar-based foods (Olson et al., 2000; Fadamiro and Heimpel, 2001; Luo et al., 2010).

Relatively to progeny production, *P. concolor* females produced high progeny when fed on glucose and artificial diet. Although many adult parasitoids, including *Braconidae*, require nutrients in the form of nectar, pollen or both for optimum progeny production that did not occur in our work. In this experiment sex ratio was high when *P. concolor* females were provided with artificial diet and fructose. Although glucose increases *P. concolor* progeny, it does not increase sex ratio as percentage of *P. concolor* females was lower in this experiment. Nevertheless sugar rich foods may enhance oviposition by parasitoids (Faria et al., 2008) and

alter the sex ration of progeny (Onagbola et al., 2007). It seems to us that artificial diet and fructose increase sex ratio of *P. concolor* in our experiments.

Our results suggest that sugar feeding can increase longevity of *P. concolor* in laboratory and can enhance female-biased progeny. Provision on sugar-based food can be utilized for to mass rearing of this parasitoid in the laboratory. That can be improved biological control by the incorporation of flowering cover crops or other sources of sugar to parasitoids in the field (Jervis et al., 1993; Landis et al., 2000). Knowing the nutrient requirements of the parasitoid *P. concolor*, we can help to improve the rearing and maintenance of this parasitoid in the laboratory and in the manipulation of habitat to ensure success in the parasitoid introduction in biological control programs.

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CHAPTER 6

Pathogenicity of *Beauveria bassiana* isolates on *Ceratitis capitata*, *Rhagoletis cerasi* and *Bactrocera oleae* pupae under laboratory conditions.

A Lagarta e Alice olharam uma para a outra durante algum tempo, em silêncio: por fim, a Lagarta tirou o cachimbo da boca e falou-lhe com uma voz lânguida e sonolenta.
- Quem és tu? – disse a Lagarta.
Estas palavras não eram lá muito encorajadoras para começar uma conversa. Alice respondeu timidamente: - Eu.. Senhor, eu agora neste momento nem sei. Sei, pelo menos, o que eu era, quando me levantei esta manhã, mas acho que devo ter mudado várias vezes desde essa altura.

Lewis Carol, Alice's Adventures in Wonderland (1865).

Coelho, V.;Baptista, P.; Mexia, A.; Bento, A. & Pereira, J.A. Pathogenicity of *Beauveria bassiana* isolates on *Ceratitis capitata, Rhagoletis cerasi* and *Bactrocera oleae* pupae under laboratory conditions. “In preparation”

Abstract

Entomopathogenic fungi, in particular Deuteromycetes, including *Beauveria bassiana*, are attractant candidates in the biocontrol of fruit flies. The aim of this work was to compare the pathogenicity of four native *B. bassiana* isolates (Bb 1M/10, Bb 2T/08, Ac93/gf09 and Ac36/gf10) on *Bactrocera oleae*, *Ceratitis capitata* and *Rhagoletis cerasi* pupae using sand-conidial suspension incorporation bioassays and select the ones with the greatest virulence and broad-host-range. All the four isolates were able to infect and kill the three fruit flies, being this effect mainly noticed on *B. oleae* and *C. capitata*. All the isolates reduced adult emergence (up to 1.2-fold when compared to control), and increased the mortality (ranging from 20 to 96%) as well as mycosis rates (ranging from 12 to 94%) in the puparia stage of these two fruit flies, depending on fungal isolate and dose applied. Susceptibility of *R. cerasi* to *B. bassiana* isolates was lower, being registered different mortality and mycosis rates, but no more than 26% and 24%, respectively. The isolate that cause both highest mortality (up to 96%) and mycosis (up to 94%) in pupae of all the fruit flies tested, was Bb 2T/08, while the highest reduction on adult's emergence of *B. oleae* and *C. capitata* was caused by isolates Ac36/gf10 (ranging from 14 to 38%) and Ac93/gf09 (ranging from 14 to 22%), respectively. Lethal concentration (LC_{50}) values were ranged from 1.6×10^{6} to 1.0×10^{0} conidia/mL, depending of the isolate and fruit fly. The isolate Bb 1M/10 had the lowest LC_{50} value, around 1.6×10^{6}, to kill *B. oleae* and *C. capitata*. Overall, isolates Bb 1M/10, and Bb 2T/08 showed great potential to be used in the biological control *B. oleae* and *C. capitata* pupae, at low concentrations. This hypothesis needs further confirmation by performing field assays.

Key words: Entomopathogenic fungi, *Beauveria bassiana* (Balsamo) Vuill., fruit flies, biological control, pathogenicity

6.1. Introduction

Fruit flies (Diptera: Tephritidae) are very dangerous pest of fruits and vegetables in different regions of the globe (Duyck et al., 2004). Among them, in Europe, three species possess high economic importance, the Mediterranean fruit fly (Medfly) *Ceratitis capitata* (Wiedemann), the olive fly, *Bactrocera oleae* (Rossi) and the European cherry fruit fly, *Rhagoletis cerasi* (L.). *C. capitata*, is a highly polyphagous species that attacks more than three hundred plant species (Liquido et al., 1991). Medfly damages result from the oviposition in fruits, followed by larvae feeding in addition to decomposition of plant tissues by invading secondary microorganisms (Bachrouch et al., 2008). *B. oleae*, is the major pest of olive crops being specific of the genus *Olea* (Daane and Johnson, 2010). The larvae are monophagous, and feed exclusively on olive fruits, resulting in about 30% loss of the olive crop in some Mediterranean countries (IOC, 2014). *R. cerasi* is the most important pest of sweet and sour cherry in Europe and in temperate regions of Asia (Stamenković et al., 2012). The major damage caused by this pest result from larval feeding in the fruit pulp. If left uncontrolled, the percentage of damaged fruits can reach up to 100% (Stamenković et al., 2012).

The control of these pests is usually based on the application of chemical insecticides (Roessler, 1989; Montiel-Bueno and Jones, 2002; Daniel and Baker, 2013), with high negative impacts on environment, and with risk of development of pest resistance and of occurrence of adverse effects on non-target organisms (Vontas et al., 2011). This practice is not compatible with sustainable production such as organic agriculture and integrated pest management. Recently, the pest management programs have encouraged the use of alternative measures to control fruit flies (Andrea et al., 2005). Those measures included mostly the use of the sterile insect technique and traps, the application of new-generation bait sprays, and the release of parasitoids (Daane et al., 2015; Kapongo et al., 2007), but they have had little impact. An alternative control of these pests could be done by using entomopathogenic fungi. Previous studies have demonstrated that some entomopathogenic fungi, such as *Beauveria bassiana* (Balsamo) Vuillemin, *Isaria fumosorosea* Wize and *Metarhizium anisopliae* (Metchnikoff) Sorokin, have potential against puparia and adults of olive fly (Konstantopoulou and Mazomenos, 2005; Quesada-Moraga et al., 2006), Mediterranean fruit fly (Ekesi et al., 2002; Konstantopoulou and Mazomenos, 2005; Beris et al., 2013) and cherry fruit fly (Ladurner et al., 2008; Daniel and Wyss, 2009). Among the fungal species tested, *B. bassiana* is considered to be one of the most promising candidates against these fruit flies (Konstantopoulou and Mazomenos, 2005; Daniel and Wyss, 2009; Beris et al., 2013). This

fungus has a cosmopolitan distribution. More than thousand isolates have been collected from most parts of the world, from arthropod pests (Oliveira et al., 2012), asymptomatically plants (as endophyte) (Guesmi-Jouini et al., 2014) and soils (Quesada-Moraga et al., 2007). However, to date it is not clear how much these fungal isolates vary in their ability to kill fruit flies. This issue was regarded as of great importance, since intraspecific variation in virulence among various strains of *B. bassiana* has been observed (Valero-Jiménez et al., 2014). Equally important is the use of indigenous strains, isolated from and adapted to a specific environment, in pest management programs. In fact, previous works have been shown that several abiotic environmental factors, such as temperature, moisture and solar radiation (Sharififard et al., 2012) play a profound role on field persistence and efficiency of these fungi. Therefore, the selection of locally adapted strains may increases the guarantee of success of a biocontrol approach.

The control of fruit flies by using entomopathogenic fungi could be performed through the application of conidia to the soil (Garrido-Jurado et al., 2011a, 2011b). This method has been shown to be particularly effective in the fruit flies biocontrol (Toledo et al., 2007), because they live part of their life cycle in soil where pupation takes place and because under soil conditions the entomopathogenic fungi might survive better (Gaugler et al., 1989). In Portugal, studies aiming to determine the pathogenicity of native entomopathogenic fungi associated with major insect pest's of crops are scarce and their use as biological control agents against fruit flies has never been performed. Thus, the aim of this work was to compare the pathogenicity of four native *B. bassiana* isolates against *B. oleae*, *C. capitata*, and *R. cerasi* pupae using sand-conidial suspension incorporation bioassays and to determine the most virulent fungal isolates against these fruit flies.

6.2. Materials and Methods

6.2.1. Beauveria bassiana

Four isolates of *B. bassiana* (Ac36/gf10, Ac93/gf09, Bb 2T/08 and Bb 1M/10) previously obtained from *B. oleae* Rossi adults and *Prays oleae* Bern. larvae collected in Mirandela region (Portugal) showing signs of infection by fungi (Oliveira et al., 2012) and deposited in the culture collection of the Laboratório de Agroecologia da Escola Superior Agrária de Bragança, were used (Table 1). Isolates from dead adults of *B. oleae* and pupae of *P. oleae* were identified under microscope observation and molecular techniques by

amplification and sequencing the internal transcribed spacer region (ITS) of the ribosomal DNA (rDNA) using universal oligonucleotide primers ITS1 and ITS4 (White et al., 1990). From Ac36/gf10, Ac93/gf09, Bb 2T/08 and Bb 1M/10 isolates a suspension of conidia was used for bioassays. Viability of each isolate was determined before bioassay by spread 0.5 µl of initial suspension 10^8 conidia/ml on PDA plates. PDA (Potato Dextrose Agar, 39g/L) plates were maintained at 25ºC and examined after 16-17 hours. Percentage germination was determined from 100 spore counts at binocular microscope at 40× magnification. Conidia were considered to have germinated if the germ tube was longer than the diameter of the conidium. Each plate was replicate three times.

6.2.2. Insects

Three species of fruit flies were used in our experiments: the Mediterranean fruit fly, the European cherry fruit fly and the olive fly. Newly pupae of each fruit fly species were uses for experiments.

Bactrocera oleae: Infested olive fruits were collected from olive groves of Mirandela region in October of 2013. Infested olive fruits were transported to laboratory and were kept under controlled conditions (24±2ºC; 60±5% HR (relative humidity) and dark: light photoperiod, 16L:8D) until exit the third instar larvae. After pupate were used for bioassays.

Ceratitis capitata: Population of *C. capitata* used in experiments have been reared in methacrylate cages (40 x 30 x 30 cm) in artificial diet for several months, since September 2012 at Laboratório de Agroecologia da Escola Superior Agrária de Bragança, under controlled conditions 24±2ºC; 60±5% HR and dark: light photoperiod, 16L:8D. Adult diet consisted of 1 part of yeast extract and 4 parts of sugar (1:4) by weight. *C. capitata* larvae have been reared on artificial diet according González-Núñez (1998).

Rhagoletis cerasi: Infested cherry fruits were collected from cherry orchards of Bragança region in July of 2013. Infested cherry fruits were transported to laboratory and were kept under controlled conditions (24±2ºC; 60±5% HR and dark: light photoperiod, 16L:8D) until pupate.

6.2.3. Bioassays

For each fungal cultures, conidia from 20-day old cultures were used and conidia suspensions were prepared by scraping conidia from petri plates into a sterile aqueous solution of 0.2% (v/v) Tween 80 in eppendorfs. Conidial suspensions were vortexed to separate the conidia into a homogeneous suspension. Conidia were quantified by direct counting with an optical microscope using a Neubauer chamber and adjusted to 10^8 conidia ml^{-1} and then diluted to prepare concentrations of 10^8, 10^7, 10^6, 10^5, 10^4 conidia/mL in Tween 80 (0.2% v/v).

For bioassays, transparent plastic cages (9 x 4 cm), each containing 30 gr of autoclaved sand, were used. In each plastic cage was previously applied 100 µL of the spore suspension of each concentration, and the cages were shaken for a five minutes to spread the spore and to produce a homogenous suspension. Five replicates of each isolate were performed. For control was applied in each cage 100 µL of distilled water containing 0.2% of Tween 80. After treatment of the spore suspension ten newly pupae were placed in each plastic cage and cages were kept at 24ºC; 70% relative humidity and dark: light photoperiod, 16L:8D. To keep the humidity inside of cages, plastic cages were covered with filter paper that was periodically wetted until adult emergence. After emergence adults, the number of hatched adults and the number of pupae not emerged were counted. Pupae that failed to emerge were surface sterilized by sodium hypochlorite (1% v/v) during 30 seconds and transferred to a humid chamber (Petri dishes with moist filter paper) to induce sporulation in order to confirm infection of *B. bassiana*. *R. cerasi* have only one generation each year and a long obligatory winter diapause (Bateman, 1972; Daniel and Grunder, 2012), thus, in bioassay with *R. cerasi*, pupae were 2 months in plastic cages, and after two months were recovered and it was followed the procedure indicated above. After one week in humid chamber, the pupae without sporulation were surface sterilized by sodium hypochlorite (1% v/v) during 30 seconds and placed on Petri dishes with PDA medium and incubated at 25±2ºC in order to induce mycelial growth and sporulation of *B. bassiana*. Pupae that showed mycelial growth were considered to have died of infection and were used to evaluate the pathogenicity of *B. bassiana*. In case of *R. cerasi* pupae, in the end of bioassay all pupae were dissected to verify dead pupae. All pupae that were brown and shriveled were considered dead.

6.2.4. Data analysis

The percentage of mortality was adjusted for natural mortality in the control using Abbott's formula (Abbott, 1925). The lethal concentrations (LC_{50}) were determined by a probit analysis using R statistical program (R Core Team, 2016). For model fitting, generalized least squares were used by applying the *glm* function of package *stats* with the probit link function (binomial family). Data on the mortality for each isolate were compared using Tukey's HSD Post-hoc test at 5% probability using the SPSS PASW Statistics 18 for Windows, IBM.

6.3. Results

In viability tests, isolates of *B. bassiana* showed a higher germination rate of conidia, ranged values from 88.3% for isolate (Bb 1M/10) to 98.3% (Bb 2T/08) after 16-17 hours (Table 6.1).

Table 6.1. Fungal isolates used in bioassays and their viability (Mean%±SD) (*n=3*).

Isolates	**Origin of isolate**	**Host-stage isolation**	**% of germination±SD**
Bb 1M/10	*Bactrocera oleae*	Adult	88.33±12.01
Bb 2T/08	*Prays oleae*	Larva	98.33±2.08
Ac93/gf09	*Prays oleae*	Larva	97.66±4.04
Ac36/gf10	*Prays oleae*	Larva	97.00±5.20

Percentage adult emergence in the control treatments were 98.0%±4.47 and 96.0%±5.47 for *B. oleae* and *C. capitata* bioassay respectively (Table 6.2). No adult emergences were recorded in *R. cerasi* bioassay.

When *B. bassiana* was applied to sand *B. oleae* adults emergence varied from 6.0%±5.48 to 80.0%±21.21 depending on fungal isolate and concentration and percentage and *C. capitata* adult emergence varied from 4.0%±8.94 to 72.0%±20.49 depending on fungal isolate and concentration In bioassays with *B. oleae* there were significant differences in *B. oleae* adults emergence between all concentrations tested for all isolates. In bioassay with *C.*

capitata were recorded significant differences in adults emergence for all isolates except for isolate Ac93/gf09 (P=0.351).

Table 6.2. Adult emergence (%) of *Bactrocera oleae* and *Ceratitis capitata* for each isolate of *Beauveria bassiana* (Bb 1M/10, Bb 2T/08, Ac36/gf10, Ac93/gf09) and control after treatment with a concentration of 10^8, 10^7, 10^6, 10^5, 10^4 conidia/mL.

Bactrocera oleae				
	Bb 1M/10	Bb 2T/08	Ac93/gf09	Ac36/gf10
Control	98.0±4.47			
$1x10^4$	80.0±21.21	60.0±21.21	52.0±13.04	38.0±4.47
$1x10^5$	42.0±10.95	30.0±20.00	54.0±19.49	40.0±15.81
$1x10^6$	48.0±16.43	22.0±19.24	40.0±14.14	30.0±18.71
$1x10^7$	22.0±13.04	16.0±18.17	16.0±8.94	12.0±8.36
$1x10^8$	10.0±7.07	6.0±5.48	6.0±8.94	14.0±11.40
P	<0.01	<0.01	<0.01	<0.01
Ceratitis capitata				
	Bb 1M/10	Bb 2T/08	Ac93/gf09	Ac36/gf10
Control	96.0±5.47			
$1x10^4$	72.0±20.49	64.0±11.40	22.0±10.95	46.0±16.36
$1x10^5$	52.0±25.88	50.0±10.00	20.0±10.00	36.0±15.17
$1x10^6$	40.0±21.21	36.0±11.40	16.0±5.48	26.0±8.94
$1x10^7$	20.0±10.00	18.0±16.43	8.0±8.37	16.0±15.17
$1x10^8$	8.0±8.37	4.0±8.94	14.0±11.40	8.0±8.36
P	<0.01	<0.01	0.351	<0.01

Tuckey's HSD post-hoc test within the same treatment, n=50.

The range of no viable pupae was between 20.0% to 94.0%, 14.0% to 88.0% and 2.0% to 32.0% in bioassays with *B. oleae*, *C. capitata* and *R. cerasi* respectively. All tested fungal isolates were able to cause mycosis to olive fly, Mediterranean fruit fly and Cherry fruit fly pupae. The entomopathogenic activity of tested isolates was confirmed by the presence of mycelial growth on pupae (Figure 6.1).

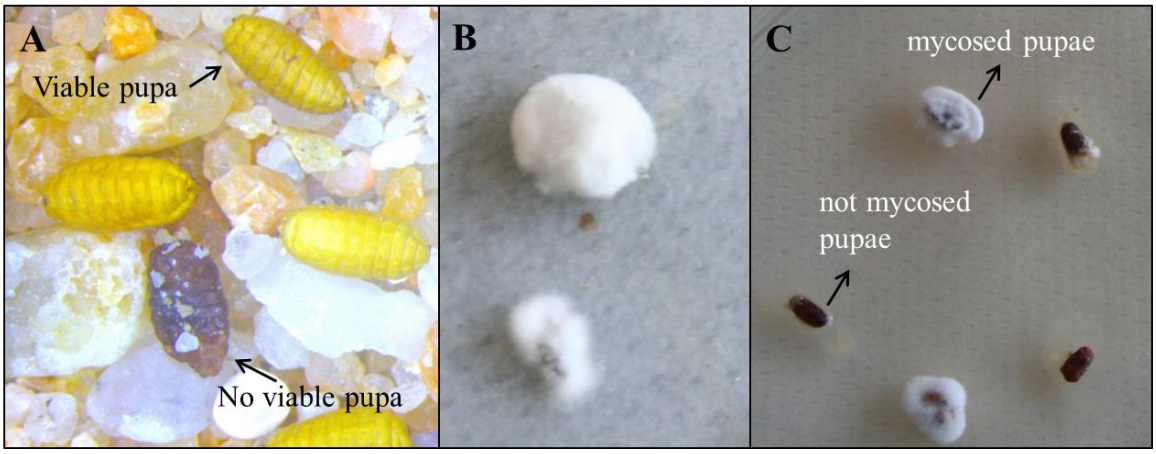

Figure 6.1. A) viable and no viable pupae of *Rhagoletis cerasi* in sand, B) mycelial growth under pupae in humid chamber, C) mycosed pupae and not mycosed pupae in PDA.

There was significant differences (*P<0.05*) on *B. oleae* pupae with mycelial growth between concentrations for all isolates tested, Bb 1M/10 (F=20.92; *P<0.01*), Bb 2T/08 (F=14.34; *P<0.01*), Ac36/gf10 (F=4.57; *P=0.01*), Ac93/gf09 (F=8.77; *P<0.01*) and was observed that mortality was significantly affected by conidial concentrations. Among the concentrations tested, was no observed significant differences between the highest concentration tested (10^8 conidia/mL and of 10^7 conidia/mL). For olive fly, the highest percentage of pupae with visible signs of mycosis was recorded with concentration of 10^8 conidia/ml for all isolates tested, except for isolate Ac36/gf10 that present the same value (78.0%±4.5 and 78.0%±13.0) at 10^8 and 10^7 concentration respectively (Table 6.3) having the isolate Bb 2T/08 present the highest value of *B. oleae* pupae with mycelial growth (94.0%±5.5) followed by isolate Bb 1M/10 (90.0%±7.1). For the concentration 10^7 conidia/mL, values of pupae with mycoses ranged from 76.0%±11.4 to 82.0%±16.4 between isolates. The lower percentage of pupae with mycelial growth was obtained with the lowest concentration (10^4 conidia/mL) for all isolates having values of pupae with visible signs of

mycosis ranged from 18.0%±20.49 to 48.0%±8.37 between tested isolates. No pupae with mycosis were found in *B. oleae* control.

Table 6.3. Non-viable pupae and pupae with mycelial growth (mean%±SD) of *Bactrocera oleae, Ceratitis capitata* and *Rhagoletis cerasi* pupae after treatment of with four isolates of *Beauveria bassiana* (Bb 1M/10, Bb 2T/08, Ac36/gf10, Ac93/gf09) in five conidial concentrations (10^8, 10^7, 10^6, 10^5, 10^4 conidia/mL).

	Bactrocera oleae		*Ceratitis capitata*		*Rhagoletis cerasi*	
	NVP (%)	WFG (%)	NVP (%)	WFG (%)	NVP (%)	WFG (%)
Control	2.00±4.47		4.00±5.48		6.00±13.42	
Bb 2T/08						
$1x10^4$	40.0±21.21c	34.0±23.02c	36.0±11.40d	14.0±5.48c	10.0±12.25a	0.0±0.00b
$1x10^5$	70.0±20.00b	62.0±13.04b	50.0±10.00cd	24.0±11.40bc	6.0±5.48a	2.0±4.47b
$1x10^6$	78.0±19.24ab	68.0±13.04b	62.0±10.95bc	40.0±7.07b	26.0±11.40ab	10.0±12.25ab
$1x10^7$	84.0±18.17ab	82.0±16.43ab	82.0±16.43ab	70.0±18.71b	32.0±16.43b	16.0±18.16ab
$1x10^8$	94.0±5.48a	94.0±5.48a	96.0±8.94a	86.0±13.42a	26.0±21.90ab	24.0±23.02a
Bb 1M/10						
$1x10^4$	20.0±21.21d	18.0±20.49c	28.0±20.49d	16.0±20.74a	4.0±5.48a	2.0±4.47a
$1x10^5$	58.0±10.95cb	46.0±11.40b	48.0±25.88cd	32.0±19.24bc	4.0±5.48a	4.0±5.48a
$1x10^6$	52.0±16.43b	50.0±18.71b	60.0±21.21bc	42.0±27.75b	2.0±4.47a	2.0±4.47a
$1x10^7$	78.0±13.04ac	76.0±11.40a	80.0±10.00ab	58.0±17.89b	12.0±4.47a	12.0±4.47a
$1x10^8$	90.0±7.07a	90.0±7.07a	92.0±8.37a	88.0±10.95a	12.0±4.47a	12.0±4.47a
Ac36/gf10						
$1x10^4$	62.0±4.47b	48.0±8.37b	54.0±16.73c	22.0±8.37b	10.0±14.14b	2.0±4.47a
$1x10^5$	60.0±15.81b	52.0±13.04b	64.0±15.17bc	26.0±11.40b	14.0±16.73ab	2.0±4.47a
$1x10^6$	70.0±18.71ab	66.0±20.74bc	74.0±8.94abc	34.0±8.94b	18.0±20.49ab	4.0±8.94a
$1x10^7$	88.0±8.37a	78.0±4.47ac	84.0±15.17ab	74.0±15.16a	12.0±8.36a	6.0±8.94a
$1x10^8$	86.0±11.40a	78.0±13.04a	92.0±8.37a	88.0±8.37a	12.0±13.04ab	12.0±13.04a
Ac93/gf09						
$1x10^4$	48.0±13.04b	42.0±13.04b	78.0±10.95a	40.0±30.82b	8.0±8.37a	0.0±0.00b
$1x10^5$	46.0±19.49b	44.0±20.74b	80.0±10.00a	44.0±20.74b	18.0±21.68a	2.0±4.47ab
$1x10^6$	60.0±14.14b	56.0±11.40ab	84.0±5.48a	60.0±25.50ab	26.0±11.40a	6.0±8.94ab
$1x10^7$	84.0±8.9a	78.0±4.47a	92.0±8.37a	80.0±7.07a	20.0±15.81a	8.0±8.36ab
$1x10^8$	94.0±8.94a	84.0±8.94a	86.0±11.40a	64.0±20.74ab	22.0±8.37a	14.0±11.40a

NVP – non-viable pupae; WFG – with mycelial growth, Data with different letters within column indicates a significant difference at $P<0.05$ according to Tuckey's HSD post-hoc test within the same treatment, n=50.

The highest percentage of Mediterranean fruit fly pupae with visible signs of mycosis was recorded with concentration of 10^8 conidia/mL for isolates Bb 1M/10 and Ac36/gf10 with 88.0%±11.0 and 88.0%±8.4 respectively followed by isolate Bb 2T/08 with 86.0±13.4% and at 10^7 conidia/mL for isolate Ac93/gf09 with 80.0%±7.1. For the concentration 10^6 conidia/mL the values of pupae with mycelial growth was lower than 50% except for isolate Ac93/gf09 that registered 60.0%±25.5 of pupae with mycelial growth. The lower percentage of pupae with mycelial growth was obtained with the lowest concentration (10^4 conidia/mL) for all isolates having values of pupae with mycelial growth ranged from 14.0±5.5% to 40.0±30.8% between all isolates. There was significant differences (*P<0.05*) on *C. capitata* pupae with mycelial growth between concentrations for all isolates tested, Bb 1M/10 (F=19.53;; *P<0.01*), Bb 2T/08 (F=4.65; *P<0.01*), Ac36/gf10 (F=25.07; *P<0.01*), Ac93/gf09 (F=5.72; *P<0.01*). No visible mycosis was found in *C. capitata* control.

The fungal isolates tested presented low pathogenicity to cherry fruit fly pupae. Only a few number of pupae exhibited superficial fungal growth. The highest percentage of cherry fruit fly pupae with mycosis was recorded with concentration of 10^8 conidia/mL for isolate Bb 2T/08 with 24.0%±2.02 and except for this isolate, values of pupae with mycelial growth were lower than 16% with all concentrations tested. There was significant differences (*P<0.05*) on mortality of cherry fly pupae for the treatments with the strains Bb 2T/08 (F=5.68; *P<0.01*), and Ac93/gf09 (F=2.72; *P=0.03*) and no differences were recorded between concentrations for isolates Bb 1M/10 (F=2.27; *P=0.06*) and Ac36/gf10 (F=1.47; *P=0.21*). No visible mycosis was found in *R. cerasi* control.

Values of corrected mortality are present in Figures 6.2, 6.3 and 6.4. A positive correlation was recorded between concentration and mortality. Suspension of 10^8 conidia/mL showed great pathogenicity against *B. oleae* and *R. cerasi* pupae. For *B. oleae* the isolate more pathogenic was the isolate Bb 2T/08 with 93.9% of corrected mortality and for *C. capitata* were the isolates Bb 1M/10 and Ac36/gf10 with 87.5% of corrected mortality. The lower values of mortality were obtained for the lower concentration (10^4 conidia/mL) for all isolates tested against *B. oleae, C. capitata* and *R. cerasi*.

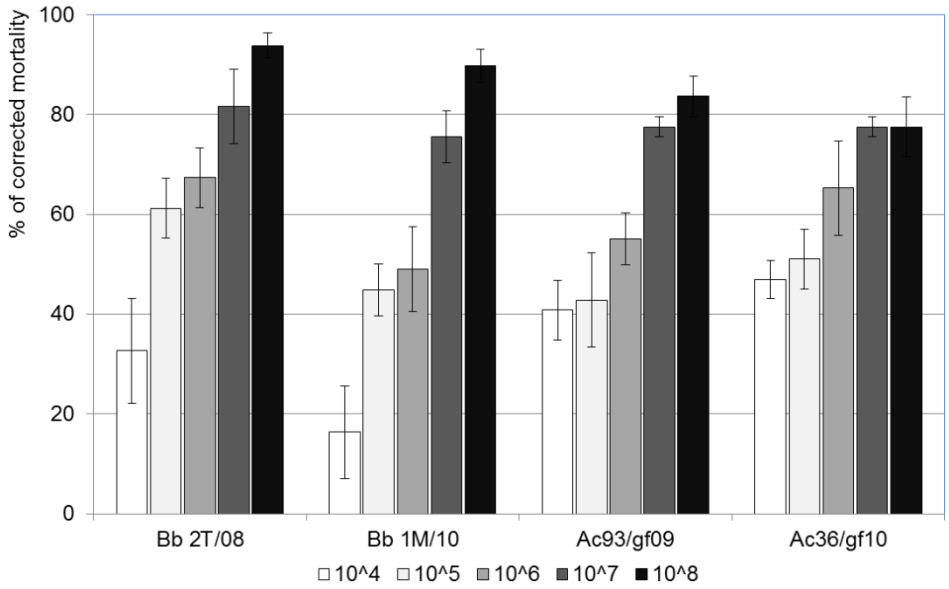

Figure 6.2. Corrected mortality (%) of *Bactrocera oleae* pupae after treatment of with four isolates of *Beauveria bassiana* (Bb 1M/10, Bb 2T/08, Ac36/gf10, Ac93/gf09) in five conidial concentrations (10^8, 10^7, 10^6, 10^5, 10^4 conidia/mL) (Vertical lines mean standard error).

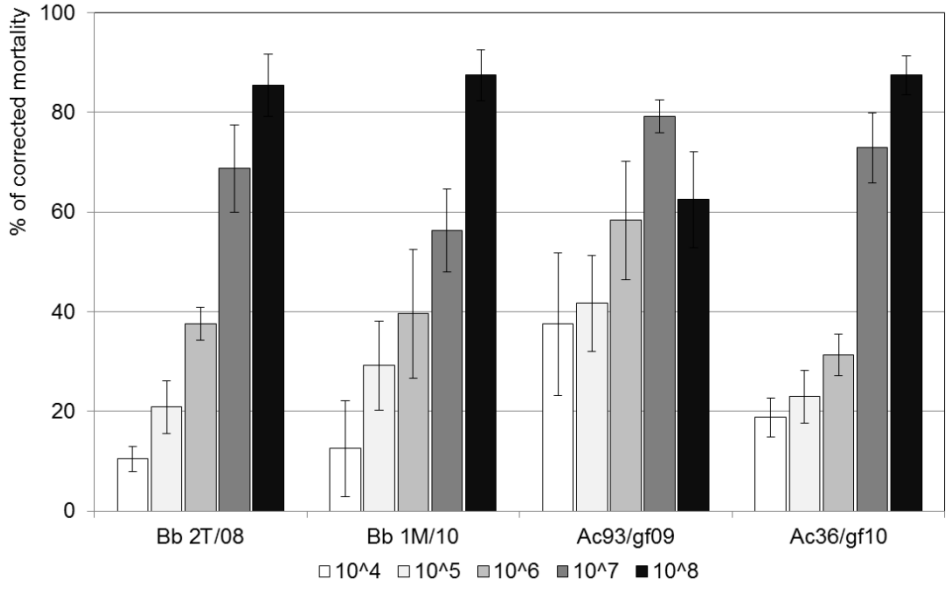

Figure 6.3. Corrected mortality (%) of *Ceratitis capitata* pupae after treatment of with four isolates of *Beauveria bassiana* (Bb 1M/10, Bb 2T/08, Ac36/gf10, Ac93/gf09) in five conidial concentrations (10^8, 10^7, 10^6, 10^5, 10^4 conidia/mL) (Vertical lines mean standard error).

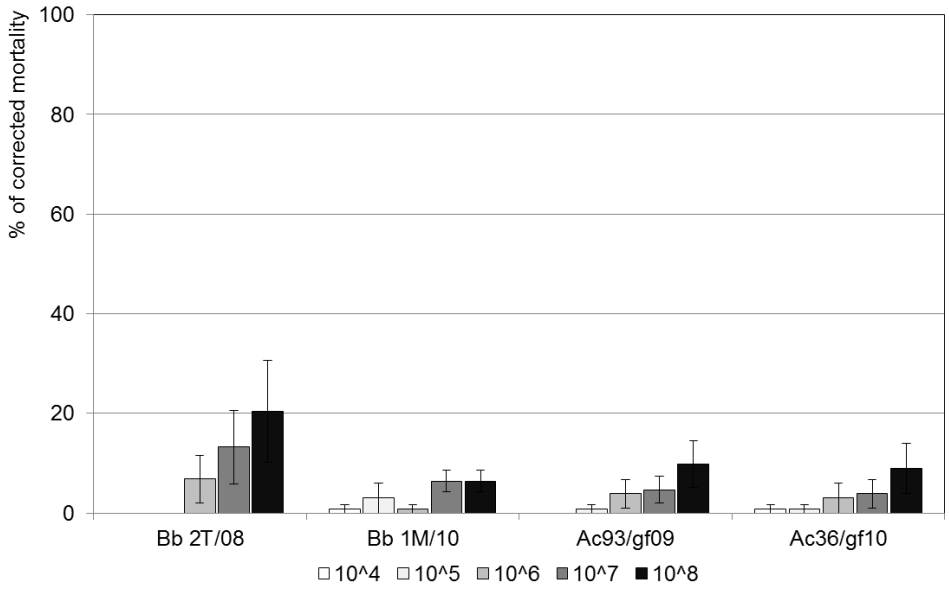

Figure 6.4. Corrected mortality (%) of *Rhagoletis cerasi* pupae after treatment of with four isolates of *Beauveria bassiana* (Bb 1M/10, Bb 2T/08, Ac36/gf10, Ac93/gf09) in five conidial concentrations (10^8, 10^7, 10^6, 10^5, 10^4 conidia/mL) (Vertical lines mean standard error).

The estimated LC_{50} values for all four isolates tested against *B. oleae* and *C. capitata* are shown in Table 6.4. The lethal concentrations (LC_{50}) of the isolates ranged from 5.9×10^4 for the most pathogenic isolate (Ac36/gf10) to 7.1×10^6 for the less pathogenic isolate (Bb 2T/08) All LC_{50} values estimated in *B. oleae* and *C. capitata* bioassays are in the interval of the studied concentrations. The isolate Ac36/gf10 was one of the most virulent isolate is bioassays showing high virulence for *B. oleae* and *C. capitata*. Bb 1M/10 isolate presented low LC_{50} values in all bioassays.

R. cerasi, has only one generation each year and a long obligatory winter (Bateman, 1972). As no adults of cherry fly emerged during the time of this study, the values of LC_{50} for this pest were not possible to calculate.

Table 6.4. Lethal concentration LC_{50} (conidia/mL) for the isolates of *Beauveria bassiana* tested (Bb 2T/08, Bb 1M/10, Ac93/gf09, Ac36/gf10); fiducial limits of LC_{50}; overall model chi-square; and parameters estimates from model fitting using *glm* function.

Isolates	LC_{50}	Fiducial limits $LL<LC_{50}<UL$	Chi-square x^2	Model coefficients	
Bactrocera oleae					
Bb 2T/08	4.4×10^{7}	$1.5\times10^{7}<LC_{50}<1.3\times10^{8}$	48.50***	b0 = -3.2667 (-4.3296; -2.5065)	b1 = 0.5163 (0.3136; 0.5802)
Bb 1M/10	1.6×10^{6}	$2.6\times10^{6}<LC_{50}<1.3\times10^{7}$	66.97***	b0 = -3.4418 (-4.3305; -2.5917)	b1 = 0.3277 (0.3811; 0.6429)
AC93/Gf09	2.1×107	5.4×106<LC50<7.8×107	30.46***	b0 = -2.3970 (-3.2078; -1.6095)	b1 = 0.2184 (0.2094; 0.4488)
AC36/Gf10	1.0×10^{8}	$1.0\times10^{7}<LC_{50}<1.0\times10^{9}$	16.44***	b0 = -1.9093 (-2.6964; -1.1399)	b1 = 0.2381 (0.1222; 0.3559)
Ceratitis capitata					
Bb 2T/08	1.6×10^{6}	$7.8\times10^{5}<LC_{50}<3.2\times10^{6}$	79.68***	b0 = -3.0199 (-4.3540; -2.6319)	b1 = 0.5605 (0.4300; 0.6967)
Bb 1M/10	1.6×10^{6}	$7.3\times10^{5}<LC_{50}<3.6\times10^{6}$	62.66***	b0 = -3.4753 (-3.8659; -2.2039)	b1 = 0.4861 (0.36032; 0.6166)
AC93/Gf09	2.0×10^{7}	$2.8\times10^{6}<LC_{50}<1.4\times10^{8}$	14.59***	b0 = -1.5933 (-2.3444; -0.8530)	b1 = 0.2184 (0.1058; 0.3323)
AC36/Gf10	2.1×10^{6}	$9.9\times10^{5}<LC_{50}<4.5\times10^{6}$	70.26***	b0 = -3.2667 (-4.1259; -2.4398)	b1 = 0.5163 (0.3897; 0.6476)

*** - *$p<0.001$*; LL – lower limit of 95% confidence interval for LC_{50}, UL – upper limit of 95% confidence interval for LC_{50}; b0 – intercept; b1 – slope; (Between parentheses are shown the 95% confidence intervals for model coefficients).

6.4. Discussion

The entomopathogenic fungi that were used can induce mortality in *B. oleae*, *C. capitata* and *R. cerasi* pupae when exposed to sand treated with conidial suspension. The overall effect of sand treated with entomopathogenic fungi led in a reduction in adults emergence with the increase of fungal concentration and significant increase of mortality in *B. oleae* and *C. capitata* pupae.

Adult emergence in control was high, 98.0% and 96.0% in *B. oleae* and *C. capitata* bioassays respectively. When it was applied *B. bassiana* to sand there was a reduction of adult emergence in all concentration tested, ranging values from 6.0% to 80.0% and 4.0% to 72.0% in *B. oleae* and *C. capitata* bioassays respectively. *B. bassiana* frequently forms white mass around the puparium inhibiting later the adult emergence if the integrity of the puparium remain intact and interior tissue was not infected (Castillo et al., 2000; Lezama-Gutiérrez et al., 2000; Ekesi et al., 2002). No *R. cerasi* adult emergences were found in *R. cerasi* bioassay. This is due because *R. cerasi* is an univoltine pest (Boller, 1966). The flies spend about 10-11 months in soil as pupa and the first flies usually appear in the orchards between mid-May and mid-June (Böhm, 1949). As cherry fruit fly has an obligate and lengthy pupal diapause, the impact of sand treated with *B. bassiana* isolates on the adults emergence cannot be assessed without an extended cold period.

The range of no viable pupae was between 20.0% to 94.0%, 14.0% to 88.0% and 2.0% to 32.0% in bioassays with *B. oleae*, *C. capitata* and *R. cerasi* respectively. The percentage values of non-viable pupae obtained in bioassay with *C. capitata* were higher than an experiment performed by Lozano-Tovar et al. (2013) using isolates of *Beauveria* spp. and *Metarhizium* spp in bioassays with *C. capitata*, where were recorded values ranged from 12.5% to 60.0%.

All tested fungal isolates were able to cause mycosis to olive fly, Mediterranean fruit fly and cherry fruit fly pupae and percentage of pupae with fungal mycelial growth differs significantly between concentrations in bioassays with all isolates tested except for isolate Ac36/gf10 in *R. cerasi* bioassay. The percentage of pupae with signs of mycosis (mycelial growth) ranged from 18.0% to 94.0% and from 14.0% to 88.0% between concentrations in bioassays with *B. oleae* and *C. capitata* respectively, having *B. oleae* bioassays presented the highest percentage of pupae with visible mycosis. Bioassays using *B. bassiana* isolates against *B. oleae* are mostly carried out at the adult stage, have been registered high adult

mortality rates (Anagnou-Veroniki et al., 2005; Konstantopoulou and Mazomenos, 2005; Blibech et al., 2012). Sookar et al. (2010) verified that when were applied *B. bassiana* suspensions to larvae of *Bactrocera zonata* (Saunders) and *Bactrocera cucurbitae* (Coquillett) the percentage of puparia with visible signs of mycosis did not exceed 40.0%. Several studies showed high mortality on *C. capitata* adults when *B. bassiana* is applied (Konstantopoulou and Mazomenos, 2005; Beris et al., 2013) and variable rate of puparia mortality and incidence of visible mycosis on pre-imaginal stages of *C. capitata* for different isolates, having been reported rates of puparia mortality and incidence of visible mycosis ranging from 3.3% to about 80.0% (Ekesi et al., 2002; Quesada-Moraga et al., 2006; Eldesouki-Arafat et al., 2007; Ali et al., 2009; Garrido-Jurado et al., 2011b; Lozano-Tovar et al., 2013). Relatively to *R. cerasi* bioassay, the incidence of visible mycosis on pupae of *R. cerasi* was low, reaching 20% of pupae with the presence of mycelial growth in the highest concentration. This results are according with Daniel (2009) having found when dipping *R. cerasi* mature larvae (L3) in a conidial suspension and putting them on moist silica sand none of the fungal isolates induce mortality more than 25% of larvae and visible mycosis ranged from 4.2% to 20.8%. Also Cossentine *et al*. (2010) achieved low mycosis on pupae of *Rhagoletis indifferens* Curran ranging between 23% and 35%. Some studies demonstrated that fruit flies species vary so much in susceptibility to EP fungi to exhibit variations in virulence. Konstantopoulou and Mazomenos (2005) demonstrated that both *B. oleae* and *C. capitata* adult fruit flies showed different degrees of susceptibility to two EP fungal species, *B. bassiana* and *Beauveria brongniartii* (Sacc.). Dimbi et al. (2003) reported considerable variation in virulence among fungal isolates tested against *C. capitata*, *Ceratitis rosa* var. *fasciventris* Karsh and *Ceratitis cosyra* (Walker).

Bb 2T/08 isolate showed high mortality (93.9%) in bioassays with *B. oleae* and the isolates Bb 1M/10 and Ac36/gf10 with 87.5% were the most pathogenic for *C. capitata* (values of corrected mortality). Natural mortality in the controls never exceeded 6%. All isolates tested against olive fly and Mediterranean fruit fly were able to kill at least 50% of pupae in the interval of concentrations studied. The isolate Bb 1M/10 showed high pathogenicity for *B. oleae* and *C. capiata*. The lethal concentrations (LC_{50}) of the isolates ranged from 1.6×10^{6} conidia/mL for the most pathogenic isolate to 1.8×10^{8} conidia/mL for the less pathogenic isolate in *B. oleae* bioassay and from 1.6×10^{6} conidia/mL to 2.0×10^{7} conidia/mL in *C. capitata* bioassay. Ekesi et al. (2002) also found that the fungal isolates used

to control *C. capitata* puparia had median lethal concentration (LC_{50}) values of between 1.7×10^5 conidia/mL to 7.7×10^6 conidia/mL.

According to Vanninen *et al*. (1999), stages of insects living in the soil may have developed high levels of resistance to infection by natural selection because fungal entomopathogens are widespread in soil. Several studies also demonstrated that pre-imaginal stages of tephritids, particularly puparia, are less susceptible to entomopathogenic fungi (Kaaya and Munyinyi, 1995; Ekesi et al., 2002, 2007; Toledo et al., 2006), may be due to the cuticle of the third stage larvae remains in the tephritids to form the puparium conferring a barrier to penetration. However, the wide variability on rates of puparia mortality and incidence of visible mycosis has demonstrated that some isolates of *B. bassiana* can be pathogenic by the high pathogenicity demonstrated, for pre-imaginal stages of fruit flies. The need to carry out the bioassays on laboratory to obtain highly infective and virulent strains is an important step for development of mycoinsecticides to control pests. This knowledge will also be very useful in improving the efficacy of these fungi as biological control agents. The results of our experiments showed that the isolates tested (Bb 1M/10, Bb 2T/08, Ac93/gf09 and Ac36/gf10) have great potential as possible biological control agents, mainly to olive fly and Mediterranean fruit fly.

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CHAPTER 7

Control of the olive fly, *Bactrocera oleae* (Rossi), in sustainable agriculture: the use of Olipe traps with different hole sizes.



Demasiado tarde. Há um momento, quase a roçar a teia, em que a mosca ainda estaria a tempo de escapar à armadilha, mas, se chegou a tocar-lhe, se o visco filou a asa doravante inútil, qualquer movimento apenas servirá para que o insecto mais se enrede e paralise, irremediavelmente condenado, mesmo que a aranha desprezasse, por insignificante, esta peça de caça.

O Evangelho segundo Jesus Cristo, José Saramago (1991)

Coelho, V.; Bento, A.; Mexia, A.; Pereira, J.A., Control of the olive fly, *Bactrocera oleae* (Rossi), in sustainable agriculture: the use of Olipe traps with different hole sizes. "in preparation"

Abstract

The olive fly, *Bactrocera oleae* (Rossi) is a key-pest of olives in the Mediterranean region, being their control usually based on the use of chemical pesticides, strategy not fully compatible with sustainable olive production systems. In this context, in the last decades, the use of Olipe traps, a bottle with holes and a fly attractant liquid, has been increasing by the growers. However, their efficacy is questionable, and needs to be improved. In this work, the effect of different bottle hole sizes on the Olipe traps efficacy was studied. The work was developed from 2009 to 2011, in an organic olive grove located in Mirandela (Northeast of Portugal), five plots of 1.5 ha, one per hole size (4, 6, 8 and 10 mm of diameter) and one as control were installed with the basis of one trap/tree. Fortnightly, in 15 traps the number of flies was counted, and the fruit infestation level was evaluated on 25 fruits/20 trees/plot. The flight curve was monitored using yellow sticky traps with sex pheromone weekly checked. The results demonstrated that the level of insect populations was the main factor that influences the efficacy of the Olipe traps and consequently the crop protection. In years of low or medium pest populations, Olipe traps decrease the infestation levels protecting the crop, nevertheless with high populations, such as in 2011, the efficacy of the traps is reduced or absent. The hole of the traps showed to be an important factor in its efficacy; traps with smaller holes diameters seem less efficient than traps with bigger hole diameter in reducing population levels. Nevertheless the limited results, due to the easy of application and very low cost of Olipe traps continuous to be an option for olive fly control in organic agriculture that should be improved.

Key-words: *Bactrocera oleae* (Rossi), Olipe traps, hole size, mass trapping.

7.1. Introduction

The olive tree is a typically Mediterranean crop that is distributed in all regions of the world where Mediterranean climate predominates. In the Mediterranean region, with around 98% of the word's cultivated olive trees, this tree is a characteristic element of the landscape, which has a great economic, ecological and social importance. This crop is attacked by many pests and diseases that reduce their yield. The olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is the major insect pest of olives worldwide.

The olive fly causes serious quantitative and qualitative damages with economic importance. The first, results from premature fall of fruit to soil that depending on the year could reach 90% of the production (Bento, 1999, 2009) and pulp destruction by larvae feeding (Neuenschwander and Michelakis, 1978; Economopoulos *et al*., 1986), ranging from 3 to 20% depending on the fruit size (Kapatos and Fletcher, 1983). The qualitative damages results from the emergence holes of adults that favor the attack of fungi and bacteria, increasing hydrolysis and oxidation, and decreasing the antioxidant compounds therefore reducing the olive oil quality (Pereira *et al*., 2004). For table olives the existence of exit holes result in total trade devaluation.

The use of pesticides has been the predominant *B. oleae* control strategy in the last seven decades which affect the food quality and safety of olive products, due to the presence of pesticide residues (Amvrazi and Albanis, 2009). On the other side, pesticide applications against olive fly have negative impact on beneficial fauna of olive groves (Cirio, 1997; Ruano *et al*., 2001), in losses of biodiversity and development of resistance in this pest (Marc *et al*., 1999; Hawkes *et al*., 2005).

The described framework is not compatible with the principles of sustainable agriculture. In sustainable olive production the olive fly control strategy should be based on the improvement of crop protection level based in knowledge of the *B. oleae* bioecology, their susceptibility factors, correct fly population monitoring systems, establishment of economic threshold levels, and the use of selected control means.

During the last decades, different control strategies against the olive fly were proposed. Such include for example the use of *Bacillus thuringiensis* (Navrozidis *et al*., 2000), application of Kaolin-based particle film (Belcari *et al*., 2005; Pascual *et al*., 2010), biological control using various parasitoids (Hepdurgun *et al*., 2009; Wang *et al*., 2009) and mass trapping (Petacchi *et al*., 2003; Ragoussis, 2005; Noce *et al*., 2009) among others.

The mass trapping has been a technique widely used in Mediterranean region to control olive fly (Broumas *et al*., 2002; Delrio, 1989). This capture technique has been developed according to different types of traps. One of these is the Olipe trap, developed in Spain in the Cooperativa Olivarera de los Pedroches, (Caballero, 2001). That consist in a translucent plastic bottle (polyethylene terephthalate) with a capacity of 1.5 liters (30 cm high, 9 cm in diameter) perforated, usually with six holes and placing inside the food attractant, generally an aqueous solution of ammonia and in some situations sex pheromone. The flies are attracted by the attractant, enter through the holes, and eventually drown in the solution.

Some works pointed that in olive growing under organic agriculture, mass trapping with Olipe traps is an option to consider, due to its low cost and effectiveness, and may reduce populations of the olive fly to levels considered acceptable (Caballero, 2002; Pavão *et al*., 2007). However, several studies have showed limited efficacy in reducing fruit infestation levels (Duatis *et al*., 2006; Tabic *et al*., 2011) and also negative impact in beneficial fauna (Seris *et al*., 2007; Porcel *et al*., 2009) that are factors that limit their use and put in evidence the need to improve the efficacy of Olipe traps.

The aim of this work was to obtain data about the biology of the olive fly in the northeast of Portugal, and to evaluate the use of Olipe traps as control method against olive fly. As bottle hole size has been considered a key point in the trap efficacy, four different trap hole sizes (4, 6, 8 and 10 mm of diameter) were evaluated.

7.2. Materials and Methods

7.2.1. Study area

The study was conducted in a commercial olive grove for oil production located near Mirandela (North Eastern Portugal) – Cedães grove. Cedães grove (41º29’18.84’’N, 7º07’36.02’’W) has been conducted following the rules of Integrated Pest Management since 2001. The olive trees, cv. Cobrançosa, are of medium size and spaced at 7 × 7 meters. The grove was non-irrigated and the soil is conducted with natural vegetation. The trees are pruned every two to three years, and no phytosanitary treatments were done during the year of the experiments.

7.2.2. Experimental design

The efficacy of mass trapping with Olipe traps with different hole sizes was evaluated during three consecutive years, from 2009 to 2011

The Olipe trap consists of a PET translucent bottle with 1.5 L capacity (30 cm high, 9 cm diameter and 825 cm^2 of outer surface) with six drilled holes 6-8 cm from the top of the bottle. Different hole sizes were evaluated, namely, 4, 6, 8 and 10 mm of diameter. And the biammonium phosphate (ammonium di-hydrogen phosphate, Panreac) at 3% was used as attractant bait (Pavão *et al.*, 2007).

In each year, the olive grove was divided in five plots of about 1.5 hectare each, where in four plots Olipe traps were placed in a branch of the tree at the rate of one trap per tree, and corresponding to each of the studied diameters (4, 6, 8 and 10 mm). The fifth plot acted as control and no Olipe traps were installed.

Olipe traps were filled to 2/3 of its capacity with 3% biammonium phosphate and placed in the inner canopy, placed about 150-190 cm of height, remaining in the field, in 2009, since the end of August until the end of October; in 2010 from mid-August to early November; and in 2011 from end of July until early November. Periodically, to avoid the traps staying empty due to evaporation the attractant solution was added.

In each plot, at fortnightly basis, 20 trees were randomly selected in the center of each plot, and in each tree, 25 olives were collected. The fruit samples were observed under the binocular microscope, and the number of immature stages, eggs, young larvae, that included the first-instar larvae (L1) and second-instar larvae (L2), mature larvae (third-instar larvae - L3) and pupae (Figure 7.1) on infested drupes was registered. Dead larvae and sterile ovipositions were also counted. With the results, for each date the pre-imaginal population of *B. oleae* was determined and the infestation index (Ii) was calculated by each plot (size hole) and date.

Figure 7.1. Pre-imaginal population (egg, young larva, mature larva, pupa) of *Bactrocera oleae* (Rossi).

The flight pattern of *B. oleae* was monitored from July to November by five yellow sticky traps with the sex pheromone of the insect (1,7-dioxaspiro[5.5]undecane). Traps were spaced in south side of the tree at least 50 m between them and in a weekly basis the number of *B. oleae* catches were registered. The results were expressed as flies per trap per week.

7.2.3. Statistical analysis

Statistical analyses were carried out with the program PASW Statistics 18, IBM. In the case of fruit infestation, analysis of variance (ANOVA) and Tukey´s honestly significant difference (HSD) test ($p < 0.05$) were used to compare the means among different plots by date ($p < 0.05$).

7.3. Results

Phenology: Data about the pre-imaginal population of *B. oleae* for the different years (2009, 2010 and 2011) along the sampling periods are shown in Table 7.1. A total of 531 *B. oleae* immature individuals alive were sampled in 2009, 178 in 2010 and 1,837 in 2011. The total number of eggs registered in 2009 was 212, have been recorded 123 eggs in 2010 and have been recorded 364 eggs in 2011. The highest average number of eggs per tree in 2009 was recorded at 24th October (1.21±0.16), that corresponds to the date before the harvest by the grower. Eggs appeared from mid-September, representing 9.3% of pre-imaginal population and their number increase until the end of October where their oviposition peak has occurred, and representing 50.4% of pre-imaginal population. In 2010, eggs were registered continuously from mid-August to early November and their oviposition peak has occurred at 24th October (0.46±0.07). It is noteworthy that in August there are only eggs. In 2011, first eggs appear in early August and were registered continuously to early November and their oviposition peak has occurred at 11th October (0.94±0.16). However it was at 27th September that the eggs reached the highest percentage (36.4%) in relation to pre-imaginal population.

Relatively to young larvae (L1+L2) were registered 278 young larvae in 2009, 50 in 2010 and 1,073 in 2011. The highest average number of young larvae per sample was recorded at 24th October in 2009 (1.08±0.16), nevertheless at 13th September the young larvae represented 86.0% of total pre-imaginal population. In 2010, the first young larvae appeared

in 13th September and were present until to the harvest. It reached their peak at 24th October (0.21±0.05). In 2011, young larvae were observed during all the sampling dates reached their peak at 11th October (2.67±0.31). The highest percentage of young larvae in this year was observed in the first sampling (88.2%).

Table 7.1. Total number of pre-imaginal individuals (eggs, young larvae, mature larvae and pupae) of *Bactrocera oleae* (Rossi) in 2009, 2010 and 2011, at different sampling times.

	2009				2010				2011			
	E	YL	L3	P	E	YL	L3	P	E	YL	L3	P
2nd Aug.	-	-	-	-	-	-	-	-	10	97	2	1
16th Aug.	-	-	-	-	5	0	0	0	17	48	16	0
30th Aug.	0	15	2	3	11	0	0	0	15	49	7	2
13th Sep.	4	37	2	0	10	3	0	0	14	51	7	8
27th Sep.	16	40	7	1	3	1	0	0	68	87	16	16
11th Oct.	71	78	8	7	29	5	0	0	94	267	91	38
24th Oct.	121	108	7	4	46	20	2	0	86	256	67	49
8th Nov.	-	-	-	-	19	21	3	0	60	218	50	30
Total	**212**	**278**	**26**	**15**	**123**	**50**	**5**	**0**	**364**	**1073**	**256**	**144**

E – Eggs, YL – Young Larvae (L1+L2), L3 – Mature Larvae, P – Pupae

The total number of mature larvae (L3) was 26 in 2009, 5 in 2010 and 256 in 2011. The highest average number of mature larvae per tree was recorded at 30th August in 2009 (0.08±0.04), at 24th October in 2010 (0.03±0.02) and at 27th September in 2011 (0.91±0.15). In 2009, mature larvae were present during all the sampling dates reached their highest percentage (11.0%) at 27th September. In 2010 mature larvae only appeared in the end of October, representing 7.0 % of pre-imaginal population. The highest percentage of mature larvae in 2011 was obtained at 16th August, representing 19.7 % of total pre-imaginal population.

The total number of pupae was 15 in 2009 and 144 in 2011. In 2010 any pupae were registered. In the first year the pupae were observed from August 30 until the end of October, and in 2011 it appears in all the sampling dates.

Flight curve: The data of *B. oleae* catches in three years of study are represented in Figure 7.2. The catches began in early July in 2009 and 2011, and in the end of July in 2010, corresponding to the period in which the traps were installed, having the *B. oleae* catches remained in olive grove until early November. There were notable differences in the number of catches according to the different years of study. Olive fly populations were higher in 2009 and 2011 than 2010. In 2009 and 2011 the mean number of catches did not exceed 20 individuals per trap before mid-September increase from that period until the end October. In 2009 it was reached a peak at 18th October (52.20±11.28) and in 2011 its peak was at 25th October (90.20±19.21). In 2010 the number of catches was always very low, not exceeding five individuals per trap during all time of the study.

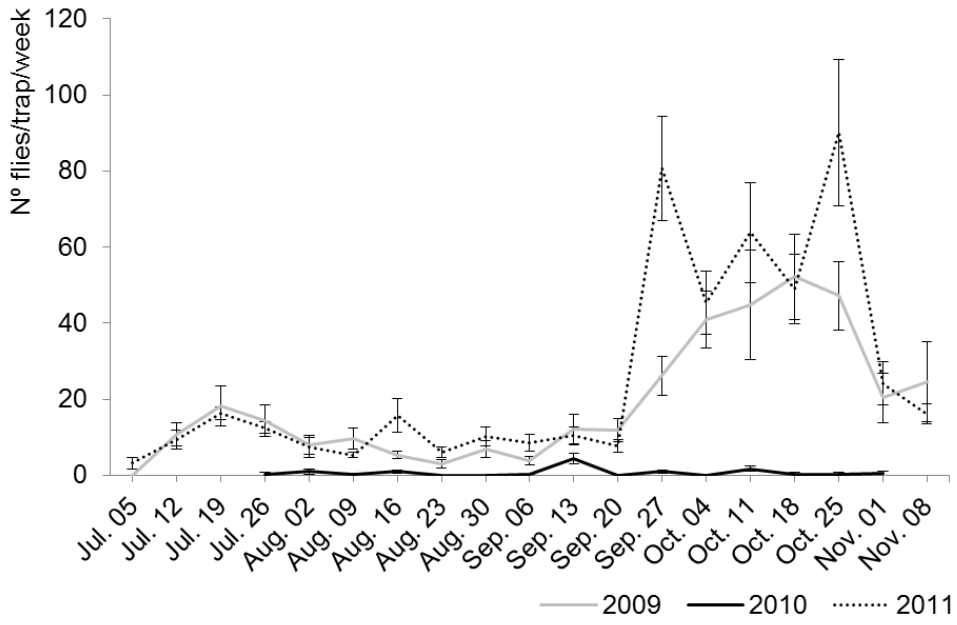

Figure 7.2. Flight curve of *Bactrocera oleae* (Rossi), in Cedães grove (2009, 2010 and 2011).

Fruit infestation: Data on the mean number of fruits with oviposition per tree in each date during the three years (2009, 2010 and 2011) and in each plot (control, 4, 6, 8 and 10 mm of hole size) are shown in Table 7.2. In 2009, the higher mean number of fruits with punctures was obtained at 24th October in all plots, the plot that acted as control reported the highest number of fruits with punctures (5.20±1.32), followed by: Olipe traps with 8 mm (3.15±0.73) > Olipe traps with 4 mm (2.65±0.45) > Olipe traps with 6 mm (1.65±0.34) > Olipe traps with

10 mm (0.85±0.21). Generally significant differences were observed between control and plot tested with Olipe traps with 4 and 6 mm of hole size ($p > 0.05$).

Table 7.2. Mean number (mean ± SE) of fruits with punctures per tree in each date during the three years studied (2009, 2010 and 2011) and in each plot (control, 4, 6, 8 and 10 mm of hole size).

2009	Control	4 mm	6 mm	8 mm	10 mm
2nd Aug.	-	-	-	-	-
16th Aug.	-	-	-	-	-
30th Aug.	0.75±0.40a	0.00±0.00b	0.00±0.00b	0.45±0.15ab	0.00±0.00b
13th Sep.	1.10±0.44a	0.00±0.00b	0.05±0.05b	1.00±0.34a	0.15±0.08ab
27th Sep.	1.90±0.49a	0.35±0.15b	0.10±0.18b	0.60±0.20b	0.60±0.18b
11th Oct.	4.15±1.20a	1.00±0.29b	1.20±0.30b	2.15±0.50ab	0.75±0.20b
24th Oct.	5.20±1.32a	2.65±0.45ab	1.65±0.34b	3.15±0.73ab	0.85±0.21b
8th Nov.	-	-	-	-	-
2010	Control	4 mm	6 mm	8 mm	10 mm
2nd Aug.	-	-	-	-	-
16th Aug.	0.05±0.05a	0.00±0.00a	0.20±0.12a	0.01±0.07a	0.05±0.05a
30th Aug.	0.20±0.12a	0.05±0.05a	0.15±0.08a	0.01±0.07a	0.10±0.07a
13th Sep.	0.45±0.18a	0.20±0.12a	0.30±0.16a	0.20±0.09a	0.20±0.09a
27th Sep.	0.20±0.09a	0.10±0.07a	1.00±0.42a	0.45±0.15a	0.55±0.32a
11th Oct.	0.80±0.24a	1.15±0.26a	1.20±0.34a	0.85±0.32a	0.65±0.18a
24th Oct.	1.85±0.39a	0.80±0.19a	1.50±0.27a	1.55±0.31a	1.25±0.25a
8th Nov.	1.70±0.29ab	0.25±0.12b	0.55±0.17b	3.15±0.64a	0.95±0.42b
2011	Control	4 mm	6 mm	8 mm	10 mm
2nd Aug.	0.45±0.18a	2.10±0.44ab	3.15±0.86b	1.45±0.31ab	1.00±0.38a
16th Aug.	0.55±0.15a	2.35±0.51b	2.15±0.69ab	1.25±0.27ab	0.80±0.21ab
30th Aug.	1.10±0.57a	1.55±0.41a	1.45±0.58a	1.25±0.35a	0.80±0.26a
13th Sep.	0.70±0.19a	1.20±0.30ab	2.60±0.83b	0.70±0.23a	0.50±0.14a
27th Sep.	1.60±0.49a	6.05±0.91b	3.45±0.97ab	1.35±0.30a	1.00±0.33a
11th Oct.	4.60±1.13a	11.40±1.30a	10.05±1.20a	2.35±0.43a	3.35±0.87a
24th Oct.	3.00±1.00ab	12.95±1.61c	8.20±1.16b	4.25±0.94b	3.70±0.54ab
8th Nov.	4.35±1.23ab	10.45±0.89c	6.70±1.07b	3.35±0.56ab	2.00±0.31a

(In the same row, mean values with different letters differ significant, $p < 0.05$)

In 2010, the higher mean number of fruits with punctures was obtained at 24th October in the following plots: control (1.85±0.39), Olipe traps with 6 mm of hole size (1.50±0.27) and Olipe traps with 10 mm of hole (1.25±0.25); and at 11th October in plot with Olipe traps with 4 mm of hole size (1.15±0.26) and at 8th November in plot with Olipe traps with 8 mm of hole size (3.15±0.64). No differences ($p > 0.05$) were registered between hole sizes until 24th October.

Relatively to 2011, it was registered a high mean number of fruits with punctures at 11th October in control (4.60±1.13) and in plot with Olipe traps with 6 mm of hole size (10.05±1.20), and at 24th October 24 in plots with Olipe traps with 4 mm of hole size (12.95±1.61), 8 mm of hole size (4.25±0.94) and 10 mm of hole size (3.70±0.54). Significant differences were observed between control and plot tested with Olipe traps with 4 and 6 mm of hole size ($p > 0.05$).

The results of three years of study have shown that the percentage of infested fruits by *B. oleae* was higher in 2011 than the other years of study, in all plots tested. In 2009, the percentage of infested fruits was high in all plots at 24th October, which corresponded to the time of harvest. In this year, the plot that acted as control obtained highest attack percentage with 19.0% of infested fruits (Figure 7.3), followed by the plots with Olipe traps with 8 mm (12.0%), 4 mm (10.2%), 6 mm (6.6%) and 10 mm (3.4%). In 2010 the fruit infestation in all plots was low. With the exception of the plot with Olipe traps with 8 mm of hole size diameter (12.6%) in all other plots the percentage of infected fruits was less than 7.0%.

In the experiment conducted in 2011, there were a higher percentage of infested fruits than the experiments conducted in previous years. The percentage of infested fruits was high in the plot that acted as control (18.4%) and in the plot with Olipe traps with 6 mm of hole size diameter (40.2%), at 11th October. In other plots it was obtained the highest percentage of infested fruits at 24th October, 51.8% in the plot with Olipe traps with 4 mm of hole size diameter, 17.0% in the plot with Olipe traps with 8 mm of hole size diameter and 14.8% in the plot with Olipe traps with 8 mm of hole size diameter.

In 2009, there was a reduction of attack at harvest in all plots tested with Olipe traps compared with the plot that acted as control, have been registered a reduction of attack at harvest of 82.1% in plot with Olipe with 10 mm of hole size, 65.3% in plot with Olipe with 6 mm, 46.3% in plot with Olipe with 4 mm and 36.8% in plot with Olipe with 8 mm. In 2010, the weather in August was characterized by high temperatures that have limited the development of the olive fly, keeping its population levels very low. The low population density of *B. oleae*, in this year, didn't allowed obtaining conclusive results about reduction of attack.

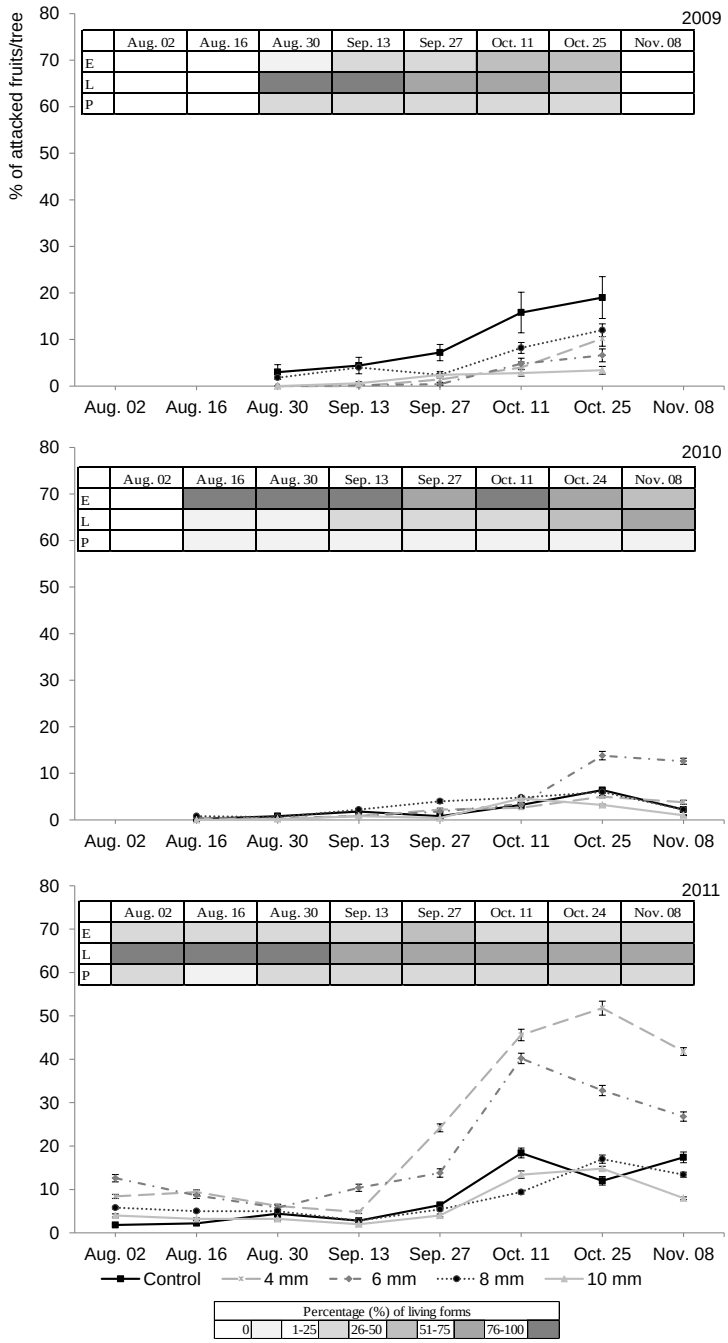

Figure 7.3. Temporal distribution of the infestation index (Ii) per tree in each plot (control, 10 mm, 8 mm, 6 mm and 4 mm) in 2010 (mean ± SE), and percentage of temporal distribution of living forms (E – eggs; L –larvae; P – pupae).

In 2011, there was a reduction of attacked fruits at harvest in plots tested with Olipe with 10 mm of hole size (54.0%) and with Olipe with 8 mm (23.0%). However, these results could be more impressive if it had not occurred low fruit infestation in plot that acted as

control. Infestation levels were below 13.0% until 13th September in all plots, and then there is a gradual increase in plots tested with Olipe traps with 4 and 6 mm of hole size.

7.4. Discussion

The olive fly is considered a monophagous frugivore once only feeds on few *Olea* species. As such, the insect survival is dependent of olives condition and availability. In the Mediterranean region, from mid-summer to late autumn the olive fly generally develops two to five generations per year. Eggs are laid under olives and larva feeds in olives mesocarp. Third larval stage moves to the fruit surface and then pupates, in the fruit or in the soil, and a new generation begin with the emerging adults.

By analyzing flight curve of olive fly it appears that this pest is present during the three years reaching a peak population in October, pattern that has been observed by Bento *et al*. (1999) and Gonçalves and Torres (2011) in the same region. In 2011 and 2009 there were higher population levels than in 2010. The differences registered between years can be partly justified by the higher temperatures recorded in 2010. In fact, in 2010, during August and during several consecutive days, the temperatures reached more than 35 ºC. Associated this fact to the very low relative humidity, provokes high mortality rates in immature stages. It is known that in hot summers, olive fly populations are low (Genç and Nation, 2008). Temperature is one of the factors that most affects the population abundance of olive fly. Adult *B. oleae* are active at temperatures between 20 and 30 ºC, but above this temperature, the flies move frantically and oviposition is thereby inhibited, whereas at 35 ºC activity ceases (Avidov, 1954). The upper development threshold for eggs has been reported ranging from 30 to 32 ºC (Tsitsipis, 1977). Although immature development could be completed at 30 ºC, up to 35 ºC no larvae are able to reach adult (Tsitsipis, 1977; Genç and Nation, 2008; Wang *et al*., 2009). The influence of relative humidity on the immature stages is important in situations where there are long periods of low humidity combined with high temperatures. In this situation the fruits wither, losing water, difficult the immature stages development (Civantos, 1999). Also several investigators have suggested high temperatures and low humidity as possible factors impeding female maturation in summer (Kapatos and Fletcher, 1986; Katsoyannos, 1992).

In Trás-os-Montes region, *B. oleae* begin laying eggs in the end of July/beginning of August (Gonçalves, 2011), period coinciding with stone hardening, the olive phenological

stage considered receptive to oviposition (Civantos, 1999) and coincident with the appearance of the first fertile female in region (Gonçalves, 2011), and usually occurs a peak abundance of eggs in the middle of October (Bento *et al*., 1999; Gonçalves, 2011). Population of eggs and larvae usually remain at low levels until October, increasing considerably after beginning of October (Bento *et al*., 1999). The increasing of infestation in this period can be explained by climatic conditions favorable to olive fly, especially by increasing humidity and moderate temperatures. In Mirandela region, the months of August and September are characterized by scarce rainfall and high temperatures (Normais climatológicas 1971-2000), factors that limit the development of pre-imaginal stages. In October, air humidity increases, reaching 60% humidity, and average temperature decrease to 15 ºC (Normais climatológicas 1971-2000), creating favorable conditions for the development of the olive fly. Data on the phenology of *B. oleae,* in all years of study, showed a gradual increase of pre-imaginal population since mid/late summer until mid/late October, the time of the year in which the *B. oleae* population is more abundant. The immature population remains low until the end of September, when there is an increase of immature population until it reaches a peak in mid/late October. This fact coincides with observations made by Bento *et al*. (1999) in olive groves in the region of Mirandela. First *B. oleae* eggs recorded in 2011 coincides with stone hardening, which is the olive phenological stage considered receptive to oviposition (Civantos, 1999). In 2010 first eggs were found in the middle of August when the study began, and they were only registered in the middle of September in 2009. In all years it was observed an oviposition peak during the month of October while the peak of young larvae occurred between early October to early November, also observed by Gonçalves and Torres (2011) in previous studies in Mirandela region. A great number of dead larvae was mainly observed in 2011, which was registered in early October with 33% of larvae mortality. This fact may be to the larvae particularly first larvae, that can suffer from high mortality when the olives are still green because they are unable to obtain adequate food or become encysted as a result of a reaction of suberiztion of gallery (Neuenschwander et al., 1986) mainly in the beginning of August. On the other hand, due to the lack of rain registered in August, September and in the beginning of October, and the values of relative humidity were low causing very high mortality larvae (Genç and Nation, 2008; Broufas *et al*., 2009; Wang *et al*., 2009). Delrio and Prota (1976) as well as Puci et al. (1985) refers that values of very low relative humidity (20% in August) simultaneously with high summer temperatures lead to sudden mortality spikes of eggs and larvae. In the case of larvae the percentage of larval mortality can reach 90%.

By observation of infested fruits, we can observe that its percentage increased progressively throughout the studied period in whole years. In 2009, the highest percentage of attack was observed, at the harvest time, in the plot which acted as control (19.0%), the double of infestation than in plots with Olipe traps. In this year the use of Olipe traps resulted in a reduction of infested fruits, at harvest time, in all plots tested with Olipe traps (12.0% in plots with Olipe traps with 8 mm hole size, 10.2% in plots with Olipe traps with 4 mm, 6.6% in plots with Olipe traps with 6 mm, 3.4% in plots with Olipe traps with 10 mm). It is noteworthy that at harvest time the infestation levels in plots tested with Olipe traps were below 12.0%. The threshold for intervention of an active infestation is recommended between 8.0 to 12.0% on cultivars for oil production (Cavaco and Marcelo, 2009). So, in this year, Olipe traps were sufficient to keep infestations below the economic threshold level at harvest time. In 2010, the low population density of *B. oleae*, did not result in conclusive results about reduction of attack, infestation levels below 13.0% in all plots have been recorded. Relatively to year 2011, the olive fruits were attacked continuously from August to early November in all plots tested with Olipe traps and in control. The highest percentage of attack was observed, at the harvest time, in plot with Olipe traps with 4 mm of hole size (41.8%) followed the plot wit Olipe traps with 6 mm (26.8%). It was observed a reduction of infested fruits in plots tested with Olipe traps with 8 and 10 mm of hole size, 13.4% and 8.0% of infested fruits respectively. The low fruit infestation observed in plot that acted as control (17.4%), may be due because no rain was registered in the summer. This plot was the most affected by lacking water and, consequently, fruits become dehydrated presenting an inadequate size for the development of olive fly life cycle. Losing water the larvae and eggs survival is compromised. However it's important to refer that being the year 2011 a year with high *B. oleae* population density, the use of Olipe traps of high size (8 and 10 mm) maintained the infestation levels below 14.0% at the time of harvest.

The hole size of Olipe traps is of great importance, once it may interfere with the number of captures of olive fly adults either the amount of volatile released from solution used as attractant (Luque and Pereda, 2003) being, according the same author, the Olipe traps with larger hole size more effective in catches of *B. oleae* adults. In this study, regarding the effect of the different hole sizes in reduction of *B. oleae* population levels, it appears that in whole years the percentage of infested fruits is low in blocks with bottles with 10 and 8 mm of diameter, also registered a reduced number of eggs and larvae at the end of the trial, at which time the population of *B. oleae* reaches peaks of flight. In sustainable olive growing,

mass trapping with Olipe traps may reduce olive fly populations to levels considered acceptable as observed in some previous studies (Duatis *et al*., 2006). Data analysis of three years, there seems to be here a positive effect of the use of bottles of larger hole size (8 and 10 mm) in reducing population levels of olive fly. Thus, the traps with largest diameters seem to us that maintain levels of infested fruits in acceptable values to olive oil production. In addition, Dimou et al. (2003) refers that fruit destined for oil pressing may have relatively high levels of infestation (e.g. 10-30%) and still be considered acceptable.

Although mass trapping with Olipe traps is able to reduce the olive fly population levels, the use of Olipe traps with larger hole size have adverse effects on beneficial fauna (Luque and Pereda, 2003.The adaptation of a net in this kind of traps, when high hole sizes are used, to prevent the passage of non-target insects may be useful to help preserve the beneficial fauna, and has been used with some success by other authors (Ros *et al.*, 2008; Seris, 2011), allowing a more effectively control of *B. oleae* population with less impact on beneficial fauna.

7.5. Conclusions

Mass trapping with Olipe traps as an alternative to conventional treatments, in sustainable olive production systems is a promising option due to their low cost and effectiveness, which may reduce the populations of the olive fly to levels considered acceptable. However, the great variability in results reported, the negative impact for beneficial fauna of olive tree and loses of efficacy during the periods that traps are in the field are all factors which limit their use, which implies the need to study some characteristics of these traps in order to make them more effective and reduce negative impacts on beneficial fauna such as hole size.

This work showed that the hole size of the Olipe traps can have influence in protecting the olive fruits against the olive fly. Traps with smaller hole diameter seem less efficient than traps with bigger hole diameter in reducing population levels. Traps of bigger diameter (8 and 10 mm) can reduce infestation levels to levels below the economic threshold level, being Olipe traps with 8 mm of hole size the best compromise to be the hole size that has less impact on beneficial fauna (data not show). The use of Olipe traps selective for beneficial fauna becomes important because in the olive groves there is a great diversity of insects that are important in biological control of many pests.

However, when there is high population density of olive fly, mass trapping with Olipe traps should be complemented with others preventive measures such as anticipation of harvesting. In organic agriculture, mass trapping with Olipe traps is a promising option and is interesting follow with such traps, due its low cost and also due to lack of alternative means of control against this pest.

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CHAPTER 8

Mass-trapping with Olipe traps against the olive fly *Bactrocera oleae* (Rossi) in organic agriculture: effect of hole size in non-target arthropods



- Ei, formiguinha, para que todo esse trabalho? O verão é para a gente aproveitar!
O verão é para a gente se divertir!
- Não, não, não! Nós, formigas, não temos tempo para diversão.
É preciso guardar comida para o inverno.
(…)

A cigarra e a formiga, Fábulas de Jean La Fontaine (1621-1695).

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Abstract

The olive fly, *Bactrocera oleae* (Rossi), is a key-pest of olives in the Mediterranean region. In organic agriculture the alternative to control this pest are few or expensive. Despite their controversy efficacy, due to their reduced price and ease of application, the use of Olipe traps have been increasing in the last decades by growers. However, their efficacy is questionable and could have negative impacts on the beneficial arthropodofauna. In this work, we aimed to evaluate the effect of hole size (4, 6, 8 and 10 mm) of Olipe traps used for olive fly mass-trapping in captures of non-target arthropods and their impact on beneficial insects. The study was developed in three consecutive years, 2009-2011, in an organic olive grove located near Mirandela (Northeast of Portugal). Four plots of 1.5 hectare, one per hole size (4, 6, 8 and 10 mm of diameter) were installed with the basis of one trap/tree. Bi-weekly, in 15 traps, the number of arthropods was counted. Three classes and 14 orders of arthropods were found. The class Insecta represented 98.4% of the total captures in 2009, 99.6% in 2010 and 99.8% in 2011, being the arthropod community numerically dominated by Formicidae family, which represented 79.6% in 2009, 78.0% in 2010 and 58.0% in 2011. The results obtained in the present work demonstrated that hole size of the traps used for *B. oleae* mass-trapping affected the number of non-target arthropods recovered. Traps with 8 and 10 mm of hole size diameter were particularly harmful for Chrysopidae adults, being the catches significantly higher ($P \leq 0.001$) in the bigger hole diameter than in smaller ones. Olipe traps with smaller hole size showed lower impact in non-target arthropods captures.

Key-words: *Bactrocera oleae* (Rossi), Olipe traps, hole size, non-target arthropods Chrysopidae.

8.1. Introduction

The olive tree is one of the oldest cultivated trees in the humanity history, and is today a symbol of the landscape of the Mediterranean region which has great economic, ecological and social importance. The olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), has long been recognized as a major pest of cultivated olive trees throughout the Mediterranean basin (Tzanakakis, 2006), and is also considered one of the most crop enemies in the Trás-os-Montes region (Portugal), responsible for major losses that may reach 80% in orchards to olive oil production (Bento *et al.*, 1999; 2009).

This pest causes serious quantitative and qualitative damages that justify the adoption of control measures every year. In organic production of olives, the use of chemical pesticides is not allowed. In fact, its use could have high negative impacts on both, on the food quality and safety of olive products with subsequent impacts on human health; and on the environment due to their impact on beneficial arthropods, development of pest resistance and also in the loss of biodiversity. So, the search of more environmental friendly control strategies was been an important domain of research in the area. Nevertheless, these alternative control strategies could not to be safe for environment and needs to be evaluated in their side-effects.

Recently, in southern of Spain, the Cooperativa Olivarera de los Pedroches, started using dispositive device against olive fly, subsequently called Olipe trap (Caballero, 2001), that is a translucent plastic bottle (polyethylene terephthalate) with a 1.5 or 2 liters of capacity (30 cm of high, 9 cm in diameter) perforated, usually with six holes about 6-8 cm from the top, and placing inside a food attractant, generally an aqueous solution of ammonia and in some situations a pheromone. The flies are attracted by the solution of ammonia, enter through the holes, and eventually drown in the solution.

In olive growing under organic agriculture, mass trapping with Olipe traps is a promising option, due to its low cost and effectiveness, and may reduce populations of the olive fly to levels considered acceptable (Caballero, 2002; Pavão *et al.*, 2007). However, several studies have showed limited efficacy in reduce fruit infestations levels (Duatis et al., 2006; Tabic *et al.*, 2011) and negative impact in beneficial fauna (Pereira *et al.*, 2007; Porcel *et al.*, 2009; Seris *et al.*, 2007) that are factors that limit their use and put in evidence the need to improve the efficacy of Olipe traps.

The aim of this work was to study the effect of different bottle hole sizes of Olipe traps used for olive fly mass-trapping on non-target arthropods and their impact in beneficial groups of olive grove.

8.2. Materials and Methods

8.2.1. Study area

The study was conducted in a commercial olive grove for oil production located near Mirandela (North Eastern Portugal) – Cedães grove. Cedães grove (41º29'18.84''N, 7º07'36.02''W) has been conducted following the rules of Integrated Pest Management since 2001. The olive trees, cv. Cobrançosa, are of medium size and spaced at 7 × 7 meters. The grove was non-irrigated and the soil is conducted with natural vegetation. The trees are pruned every two to three years, and no phytosanitary treatments were done during the year of the experiments.

8.2.2. Experimental design

The effect of mass trapping with Olipe traps on non-target arthropods was evaluated during three consecutive years, from 2009, to 2011. Fieldwork was carried out from the end of August to the end of October in 2009, from middle August to early November in 2010 and from late July to early November in 2011.

The Olipe trap consists of a PET translucent bottle with 1.5 L capacity (30 cm high, 9 cm diameter and 825 cm^2 of outer surface) The attractant used as bait was 3% biammonium phosphate (Ammonium di-Hydrogen Phosphate, Panreac). Each Olipe trap had six holes to increase capture, and four hole sizes were evaluated, namely 4, 6, 8 and 10 mm of diameter.

In each year, the olive grove was divided in four plots of about 1.5 hectare each, where in four plots Olipe traps were placed in a branch of the tree at the rate of one trap per tree, and corresponding to each of the studied diameters (4, 6, 8 and 10 mm). Within each block were selected and marked 15 Olipe traps that were changed every fortnight.

Olipe traps were filled to 2/3 of its capacity with 3% biammonium phosphate and placed in the inner canopy, placed about 150-190 cm of height, remaining in the field, in 2009, since the end of August until the end of October; in 2010 from mid-August to early

November; and in 2011 from end of July until early November. Periodically, to avoid the traps staying empty due to evaporation the attractant solution was added.

For each hole size, 15 Olipe traps were collected biweekly and carried out in boxes to the laboratory. After collected, each site was replaced with new Olipe traps. The trap content was filtered with a nylon mesh obtaining the arthropods captured and then having proceeded to count the arthropods. All arthropods were collected, counted and preserved in 70% ethanol until their identification. The individuals were taxonomically classified up to level order, family or species taxa. Formicidae, Coccinellidae, Syrphidae and Chrysopidae family was identified to level species. Formicidae family was identified according to Collingwood and Price (1998). Coccinellidae species were identified according Raimundo and Alves (1986). Syrphidae species were identified based on dichotomous keys produced by Seguy (1961) and Gilbert (1986). Beetles were identified up to the level of family following Borror and Delong (1988) and then, were separated by morphospecies under a binocular microscope. The mean number of catches per trap were calculated and richness (S), evenness (E), diversity (H', D and 1-D), and Morisita index (I_M) were used as biodiversity descriptors.

8.2.3. Statistical analysis

Results of arthropods capture are presented as means (±SD). Statistical analyses were carried out with the program SPSS PASW Statistics 18 for Windows, IBM. Data on the number of captures of each taxon in each Olipe trap were evaluated for normality with Kolmogorov-Smirnov test and proceeded to the mathematical transformation to normalize the variable using a logarithmic scale $\log_{10}(x+1)$. Independent samples Kruskal-Wallis test (non-parametric) ($P<0.05$) was used to compare means among different hole size.

8.3. Results

8.3.1. General entomofauna analysis

For a best understanding of the results, this section was divided in two main points. In the first, the total entomofauna were presented and some of the identified groups were shown with more details, in the second point the effect of Olipe traps hole size on beneficial arthropods were compared.

The arthropods recovered were classified into three classes, Insecta, Arachnida and Entognatha and 14 orders: Acari, Araneae, Collembola, Coleoptera (larvae and adults of Coccinellidae and others Coleoptera), Dermaptera, Diptera (Syrphidae, *B. oleae* and others Diptera), Hemiptera, Heteroptera, Hymenoptera (Formicidae, Hymenoptera parasitoids, bees and others Hymenoptera), Lepidoptera (*Prays oleae* Bern., and others Lepidoptera), Neuroptera (larvae and adults of Chrysopidae), Odonata, Psocoptera and Thysanoptera.

A total of 39108 arthropods were captured in the three years, 3066 in 2009, 13852 2010 and 22190 in 2011 (Table 8.1).The class Insecta represented 98.4% of the total captures in 2009, 99.6% in 2010 and 99.8% in 2011. The class Arachnida represented 1.4% of total captures in 2009 and less of 1.0% in 2010 and 2011. The captures of class Entognatha were very low in all years (<0.1%).

Into the class Insecta, the most abundant taxa was the Hymenoptera order, with 83.1%, 79.0% and 58.4%, followed by order Diptera with 2.0% 14.5% and 38.0% respectively in 2009, 2010 and in 2011. The large percentage of Diptera observed in 2011 its due the high captures of *B. oleae* observed this year. From this captures it is noteworthy that in all years, arthropod community was numerically dominated by Formicidae family, which represented 79.6% in 2009, 78.0% in 2010 and 58.0% in 2011 of total Insecta captured. The order Neuroptera represented 2.5%, 3.2% and 1.6% in 2009, 2010 and 2011 respectively, and was the third taxa with more captures.

Order Neuroptera, with a total of 840 adults Chrysopidae captured along the three years, were the second in order of importance. In this order, 66 (10.0% of total non-target arthropods, excluding Formicidae) were captured in 2009, 434 (14.5%) in 2010 and 340 (21.7%) in 2011.

For Coleoptera, a total of 191 individuals were captured during the work and 13 families were identified, namely: Bruchidae (52.4%), Coccinellidae (12.6%), Tenebrionidae (9.4%), Phalacridae (4.7%), Chrysomelidae (4.2%), Bostrichidae (3.7%), Carabidae (3.7%), Curculionidae (3.1%), Apionidae (2.1%), Melyridae (2.1%), Anthicidae (0.5%), Elateridae (0.5%) and Staphylinidae (0.5%).

Table 8.1. Cumulative number of arthropods captured in Olipe traps in 2009 (*n=240*), 2010 (*n=360*) and 2011 (*n=420*).

Taxa			2009	2010	2011	Total
Insecta						
	Hymenopera					
		Parasitoids	105	128	78	311
		Formicidae	2402	10770	12845	26017
		Bees	0	7	0	7
		Others Hymenop.	0	0	10	10
	Coleoptera					
		Coccinellidae (L)	0	2	2	4
		Coccinellidae (A)	1	10	13	24
		Others Coleoptera	13	23	130	166
	Neuroptera					
		Chrysopidae (L)	10	13	10	33
		Chrysopidae (A)	66	434	340	840
	Diptera					
		Bactrocera oleae	7	91	7780	7878
		Syrphidae	24	7	6	37
		Others Diptera	28	1898	613	2539
	Lepidoptera					
		Prays oleae	0	9	89	98
		Others Lepidoptera	215	170	37	422
	Thysanoptera		30	1	3	34
	Dermaptera		4	61	99	164
	Psocoptera		0	1	70	71
	Heteroptera		5	5	4	14
	Hemiptera		107	168	9	284
	Odonata		0	2	2	4
Arachnida						
	Araneae		44	52	39	135
	Acari		0	4	3	7
Entognatha						
	Collembola		3	2	2	7
Others			2	2	8	12
Total			3066	13852	22190	39108

L – larvae, A – adults

The family Coccinellidae is presents in low number. Only 24 individuals were identified, belonging to the species *Scymnus (Sc.) interruptus* Goeze (14), *Rhyzobius chrysomeloides* (Herbst.) (4), *Chilocorus bipustulatus* L. (2); *Scymnus (Pullus) mediterraneus* Khnz. (2), *Platynaspi luteorubra* Goeze (1) and *Scymnus apetzi* Muls (1). The other Coleoptera (excluding Coccinellidae family) represented 2.0% of non-target arthropods

captures in 2009 (excluding Formicidae), 0.8% in 2010 and 8.3% in 2011. That belongs to eight, nine and 10 families in 2009, 2010 and 2011 respectively.

For Diptera order, 37 Syrphidae were collected belonging to six species namely: *Episyrphus balteatus* (De Geer, 1776) (25), *Episyrphus (Meliscaeva) auricollis* (Meigen, 1822) (5), *Eupeodes corollae* (Fabricius, 1794) (3), *Syrphus vitripennis* (Meigen, 1822) (2), *Melanostoma scalare* (Fabricius, 1794) (1), *Metasyrphus corollae* (Fabricius, 1794) (1). In 2011 there is an individual that was not possible to identify the species.

Araneae represents 6.7% in 2009 and 1.7% in 2010 and 2.5% in 2011 of total non-target arthropods (excluding Formicidae).

Due its higher number, Formicidae family were analyzed apart from the others groups. A total of 26017 specimens of Formicidae belonging to three subfamilies, 10 genera and 19 species were collected in the three years. The identified species in decreasing order of abundance were: *Crematogaster auberti* (Emery, 1869); *Crematogaster scutellaris* (Olivier, 1792); *Leptotorax* sp., *Camponotus lateralis* (Olivier, 1792); *Camponotus piceus* (Leach, 1825); *Plagiolepis pygmaea* (Latreille, 1798); *Camponotus sylvaticus* (Olivier, 1792); *Camponotus aethiops* (Latreille, 1798); *Messor barbarus* (Linnaeus, 1767); *Tapinoma nigerrimum* (Nylander, 1856); *Cataglyphis* sp2; *Formica subrufa* (Roger, 1859); *Cataglyphis hispanicus* (Emery, 1900); *Cataglyphis* sp1*; Camponotus fallax* (Nylander, 1910); *Tetramorium forte* (Forel, 1904); *Camponotus cruentatus* (Latreille, 1802); *Messor lusitanica* (Tinaut, 1985) and *Camponotus truncatus* (Ito, 1914). The most represented were *C. auberti* and *C. scutellaris* with 46.6% and 43.3% respectively, representing in total 89.9% of total Formicidae catches.

In 2009 were collected 2402 individuals of Formicidae (Table 8.2). *C. auberti* was the most abundant, represented 88.1% of total captured, followed by *P. pygmaea* with 4.8%, *Leptotorax* sp. (2.6%) and *C. scutellaris* (2.5%). Others species represented less than 1% of the total recovered.

In 2010, 10770 individuals of Formicidae were collected. *C. auberti* was the most abundant represented 85.1% of total captured, followed by *Leptotorax* sp. (11.3%) and *C. scutellaris* (2.0%). In 2011, 12845 individuals of Formicidae were collected and *C. scutellaris* was the most abundant, represented 85.2% of total Formicidae, followed by *C. auberti* (7.1%), *C. lateralis* (3.3%), *C. piceus* (1.9%) and *C. sylvaticus* (1.2%). All other species, their number was lower of 1.0%.

Table 8.2. Cumulative number of Formicidae species captured in Olipe traps in different years (2009, 2010 and 2011).

Subfamily and species of ants	**2009**	**2010**	**2011**
Subfamily Dolichorinae			
Tapinoma nigerrimum (Nylander, 1856)	8	8	13
Subfamily Formicinae			
Camponotus lateralis (Olivier, 1792)	7	14	422
Camponotus piceus (Leach, 1825)	0	3	239
Camponotus sylvaticus (Olivier, 1792)	5	4	150
Camponotus aethiops (Latreille, 1798)	0	0	96
Camponotus fallax (Nylander, 1856)	0	0	11
Camponotus cruentatus (Latreille, 1802)	0	0	3
Camponotus truncatus (Spinola, 1808)	0	0	1
Cataglyphis hispanicus (Emery, 1900)	3	11	2
Cataglyphis Sp1	13	0	1
Cataglyphis Sp2	4	0	15
Plagiolepis pygmaea (Latreille, 1798*)*	115	67	1
Formica subrufa (Roger, 1859)	0	1	16
Subfamily Myrmicinae			
Crematogaster auberti (Emery, 1869)	2117	9167	914
Crematogaster scutellaris (Olivier, 1792)	61	213	10952
Messor barbarus (Linnaeus, 1767)	1	56	4
Messor lusitanica (Emery, 1915)	1	0	0
Leptotorax sp.	62	1222	4
Tetramorium forte (Forel, 1904)	4	4	0
Others	1	0	1
Total	**2402**	**10770**	**12845**

8.3.2. Effect of Olipe trap hole size on beneficial arthropods

For the analysis of the effect of Olipe trap hole size, the Formicidae were excluded. In general, Olipe traps with largest diameter present high values of richness (Table 8.3), mainly in 2010 and 2011.

Table 8.3. Richness (S), evenness (E), diversity (H', D and 1-D), and community similarity (I_M) indices of arthropods captured in different plots with Olipe traps (4, 6, 8 and 10 mm) and in different years (2009, 2010 and 2011).

Year	Plot	S	H	E	D	1-D	I_M
2009	4 mm	14	1.98	0.75	0.19	0.81	0.19
	6 mm	15	2.04	0.75	0.20	0.80	
	8 mm	14	2.09	0.80	0.16	0.84	
	10 mm	12	1.94	0.78	0.19	0.81	
2010	4 mm	15	0.93	0.34	0.64	0.36	0.49
	6 mm	17	1.58	0.56	0.33	0.67	
	8 mm	16	1.74	0.63	0.28	0.72	
	10 mm	22	1.56	0.50	0.34	0.66	
2011	4 mm	15	1.14	0.42	0.49	0.51	0.42
	6 mm	19	0.75	0.26	0.72	0.28	
	8 mm	19	0.70	0.24	0.73	0.27	
	10 mm	20	0.69	0.23	0.74	0.26	

Table 8.4 shown the mean number of arthropods recovered in the Olipe traps with different hole size (4, 6, 8 and 10 mm). According the year, there are significant differences in the catches number for the different groups. In 2009, parasitoids ($p = 0.050$), Formicidae ($p = 0.013$), Chrysopidae ($p \leq 0.001$) and Syrphidae ($p = 0.001$) differ among hole size (Table 3). While in 2010, it was found differences among hole size in the captures of Formicidae ($p = 0.013$), Chrysopidae ($p \leq 0.001$), Syrphidae ($p = 0.001$), Araneae ($p = 0.009$), *P. oleae* ($p \leq 0.001$), Hemiptera ($p = 0.005$) and others Diptera ($p \leq 0.001$). On the other side, in 2011 Chrysopidae ($p \leq 0.001$) Formicidae ($p \leq 0.001$), *P. oleae* ($p \leq 0.001$), Dermaptera ($p = 0.030$), others Lepidoptera ($p = 0.006$) and Odonata ($p = 0.007$) differ according the hole size.

Table 8.4. Mean number (Mean ± Standard Deviation of the mean) of arthropods captured in each plot with Olipe traps (4, 6, 8 and 10 mm) in an organic olive grove of Mirandela region in 2009, 2010 and 2011. An asterisk indicates a significant difference in the catches number among different hole sizes.

	2009 (*n=60*)					2010 (*n=90*)					2011 (*n=105*)				
	4 mm	6 mm	8 mm	10 mm	P	4 mm	6 mm	8 mm	10 mm	P	4 mm	6 mm	8 mm	10 mm	P
Insecta															
Hymenopera															
Parasitoids	0.48±0.68	0.33±0.63	0.48±1.10	0.18±0.43	0.050*	0.49±0.90	0.34±0.77	0.30±0.59	0.29±0.64	0.338	0.45±1.17	0.19±0.44	0.13±0.34	0.24±0.55	0.131
Formicidae	18.93±49.47	4.57±15.74	4.38±21.23	12.18±35.50	0.013*	44.46±144.59	15.79±52.63	56.99±199.49	2.43±9.58	0.013*	35.67±148.13	12.06±43.81	37.50±92.08	37.10±99.04	≤0.001*
Bees	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	-	0.00±0.00	0.03±0.23	0.03±0.23	0.01±0.11	0.525	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	-
Others Hym.	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	-	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	-	0.00±0.00	0.00±0.00	0.02±0.13	0.04±0.19	0.06
Coleoptera															
Coccinellidae	0.02±0.13	0.00±0.00	0.00±0.00	0.00±0.00	0.392	0.03±0.18	0.04±0.21	0.02±0.15	0.01±0.11	0.562	0.05±0.21	0.01±0.10	0.04±0.42	0.03±0.22	0.359
Others Col.	0.10±0.30	0.05±0.22	0.05±0.29	0.02±0.13	0.186	0.11±0.44	0.04±0.21	0.06±0.27	0.02±0.15	0.360	0.30±0.60	0.26±0.54	0.30±0.60	0.38±0.76	0.933
Neuroptera															
Chrysopidae	0.00±0.00	0.18±0.47	0.50±1.07	0.42±1.14	≤0.001*	0.13±0.62	1.63±2.61	1.48±2.33	1.58±3.34	≤0.001*	0.01±0.10	0.53±0.91	1.29±2.21	1.41±2.02	≤0.001*
Diptera															
Syrphidae	0.00±0.00	0.03±0.18	0.20±0.44	0.17±0.49	0.001*	0.00±0.00	0.02±0.15	0.02±0.15	0.03±0.23	0.567	0.00±0.00	0.01±0.10	0.01±0.10	0.04±0.19	0.108
Others Diptera	0.02±0.13	0.17±0.49	0.13±0.34	0.15±0.36	0.071	9.48±16.12	5.07±8.47	3.04±4.48	3.50±7.56	≤0.001*	2.10±4.55	1.13±0.30	1.41±3.13	0.92±1.59	0.225
Lepidoptera															
Prays oleae	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	-	0.00±0.00	0.00±0.00	0.00±0.00	0.10±0.34	≤0.001*	0.00±0.00	0.30±0.74	0.28±0.64	0.27±0.70	≤0.001*
Others Lep.	0.67±0.77	1.22±1.39	0.92±1.08	1.05±1.31	0.057	0.51±1.21	0.62±1.17	0.38±0.08	0.29±0.71	0.187	0.05±0.21	0.02±0.14	0.16±0.44	0.12±0.41	0.006*
Thysanoptera	0.10±0.35	0.13±0.39	0.12±0.37	0.15±0.52	0.949	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.11	0.392	0.01±0.10	0.02±0.14	0.00±0.00	0.00±0.00	0.298
Dermaptera	0.03±0.26	0.03±0.62	0.00±0.00	0.00±0.00	0.571	0.21±0.66	0.22±0.63	0.19±0.56	0.06±0.27	0.103	0.55±1.33	0.10±0.48	0.19±0.67	0.10±0.29	0.003*
Psocoptera	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	-	0.01±0.11	0.00±0.00	0.00±0.00	0.00±0.00	0.392	0.19±0.50	0.29±0.76	0.07±0.29	0.12±0.36	0.052
Heteroptera	0.02±0.13	0.05±0.26	0.02±0.13	0.00±0.00	0.564	0.01±0.11	0.02±0.15	0.00±0.00	0.02±0.15	0.527	0.00±0.00	0.01±0.10	0.00±0.00	0.03±0.16	0.110
Hemiptera	0.13±0.34	0.53±2.63	0.22±0.72	0.90±1.88	0.059	0.51±1.17	0.97±2.36	0.21±0.57	0.18±0.49	0.005*	0.01±0.10	0.02±0.14	0.02±0.14	0.04±0.19	0.541
Odonata	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	-	0.00±0.00	0.00±0.00	0.00±0.00	0.02±0.15	0.111	0.00±0.00	0.00±0.00	0.00±0.00	0.04±0.19	0.007*
Arachnidae															
Araneae	0.15±0.36	0.17±0.38	0.22±0.45	0.20±0.45	0.885	0.22±0.44	0.09±0.29	0.20±0.52	0.07±0.29	0.009*	0.09±0.31	0.11±0.38	0.09±0.31	0.09±0.31	0.939
Acari	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	-	0.00±0.00	0.01±0.11	0.01±0.11	0.02±0.15	0.569	0.00±0.00	0.00±0.00	0.01±0.01	0.02±0.14	0.298
Entognatha															
Collembola	0.02±0.13	0.00±0.00	0.03±0.18	0.00±0.00	0.296	0.01±0.11	0.00±0.00	0.00±0.00	0.01±0.11	0.571	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	-

With the exception of 2009, hole size influenced significantly ($P<0.05$) the catches of Olipe traps for beneficial groups, that includes predators and hymenoptera parasitoids, and excluding ants. In 2009, the number of non-target arthropods captured were, 46.8%, 26.8%, 50.6% and 30.1% in Olipe traps with 4, 6, 8 and 10 mm of hole size, respectively. For the same order of hole size, the beneficial insects represented 7.8%, 23.7%, 34.1% and 32.0%; and in 2011, these groups represented 11.5%, 27.8%, 38.6% and 45.6%. In the last two years of the work (2010 and 2011) the negative effect of the Olipe traps was well evident, being this effect more clear for larger diameter (8 and 10 mm).

There was significant differences among hole size in total of Formicidae catches in 2009 ($p = 0.013$), 2010 ($p = 0.013$) and 2011 ($p \leq 0.001$). In average, the total of captures in 2009 was 18.93±6.39, 4.57±2.03, 4.38 and 12.18±4.58, in 2010 was 44.46±15.24, 15.79±5.55, 56.99±21.03 and 2.43±1.01 and 35.67±14.46, 12.06±4.28, 37.50±8.98 and 37.10±9.67 respectively for Olipe traps with 4, 6, 8 and 10 mm of hole size.

In the order Neuroptera, there were significant differences among hole size of Olipe traps in total catches of Chrysopidae adults in 2009 ($p \leq 0.001$), in 2010 ($p \leq 0.001$) and in 2011($p \leq 0.001$). In 2009, the highest percentage of Chrysopidae adults captured was recorded at September 07 with 77.3% of total Chrysopidae adults captured, corresponding to the date of the begin of study, being Olipe traps with 8 mm of hole size (1.27±0.28) and Olipe traps with 10 mm of hole size (1.53±0.49) those that recorded the highest average number of individuals captured (Figure 8.1).

In 2010, 85.3% of total Chrysopidae adults were captured in September, 51.4% in a single date, September 21st, and 33.9% at September 07th. The highest average number of individuals captured (7.93±1.06) was recorded at September 21 in Olipe traps with 10 mm of hole size. On the contrary the observations recorded in 2009 and 2010, the most of captures of Chrysopidae adults in 2011 occurred in October, with 33.5% at October 19 and 26.8% at October 05th. The highest average number of individuals captured (4.27±1.04) was recorded at October 18th in Olipe traps with 8 mm of hole size.

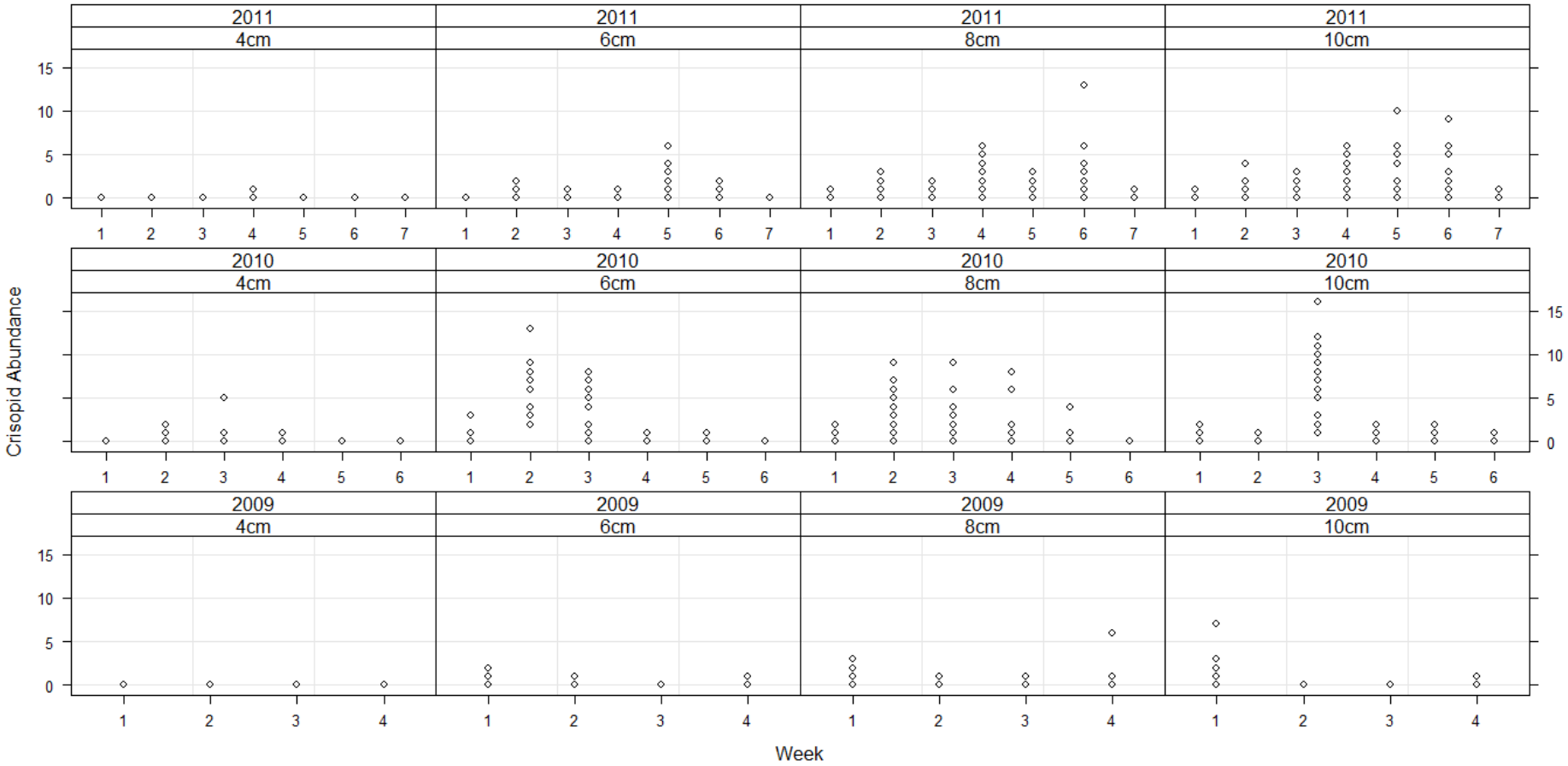

Figure 8.1. Temporal distribution of Chrysopidae caches in olive groves, mean number of 15 traps, in Olipe traps with different hole sizes (4, 6, 8 and 10 mm) in olive 2009, 2010 and 2011. Mirandela.

In all years, the use of bottles with 8 and 10 mm of diameter was particularly harmful for the Chrysopidae adults, which represented (excluding Formicidae) up to 14% of total recovered individuals in these bottles in 2009, 24% of total recovered individuals in 2010 and 33% of total recovered individuals in 2011. In this study were recorded 27.0% of *C. carnea* in 2009, 35.0% in 2010 and 11.0% in 2011. The number of Chrysopidae larvae was lower in all years of this study and no differences were recorded among hole size.

For order Coleoptera, 2009 and 2010, Olipe traps with 4 mm of hole size captured more Coleoptera, 0.12±0.04 and 0.14±0.05 respectively (Figure 2), and in 2011 the higher number of catches it was observed in Olipe traps with 10 mm of hole size (0.48±0.08). For the family Coccinellidae, no significant differences were observed between hole sizes. The presence of Coccinellidae larvae was very low with only two individuals in 2010 and 2011. For other Coleoptera no significant differences were recorded among hole size of Olipe trap in the three years, $p = 0.186$ (2009), $p = 0.360$ (2010) and $p = 0.933$ (2011).

In the order Diptera, there was significant differences among hole size in total catches in 2009 ($p = 0.040$). Nevertheless no significant differences among hole size were observed ($p = 0.567$ in 2010 and $p = 0.108$ in 2011) were observed in the other years. In 2009, the use of bottles of larger hole size (8 and 10 mm) was particularly harmful with catches of 6.8% in bottles with 8 mm of hole size and 5.1% in bottles with 10 mm of hole size in total of non-target arthropods captured (excluding ants). No syrphid was captured in Olipe traps with 4 mm of hole size. The other Diptera represented 4.3% in 2009, 63.3% in 2010 and 39.1% in 2011 of total non-target arthropods, and it was found significant differences ($p \leq 0.001$) in 2010.

For the order Araneae only in 2010 significant differences ($p < 0.05$) were found among hole sizes, having been captured more Araneae in Olipe traps with 4 and 8 mm of hole size.

In 2009, Olipe traps with 4 and 8 mm of hole size captured the highest number of parasitoids 0.62±0.10 and 0.55±0.15 respectively, followed by Olipe traps with 6 mm of hole size (0.37±0.09) and Olipe traps with 10 mm of hole size (0.18±0.11) (Figure 3). Among hole sizes, statistical differences were found ($p = 0.050$). In 2010, Olipe traps with 4 mm of hole size captured the highest number (0.49±0.10) of parasitoids followed by Olipe traps with 6 mm (0.34±0.08), 8 mm (0.30±0.06) and 10 mm (0.29±0.07). In 2011, Olipe traps with 4 mm of hole size captured the highest number (0.45±0.11) of parasitoids followed by Olipe 10 mm (0.24±0.05), 6 mm (0.19±0.04) and 8 mm (0.13±0.03). In 2010 (P=0.338) and 2011

(P=0.131) no statistical differences was found among hole size. In all years the Olipe traps with 4 mm of hole size showed higher captures compared with other evaluated hole sizes, capturing in average 0.48±0.09 parasitoids in 2009, 0.49±0.10 parasitoids in 2010 and 0.45±0.11 parasitoids in 2011.

Into the order Hymenoptera, bees were captured only in 2010 but in low number, representing 0.2% of total non-targeted insects.

The order Dermaptera represented 0.6% in 2009, 2.0% in 2010 and 6.3% in 2011 of total non-target arthropods, and significant differences were recorded in 2011 (P=0.003).

Amongst other arthropods with less importance, the order Hemiptera represented 16.3% in 2009, 5.6% in 2010 and 0.6% of total non-target arthropods. In the order Lepidoptera, *P. oleae*, presents less of 1.0% of total non-target arthropods in 2010 and 5.7% in 2011. The other Lepidoptera were present in 2009 with 32.7% in 2009, 5.7 in 2010 and 2.4% in 2011 of total non-target arthropods.

The order Thysanoptera was present in 2009 with 4.6% of total non-target arthropods and in 2010 and 2011 with less of 1.0% of total non-target arthropods. The order Psocoptera was present in 2010 with less of 1.0% and 2011 with 4.5% of total non-target arthropods. The orders Acari, Collembola, Heteroptera and Odonata, were present in lower number representing less of 1.0% of total non-target arthropods in each year.

8.4. Discussion

Mass-trapping, using different types of traps, has been a technique used in Mediterranean region to control *Ceratitis capitata* (Wiedemann) and *B. oleae* (Broumas *et al*., 2002). In olive groves conducted under sustainable production systems, mass trapping with Olipe traps could be an interesting option. Nevertheless, different studies showed Olipe traps negative impact in beneficial fauna that can change arthropod community, its environmental impact is considered low in comparison to chemical control (García-Rojas *et al*., 2002; Luque and Pereda, 2003; Pereira *et al*., 2007; Seris *et al*., 2007; Porcel *et al*., 2009). In the present work, a high number of specimens belonging to different taxa were captured in Olipe traps. Captures were numerically dominated by class Insecta that represented more than 98% of the total captures in the three years, and the most representative orders were Hymenoptera and Diptera.

If the recovered material were divided according the different trophic level, beneficial insects represented between 21,5% and 38.1%, values higher than the 17% observed by Porcel *et al*. (2009) when studied the side effects of Olipe traps.

Formicidae family was the group more recovered in Olipe traps that was in line of previous observations in Spain (Luque and Pereda, 2003; Porcel *et al*., 2009) and in Portugal (Pereira *et al*., 2007) probably due to the attractantness of biammonium phosphate in this group. Formicidae have predatory activity on *B. oleae* larvae and pupae (Arambourg, 1986; Katsoyannos, 1992) and other phytophagous species (Varela and González, 1999; Pereira *et al*., 2002) and the reduction of theirs population can affect phytophagous population levels. Nineteen species were captured, dominated in number by *C. auberti* (46.6% of the total) and *C. scutellaris* (43.3%). In general *C. scutellaris* was one of the most abundant ants in olive groves (Morris, 1997; Martínez and Ruíz, 1999; Morris *et al.*, 1998) jointly with *T. nigerrimum* (Pereira *et al*., 2002).

Amongst Diptera, the second taxa most captured, Olipe traps had a negative effect in Syrphidae, represented mainly by *E. balteatus* (64.9% of total) and *M. auricollis* (13.5%). The larvae of syrphids, and particularly the recovered species, are well known predators of the olive psyllid, *Euphyllura olivina* Costa (Ksantini, 2003).

Neuroptera was the third taxa most captured in Olipe traps. Chrysopidae represented from 10.0% to 21.7% of total non-target arthropods (excluding Formicidae) being captured mainly in September. Chrisopids are one of the most important predators associated to olive grove, dominated by *C. carnea* (Neuenschwander and Michelakis, 1980; Campos and Ramos, 1983; Alrouechdi, 1980; Bento, 1994; Campos and Ramos, 1983; Pantaleoni *et al*., 2001). High size holes (8 and 10 mm) of Olipe traps were particularly harmful for Chrysopidae adults, with reduced action on larvae, and the traps needs to be improved to reduce the captures of this important predator.

In the Coleoptera, some species of Coccinellidae, Carabidae and Staphylinidae have predatory action on olive groves, in particular some Coccinellidae (Santos *et al*., 2007). Olipe traps seems to be reduced effect in this group, with some captures of adults dominated by *S. interruptus*. Due the lower number of individuals captured in Olipe traps, it seems for us that Olipe traps have lower impact in order Coleoptera.

Araneae are one of the most abundant groups of generalist predators in olive groves (Gonçalves and Pereira, 2012). In this work, Araneae was present in lower number (<1.0% of

total non-target arthropods in each year) being is not clear the effect of hole size in Araneae captures.

In the Hymenoptera, parasitoids represent an important beneficial group in olive agroecosystem and have a principal role in the biological control of some olive key pests in Trás-os-Montes region (Bento *et al*., 1998; Pereira *et al*., 1998). Parasitoids represented between 4.3% to 16.0% of total non-target arthropods (excluding Formicidae). Olipe traps with 4 mm of hole size were those with highest captures in all years. Within order Hymenoptera, the impact of Olipe traps in bees (Apidae family), important insects for their pollination activity, is lower as was showed by the observed captures. This may be considered a positive aspect in the use of Olipe traps and also verified by Seris (2011) using the same method.

Dermaptera, dominated by *Forficula auricularia* L., appeared in great number in 2010 and 2011, however only in 2011 there was significant differences among hole size.

Relatively to other arthropods that were not regarded as beneficial insects, the other Diptera (not included *B. oleae* and Syrphidae) was a group with high catches. Some authors refer this order as the most abundant in the olive grove ecosystem (Petacchi and Minnocci, 1993; Ruano *et al*., 2004). The order Lepidoptera had high captures in 2009. Ruano *et al*. (2004) refer great abundance of this order in olive groves under organic farmer mainly in June. However, our results showed great abundance of Lepidoptera in September and October, mainly in 2009. In this order it is noteworthy the catches of *P. oleae* mainly in 2011, which is not a target arthropod but is a key pest in region, the use of Olipe traps can be a positive effect on reducing population levels of this pest. With the exception of order Hemiptera in 2009 (16.3% of total arthropods excluding Formicidae), all the other orders presented lower captures of individuals in all plots tested.

8.5. Conclusions

In conclusion, the results obtained in the present work demonstrated that hole size of the bottle used for *B. oleae* mass-trapping affects the number of non-target arthropods recovered. Although the Olipe traps of larger diameters increase the *B. oleae* captures it also increases the capture of non-target arthropods, in special the beneficial insects, making it more effective traps but less selective. Olipe traps with 4 mm of hole size showed lower impact in non-target arthropods. The use of Olipe traps with holes size with smaller diameters is an option to

consider since they have less impact on beneficial insects. It is noteworthy that interest in using this type of trap is reinforced by the fact that there are no alternatives to chemical control against the olive fly in olive growing under organic production, and that these traps either by its low cost and demonstrated efficacy will be an alternative to consider.

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CHAPTER 9

9.1 General conclusions

Currently, there is a great interest in the management of agricultural ecosystems aiming to optimizing the action of natural enemies. The maintenance of spontaneous vegetation into the orchards is a kind of ecological infrastructure designed to improve the action of natural enemies (Franco, 2010). The results show that the maintenance of spontaneous vegetation was promoter of biodiversity in the olive groves studied either by the abundance and richness of carabid (Chapter 4) either by the diversity of arthropods presents in *C. juncea* (Chapter 3). In this work it was observed a great abundance of carabids in the olive groves studied (Chapter 4). In both groves, *C. granatensis* was the most abundant species and the peak of abundance of this species occurred between late summer and middle autumn, which coincides with a gradual increase of olive fly pupae on the ground, especially the autumn generations. The occurrence of *C. granatensis* between the end of summer and autumn may contribute to the natural biological control of olive fly through predation of pupae found on soil. *C. juncea* is naturally spread in olive groves of Trás-os-Montes region and a great number of specimens belonging to different taxa were collected in this plant during the work (Chapter 3). It was found that this plant is a source of preys for many natural enemies by the number of aphids and thrips found on plant. The Diptera immature found on *C. juncea* can serve as alternative host to parasitoids which may act as natural enemies of olive fly. Thus, the presence of preys and alternative hosts can contribute to maintaining or enhancing parasitoid and predator populations on olive grove, resulting in improved pest control.

In laboratory, different food sources were studied to evaluate the effect on the *P. concolor* longevity (Chapter 5). Our results suggest that sugar feeding (sucrose and fructose) can increase longevity of *P. concolor* in laboratory and can enhance female-biased progeny. According Wäckers (2001) some species of parasitoids are strongly stimulated by certain sugar sources such as sucrose, glucose and fructose and food rich on them increase parasitoids

longevity. Provision on sugar-based food can be utilized for to mass rearing of this parasitoid in the laboratory. Thus, knowing the energy requirements of the parasitoid *P. concolor*, we can help to improve the rearing and maintenance of this parasitoid in the laboratory and in the manipulation of habitat to ensure success in the parasitoid introduction in biological control programs.

Among the direct strategic measures of protection against olive fly compatible in organic production, the biological control using entomopathogenic fungi agents has shown great potential against agricultural pests. Previous studies have demonstrated that the entomopathogenic fungi, *B. bassiana* have shown potential against puparia and adults of olive fly (Anagnou-Veroniki et al., 2005; Konstantopoulou and Mazomenos, 2005). In our laboratory studies it was shown that the strain of *B. bassiana*, Bb2T/08, indicating high mortality (93.9%) in bioassays with *B. oleae* pupae showing potential in biological control against the pest (Chapter 6).

The mass trapping has been a technique widely used in Mediterranean region to control olive fly (Broumas et al., 2002). In olive growing under organic agriculture, mass trapping with Olipe traps is a promising option, due to its low cost and effectiveness, and may reduce populations of the olive fly to levels considered acceptable (Caballero, 2002; Pavão et al., 2007). This work showed that the hole size of the Olipe traps can have influence in protecting the olive fruits against the olive fly. Traps with smaller hole diameter seem less efficient than traps with bigger hole diameter in reducing population levels. Traps of bigger diameter (8 and 10 mm) can reduce infestation levels to levels below the economic threshold level (Chapter 7). The results obtained in the present work demonstrated too that hole size of the bottle used for *B. oleae* mass-trapping affects the number of non-target arthropods catches (Chapter 8). Although the Olipe traps of larger diameters increase the *B. oleae* captures, it also increases the capture of non-target arthropods, in special the beneficial insects, making it more effective traps but less selective. Olipe traps with 8 mm of hole size proved to be the best compromise to be the hole size that has less impact on beneficial fauna. The use of Olipe traps selective for beneficial fauna becomes important because there is a great diversity of insects in the olive groves that are important in biological control of many pests.

9.2. References

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