Universidade de Aveiro Departamento de Biologia
2007

Sónia Alexandra Paiva dos Santos

Acção de predadores sobre a cochonilha-negra, *Saissetia oleae* (Oliv.) no olival transmontano

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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Prof. Doutor António José Arsénia Nogueira, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro e da Prof. Doutora Laura Monteiro Torres, Professora Catedrática do Departamento de Protecção de Plantas da Universidade de Trás-os-Montes e Alto Douro.

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Doutor Manuel Augusto Marques da Silva
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Doutor António José Arsénia Nogueira (orientador)
Professor Associado com Agregação da Universidade de Aveiro

Doutora Maria da Conceição Lopes Vieira dos Santos
Professora Associada da Universidade de Aveiro

Doutora Mónica João Barros Amorim André
Investigadora Auxiliar do CESAM – Departamento de Biologia da Universidade de Aveiro

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palavras-chave

protecção integrada, agricultura biológica, cochonilha-negra, coccinelídeos, inimigos naturais, fumagina.

resumo

A artropodofauna indigena deve ter um papel relevante na protecção das culturas como limitador natural das populações de espécies fitófagas. O seu conhecimento específico e as suas relações nos ecossistemas agrários são fundamentais no âmbito de uma agricultura sustentável. Neste trabalho pretendeu-se estudar a relação trófica entre predadores, nomeadamente coccinelídeos, e cochonilha-negra, *Saissetia oleae*, uma importante praga da oliveira na região de Trás-os-Montes, bem como saber quais os efeitos ao nível da planta desta praga através da investigação paralela em campo e em laboratório. O trabalho foi realizado em dois olivais orientados sob dois regimes de protecção da cultura para averiguar diferenças ao nível da abundância e diversidade de artrópodes. As diferenças de abundância e diversidade encontradas estão relacionadas com o tipo de sistema de protecção da cultura utilizados nos dois olivais em estudo. Quatro espécies de coccinelídeos, *Chilocorus bipustulatus*, *Scymnus (Scymnus) interruptus*, *Scymnus (Pullus) subvillosus* and *Scymnus (Mimopullus) mediterraneus*, mostraram ser as espécies mais comuns no olival transmontano e também possíveis candidatos a agentes de controlo natural contra a cochonilha-negra no olival devido quer à sua abundância quer à percentagem de respostas positivas obtidas nos testes ELISA. Assim, foram dados passos importantes no sentido de perceber quais as principais espécies predadoras de cochonilha-negra e ao mesmo tempo alertar para a importância que o sistema de protecção da cultura tem na artropodofauna predadora do olival, com especial relevância para os coccinelídeos.

keywords

Integrated protection, organic farming, black-scale, coccinellids, natural enemies, sooty mold.

abstract

The indigenous arthropod fauna, as natural control agents of phytophagous species, can have a relevant function in plant protection. Their specific knowledge and their relationships in the agro-ecosystems are in the scope of a sustainable agriculture. This work pretended to study the trophic relationship between predators, with a special emphasis of coccinellids, and the black-scale, *Saissetia oleae*, an important pest of the olive tree in the Trás-os-Montes region as well as to know the effects of this pest to the olive tree, by integrating both field and laboratory research. The field work was conducted in two olive groves with different agricultural systems in order to investigate the effect of the management regimes in the abundance and diversity of arthropods. Differences found for abundance and diversity were related with the agricultural management regime in the olive groves. Four coccinellid species, *Chilocorus bipustulatus*, *Scymnus (Scymnus) interruptus*, *Scymnus (Pullus) subvillosus* and *Scymnus (Mimopullus) mediterraneus*, showed to be the most common species in the olive grove of Trás-os-Montes region either due to their abundance or the percentage of positive responses obtained in the ELISA tests, being also potential candidates of natural control agents against the black-scale. Important steps were made in the direction of understanding which are the principal species of predators of the black-scale and, at the same time, to alert for the extreme importance of the effect of the management regime to the predator arthropod fauna of the olive grove, with special relevance for the coccinellids.

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Chapter 1

1. GENERAL INTRODUCTION

The olive tree (*Olea europaea* L.) is a typical and emblematic tree in the Mediterranean countries (Loumou and Giourga, 2003). Trás-os-Montes region accounts for 20.8% of the Portuguese olive cultivation and represents the second production region of the Portuguese olive oil (Anonymous, 2007) where it has a multiple importance particularly in that concern to economic, social, cultural and landscape aspects. The olive groves, which grow mostly on inclined, shallow and low fertility soils, have limited watering requirements and sustain the fragile natural resources of the region.

Within the scope of the agri-environmental measures, which have been supported by the European Union since 1992, farmers have been encouraged to promote environmentally friendly agricultural practices by protecting and enhancing the status of the agro-ecosystem and its biodiversity (de Lacroix, 2004). Simultaneously, the increasing consumer awareness of food production methods and environmental and health concerns have contributed to a rapid growth of a more sustainable farming. As a consequence, pest management systems like integrated protection (Boller *et al.*, 2004) and organic farming (Council Regulation (EEC) no. 2092/91 of 24 June 1991) gained a widespread interest by olive farmers.

In the core of those two management systems is the reduction or the eradication of the use of synthetic pesticides. Chemical insecticides are generally intended for particular pests at a particular site. Nevertheless, problems often arise because these chemicals are usually toxic to a much broader range of organisms and also persist in the environment (Walker et al, 1996). Of particular importance are the effects of insecticides on the arthropod natural enemies of insect pests. Target pests rapidly increase in abundance following some time after the initial drop caused by the insecticide application. This rebound effect occurs when treatment kills not only large numbers of the pest but large numbers of their natural enemies too. Then, any pest individuals that survive or that migrate into the area find an abundant food resource but few natural enemies and a population outbreak is the likely outcome (Begon *et al.*, 1996).

The target species selected for this study is among the major pests of the olive grove, the black-scale, *Saissetia oleae* (Olivier) (Hemiptera: Coccidae). This pest is an

oviparous species with parthenogenetic reproduction since males are very rare or absent (Passos-Carvalho *et al.*, 2003). Females have three nymphal stages before the adult stage that presents two distinct phases, the immature female or fourth instar nymph stage and the mature or ovipositioning female. In Trás-os-Montes, *S. oleae* is a univoltine species which winters on immature stages, mainly on second and third-instar nymphs. However, under favourable conditions, a partial or even a complete second generation can take place (Pereira, 2004). Third-instar nymphs usually migrate from leaves to younger sprout in spring. During April and May, the third-instar nymphs give origin to young females, which mature and lay eggs from May to the end of July (Passos-Carvalho *et al.*, 2003; Pereira, 2004).

In the olive tree, *S. oleae* feeding causes both direct damages by sucking plant sap and indirect damages by favouring the development of fungi such as the sooty mold (Noguera *et al.*, 2003). Sooty mold is the common name applied to several genera of fungi (e.g. *Capnodium*, *Cladosporium* and *Fumago*) that grow on honeydew secretions that accumulates on different plant parts (leaves, stems and fruits). Honeydew is a sweet and sticky liquid that is excreted by plant-sucking insects as they ingest large quantities of sap from the plant. It is consensual that sooty mold do not infect plants, although they can indirectly damage the plant by coating the leaves to the point that sunlight absorption is reduced or inhibited. Without adequate sunlight, the plant's ability to carry on photosynthesis is reduced resulting in a decrease of the quantity and quality of the yield (Cozzi *et al.*, 2002). Coated leaves may also prematurely senesce and die, causing premature leaf drop.

The development of an environmentally sustainable pest management program to control *S. oleae* should include the use of natural enemies, maximizing the effectiveness of predation of as many of the natural enemies of this pest as possible and abolish the use of synthetic pesticides. To accomplish this objective, background knowledge about the biology of indigenous predators as potential natural control agents of this pest is required. Information concerning the identity, abundance and diversity of predators, synchronization of predator-pest life cycles and ability to predate *S. oleae* are also essential aspects when natural control of *S. oleae* is required.

Field data on predator-prey relationships can be obtained by different ways, including (1) field surveys, (2) gut content analysis and (3) laboratory experimentation.

Field surveys in the olive grove are very useful but sometimes difficult to conduct because predators will most often be in the vicinity of their prey for only a short period. They play an important role in the identification of species with the narrowest range out of a pool of species and provide guidance regarding which species should be included in prey specificity tests (Babendreier *et al.*, 2005).

The difficulty of directly observing predation prompted the development of techniques to identify the presence of prey within the gut of predators (Sopp *et al.*, 1992), which can be done using, for example, an enzyme-linked immunosorbent assay (ELISA) (Fichter and Stephen, 1981; Symondson and Liddell, 1993; Hagler and Naranjo, 1997; Morris *et al.*, 1999) or laboratory experiments where the voracity of predators to consume a specific prey is investigated (Sahayaraj and Paulraj, 2001; Sengonca *et al.*, 2005).

The key objectives of this study were:

(*i*) to study the effect of two different management regimes, organic farming and integrated protection, on the olive tree canopy arthropod community (Chapter 2).

(ii) to emphasize the effect of the different management regimes, organic farming and integrated protection, on the structure and biodiversity of the coccinellid community (Chapter 3).

(*iii*) to study the temporal synchrony between the abundance of different coccinellid species and the abundance of each phenological stage of *S. oleae* (Chapter 4).

(*iv*) to establish trophic relationships between coccinellids and *S. oleae* using an indirect – ELISA (Chapter 5).

(v) to study the voracity of selected coccinellid species against different phenological stages of *S. oleae* (Chapter 6).

(*vi*) to identify the indirect effects caused by the target pest, *S. oleae*, to the olive tree through the analysis of histological and biochemical parameters in leaves covered with sooty mold (Chapter 7).

Thus, this dissertation includes six papers that have been submitted to or published in international scientific journals with referees and is planned in eight chapters where the key objectives will be addressed:

- **Second chapter** - the effect of two different management regimes, organic farming and integrated protection, used to control the olive moth, *Prays oleae* Bern., was studied on the overall canopy arthropod community of the olive tree. The effects of the management system on abundance of the different taxa and functional groups found on the olive tree canopy were highlighted.
- **Third chapter**, the effect of two different management regimes, organic farming and integrated protection, was studied on the structure of the coccinellid community. Aspects like abundance and species diversity found in the two olive groves were analyzed.
- **Fourth chapter**, the temporal synchrony between the abundance of the most important coccinellid species found in both olive groves were correlated with the abundance of the target pest species, the black scale *S. oleae*.
- **Fifth chapter**, the identification of the most important coccinellid species that predates *S. oleae* was carried out using an indirect-ELISA, after the development and characterization of a polyclonal antiserum against the pest.
- **Sixth chapter**, the ability of selected coccinellid species to use different phenological stages of *S. oleae* as food items was tested under controlled conditions.
- **Seventh chapter**, the main indirect effect of *S. oleae*, i.e. the consequent development of sooty mold on olive leaves and its effects, was investigated by comparing histological and biochemical parameters between healthy and attacked leaves.
- **Eighth chapter**, a general discussion and conclusions deals with the relevance of the results and with their implications to control the black scale.

All these chapters focus on specific issues that are important for the final goal of this work that is to know the action of predators against the black scale in the olive grove, so that these results can be useful to develop rational pest control and increase sustainability of this agro-ecosystem.

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Chapter 2

2. EVALUATION OF THE EFFECTS, ON CANOPY ARTHROPODS, OF TWO AGRICULTURAL MANAGEMENT SYSTEMS TO CONTROL PESTS IN OLIVE GROVES FROM NORTH-EAST OF PORTUGAL

Sónia A.P. Santos, José A. Pereira, Laura M. Torres & António J.A. Nogueira

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ABSTRACT

This study aims to investigate the effect of the management regime on canopy arthropod community of the olive tree (*Olea europaea* L.). Field studies were performed in two successive years, 2002 and 2003, in two olive groves, one under organic farming and the other under integrated protection. Integrated protection grove was sprayed once a year in June, with dimethoate, to control the anthophagous generation of the olive moth, *Prays oleae* (Bern.). From April to November of each year, the canopy arthropods were sampled weekly. PRC method was used to analyse the effect of management regime at the community level and results showed that taxa responded differently to insecticide application suggesting that the organic grove was a more suitable habitat for the arthropods than the integrated protection grove. Abundance of arthropods peaked in May and June for both years but, after spraying with dimethoate, decreased significantly in integrated protection grove, recovering very slowly thereafter. Psocoptera, Miridae, Formicidae and Coccinellidae were the most sensitive taxa to insecticide application. Their decreasing in abundance was more evident in the second year of the trial. On the other hand chrysopids showed some tolerance to insecticide applications. These results suggest that the timing of spray is of utmost importance in reducing the side effects of spraying on beneficial arthropods. Moreover, differences in population susceptibility as well as in life cycle patterns must be considered.

Key-words: Functional groups; Integrated protection; Organic farming; Principal Response curves.

2.1. Introduction

Olive grove is an agroecosystem that supports a wide range of diverse and functionally important groups of arthropods (Morris *et al.*, 1999; Rodríguez *et al.*, 2003; Ruano *et al.*, 2004). The canopy arthropod community has a complex structure that includes functional groups with phytophagous, predators, parasitoids and detritivores species (Rodriguez *et al.*, 2003; Ruano *et al.*, 2004). The maintenance of the steady state between these groups is important because functional biodiversity performs key ecological services and can bring sustainability to agroecosystem (Altieri, 1999). However, agricultural management practices, specially the use of pesticides, disturb the natural balance between guilds and some phytophagous species increase rapidly resulting in pest outbreaks. These outbreaks of pest populations after chemical treatment may be induced by the reduction of predators or by the development of resistance in pest population to certain insecticides (Marc *et al.*, 1999; Hawkes *et al.*, 2005).

In the last decades, European Union developed policies to regulate pest management under more environmentally sensitive farming practices and has led to a widespread interest in integrated protection (Boller *et al.*, 2004) and in organic farming (Council Regulation (EEC) no. 2092/91 of 24 June 1991). Both, integrated protection and organic farming, are management regimes that promote the protection of the environment, specifically biodiversity, soil and water and the achievement of high-quality agricultural products (Guillou and Scharpé, 2000; Malavolta *et al.*, 2002; Boller *et al.*, 2004). To date, insecticides such as, dimethoate, methidathion, phosmet, fenthion and summer oil (1-2%), as well as formulations of *Bacillus thuringiensis* Berliner are allowed in olive groves in integrated pest management in Portugal, although its uses is recommended only when economic threshold is reached, and in some cases in one only application (Gomes and Cavaco, 2003). However, in organic farming only naturally derived pesticides such as *B. thuringiensis* can be used; synthetic pesticides and fertilizers are excluded. Instead of synthetic inputs, compost and animal and green manures are used to build up soil fertility. Biological control through the use of mating disruption, traps and releases of insect predators or parasitoids is especially encouraged for both agricultural systems (Luck *et al.*, 1999).

In integrated protection, the use of authorized chemicals to control olive pests can also affect beneficial arthropods changing the equilibrium of the agroecosystem (Petacchi and Minnocci, 1994; Rodríguez *et al.*, 2003; Ruano *et al.*, 2004). For this, it is essential to improve knowledge on the effects of agricultural practices on arthropod community (pests and beneficial species). Therefore, the objective of this investigation was to study the effect of two different management regimes, organic farming and integrated protection, on canopy arthropod community.

2.2. *Material and Methods*

2.2.1. Study areas

The study areas were located in two olive groves near Mirandela (Portugal), Valbom-dos-Figos and Paradela groves, with different agricultural systems.

Valbom-dos-Figos (41º 33' 4'' N, 7º 8' 43'' 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 10 × 10 m apart. The predominant cultivars are Cobrançosa and Verdeal Transmontana. No sprays were done against pests or diseases and soil was fertilized with organic nutrients two times a year.

In Paradela (41º 32' 38" N, 7º 7' 29" W) olive grove plant protection was done according to the principles of Integrated Pest Management since 2001. The predominant olive cultivar is Cobrançosa and in low numbers Verdeal Transmontana, Madural and Borrenta. The grove covers an area of 3 ha, the planting density is of 9 × 9 meters and the trees are about 50 years old. According to farmer's information, a dimethoate spray (150 mL hL^{-1} of the formulation at 42.8% (W/V)) against the anthophagous generation of the olive moth, *Prays oleae* (Bern.), was applied in June: 13 and 16 June of 2002 and 2003, respectively; soil was fertilized with organic and mineral nutrients two times a year. Both olive groves were not irrigated and soil was ploughed superficially with a scarifier two to four times a year to control weeds. Trees were pruned every two or three years. In this work, Valbom-dos-Figos is referred as organic and Paradela as integrated grove.

2.2.2. Survey of canopy arthropods

Sampling was carried out weekly, between April and November of two consecutive years, 2002 and 2003. Arthropods were collected by the beating technique. In every sampling period, five samples per olive grove were collected. Each sample has the beatings of ten branches randomly selected. All captured individuals were frozen, sorted and identified under binoculars until Orders, Families or species taxa and the total number of each taxon was recorded. Each taxon was further classified by their trophic role based on personal observation and literature review (Chinery, 1993; Iperti, 1999; Fauvel, 1999; Sommaggio, 1999, Stelzl and Devetak, 1999). Whenever larvae and adults belonged to different functional groups they were counted and classified independently. Phytophagous, predators and detritivores were classified based on whether they ate primarily plants, animals, and dead matter or fungi, respectively. Parasitoids were host feeders; omnivorous had different food sources such as pollen, yeast, fungus, honeydew and small arthropods; and indeterminate included taxa with phytophagous, predators or parasitoids species.

2.2.3. Statistical analysis

Univariate statistical analyses were performed using the Statistica statistical package, version 7.0 (StatSoft, 2004). Data were evaluated for normality and homogeneity of variances with Kolmogorov-Smirnov test and Bartlett's test, respectively and when necessary, the transformation $\log_{10}(x + 1)$ was used to normalise the data. The abundance of individuals from different taxa and trophic guilds captured in both olive groves over different times was compared by repeated measures analysis of variance (Zar, 1996).

Multivariate statistical analyses were performed using CANOCO for Windows 4.5 (Ter Braak and Šmilauer, 2002). Multivariate techniques were applied to analyse the response of the whole community. Principal response curves (PRC) analysis is derivative of the multivariate ordination technique redundancy analysis (RDA) and was especially designed for time series obtained from experiments performed with communities (Van den Brink and Ter Braak, 1999; Van den Brink *et al.*, 2003). The resulting PRC diagram displays a curve for the treatment that can be interpreted as the principal response of the community. By definition, the reference is zero in every date and, at each sampling period

t, the deviation (given by a basic response pattern or C_{dt}) of the treatment curve *d* compared to reference is proportional to the effect of insecticide spraying. The advantage of this method over other methods is that it is able to focus on the part of the variance explained by the treatment (here integrated grove) when compared with the reference site (here organic grove). The objective of a PRC diagram is to maximize the amount of variance that is displayed by the effect of treatment; when the displayed variances are large then the fitted relative abundance of individual taxa inferred from the diagram matches very well the observed relative abundance (Ter Braak and Šmilauer, 2002). The species weight (b_k) indicates how closely the response of species *k* matches the overall community response as displayed in the PRC diagram. Taxa with a positive weight are expected to decrease in abundance, relative to reference while taxa with negative weight are expected to increase. Taxa with species weights between 0.5 and -0.5 show a weak response.

Expression (1) can be applied for each species *k* at treatment *d* and sampling date *t* to evaluate quantitatively the PRC. The value obtained times the geometric mean in the reference gives the abundance in the treatment for species *k*.

$$\exp(b_k * C_{dt}) \quad (1)$$

2.3. Results

A total of 35346 and 14540 arthropods were captured in the organic and integrated groves, respectively over the two years of the study. Arthropods were classified into 12 orders: Acari, Araneae, Coleoptera, Dermaptera, Diptera, Heteroptera, Homoptera, Hymenoptera, Lepidoptera, Neuroptera, Psocoptera and Thysanoptera. From those, five families (Formicidae, Coccinellidae, Miridae, Chrysopidae and Anthocoridae) and two pest species were identified, the olive moth *P. oleae*, the most important pest of the olive tree in the region and the olive psylla *Euphyllura olivina* Costa, a secondary pest. The overall taxa abundance varied between olive groves and years and was significantly higher in organic than in integrated grove ($F_{1, 8} = 30.76$, $p<0.001$ for 2002 and $F_{1, 8} = 764.26$, $p<0.001$ for 2003) and in 2003 than in 2002 ($F_{1, 548} = 46.47$, $p<0.001$).

Table 2.1. Total abundance (N) and mean ± standard error of the mean (SE) of taxa and functional groups captured in total samples in organic and integrated protection olive groves in 2002.

Group	Organic 2002 (n=145)		Integrated 2002 (n=145)		$F_{1,8}$	p[1]	a[2]
	N	Mean ± SE	N	Mean ± SE			
Phytophagous							
Homoptera							
E. olivina	21	0.14 ± 0.03	28	0.19 ± 0.04	0.83	ns	2
Lepidoptera							
P. oleae (Larvae)	14	0.10 ± 0.05	70	0.48 ± 0.12	41.53	***	2
Thysanoptera	226	1.56 ± 0.17	264	1.82 ± 0.24	0.07	ns	1
Predators							
Araneae	811	5.59 ± 0.32	495	3.41 ± 0.26	33.49	***	6
Heteroptera							
Miridae	382	2.63 ± 0.26	183	1.26 ± 0.23	26.31	***	10
Anthocoridae	30	0.21 ± 0.11	10	0.07 ± 0.02	1.75	ns	1
Coleoptera							
Coccinellidae	1203	8.30 ± 0.61	530	3.66 ± 0.35	40.30	***	14
Neuroptera							
Chrysopidae (Larvae)	57	0.39 ± 0.07	116	0.80 ± 0.10	9.86	*	5
Hymenoptera							
Formicidae	1756	12.11 ± 1.05	2741	18.90 ± 3.29	11.67	**	16
Parasitoids							
Hymenoptera	728	5.02 ± 0.45	683	4.71 ± 0.48	1.01	ns	12
Detritivores							
Dermaptera	15	0.10 ± 0.03	16	0.11 ± 0.04	0.08	ns	0
Psocoptera	1073	7.40 ± 0.63	1037	7.15 ± 1.30	35.98	***	18
Omnivorous							
Neuroptera	10	0.07 ± 0.02	4	0.03 ± 0.01	2.79	ns	0
Chrysopidae (Adults)	35	0.24 ± 0.04	39	0.27 ± 0.04	1.14	ns	3
Lepidoptera							
P. oleae (Adults)	58	0.40 ± 0.07	40	0.28 ± 0.06	3.84	ns	5
Indeterminate							
Acari	1805	12.45 ± 2.10	221	1.52 ± 0.30	117.08	***	21
Coleoptera	902	6.22 ± 0.43	460	3.17 ± 0.28	36.67	***	12
Diptera	941	6.49 ± 0.82	515	3.55 ± 0.55	21.82	**	9
Total	10067	69.87 ± 3.41	7452	51.99 ± 4.68	30.76	***	20
Trophic Guild							
Phytophagous	261	2.24 ± 0.19	362	3.10 ± 0.35	0.83	ns	5
Predators	4239	29.23 ± 1.36	4075	28.10 ± 3.44	17.13	**	20
Parasitoids	728	5.02 ± 0.45	683	4.71 ± 0.48	1.01	ns	12
Detritivores	1088	7.50 ± 0.63	1053	7.26 ± 1.35	32.77	***	19
Omnivorous	103	0.71 ± 0.08	83	0.57 ± 0.07	5.21	ns	4
Indeterminate	3648	25.16 ± 2.45	1196	8.25 ± 0.74	94.88	***	16

n = total number of samples; [1] ns- non significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$; [2] Number of sample dates (out of 29 total) on which the F-value was significant $p<0.05$.

In 2002, arthropod community in organic grove was numerically dominated by Acari and Formicidae (17.9% and 17.4% respectively), followed by Coccinellidae (12%) and Psocoptera (10.6%); in integrated grove, community was mainly dominated by Formicidae (36.8%), followed by Psocoptera (13.9%). Predators were the most abundant functional group in both groves (Table 2.1). Average abundance of seven taxa out of 19 were significantly lower in integrated grove ($p<0.05$) compared with organic, including predators such as Araneae, Miridae and Coccinellidae. On the other hand, average abundance of Formicidae was significantly higher in integrated than in organic grove but the greatest peaks of abundance were recorded before the application of the insecticide, decreasing thereafter. For phytophagous species, only for the target, *P. oleae*, were found significant differences ($p<0.05$) between groves with a higher abundance in integrated than in organic grove. No significant differences ($p>0.05$) were observed for omnivorous and parasitoids between groves (Table 2.1).

Before sprays against *P. oleae*, the abundance of trophic groups was generally higher in integrated than in organic grove. However, spray caused a decrease in the abundance of arthropods (Fig. 2.1). Predators and detritivores were the most affected groups. This situation was observed in sampling dates immediately after the insecticide application, when significant differences between groves were found ($p<0.05$). The effect of spray on detritivores remained until the end of sampling period and no recovery was observed, while parasitoids recovered, increasing in abundance in the mid summer.

In 2003, Psocoptera largely dominated the arthropod community in organic grove with 50.8% of total specimens captured, followed by Diptera (8.6%), Coccinellidae (6.9%) and Formicidae (6.3 %); in integrated grove, Psocoptera accounted 21.9% of total captures, followed by Diptera (17.0%), Araneae (9.8%) and Formicidae (8.8%). The dominant trophic group was different for organic and integrated groves. Detritivores dominated the former whereas predators prevailed in the second (Table 2.2). In this year, the differences between management regimes increased. Thus, average abundance of 15 taxa out of 19 was significantly lower in integrated grove ($p<0.05$) compared with organic including all phytophagous, predators, parasitoids and indeterminate taxa.

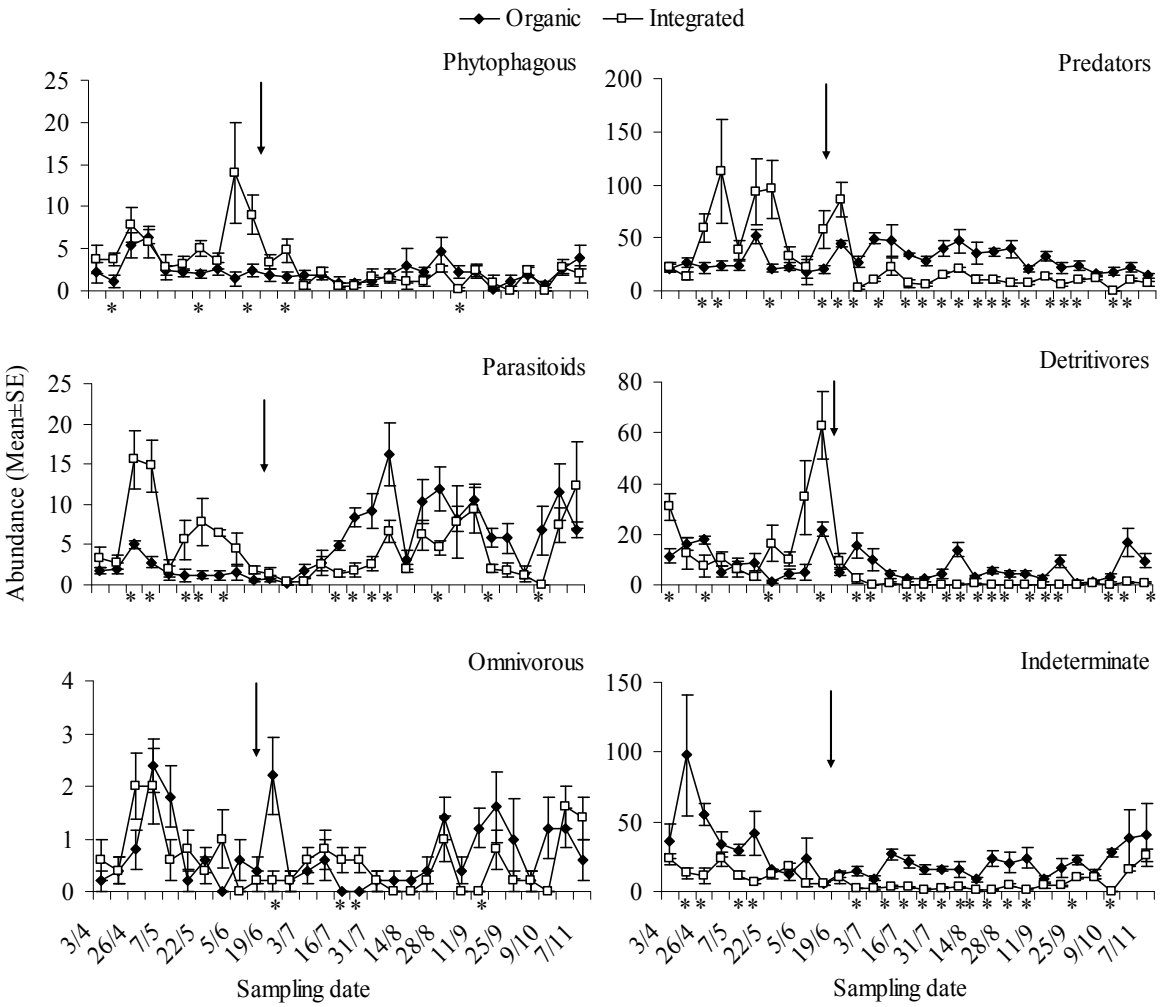

Figure 2.1. Abundance (mean ± SE) of different trophic groups in each sampling date during 2002 (n = 5). Asterisks denote dates on which significant differences were observed ($p<0.05$) between olive groves. Arrows indicate spray date in integrated protection grove. Different axis scales were used.

Unlike 2002, during 2003 the seasonal abundance of trophic groups was commonly higher in organic than in integrated grove with the difference being magnified after spraying (Fig. 2.2). The greatest differences were observed on predators and detritivores which reached high levels of abundance in organic grove in the beginning of summer. In integrated grove the abundance decreased after insecticide application and significant differences between the two groves were found in 15 out of 17 sampling dates for predators and 16 out of 17 dates for detritivores. Once more, parasitoids recovered easily after spraying.

Table 2.2. Total abundance (N) and mean ± standard error of the mean (SE) of taxa and functional groups captured in total samples in organic and integrated protection olive groves in 2003.

Group	Organic 2003 (n=130)		Integrated 2003		$F_{1,8}$	p^1	a[2]
	N	Mean ± SE	N	Mean ± SE			
Phytophagous							
Homoptera							
*E. olivin*a	593	4.56 ± 0.37	238	1.83 ± 0.20	105.86	***	11
Lepidoptera							
P. oleae (Larvae)	320	2.46 ± 0.96	191	1.47 ± 0.45	6.59	*	3
Thysanoptera	308	2.37 ± 0.23	246	1.89 ± 0.34	10.41	*	9
Predators							
Araneae	1010	7.77 ± 0.47	696	5.35 ± 0.30	114.79	***	9
Heteroptera							
Miridae	1367	10.52 ± 1.46	53	0.41 ± 0.08	306.96	***	16
Anthocoridae	80	0.62 ± 0.09	5	0.04 ± 0.02	182.19	***	4
Coleoptera							
Coccinellidae	1731	13.32 ± 1.04	332	2.55 ± 0.20	202.91	***	16
Neuroptera							
Chrysopidae (Larvae)	326	2.51 ± 0.22	212	1.63 ± 0.17	21.39	**	2
Hymenoptera							
Formicidae	1584	12.18 ± 0.80	623	4.79 ± 0.81	439.24	***	16
Parasitoids							
Hymenoptera	645	4.96 ± 0.36	584	4.49 ± 0.42	14.49	**	10
Detritivores							
Dermaptera	7	0.05 ± 0.02	48	0.37 ± 0.07	23.49	**	1
Psocoptera	12840	98.77 ± 10.38	1556	11.97 ± 2.30	1078.20	***	24
Omnivorous							
Neuroptera	8	0.06 ± 0.02	20	0.15 ± 0.04	17.11	**	0
Chrysopidae (Adults)	54	0.42 ± 0.08	43	0.33 ± 0.05	0.31	ns	2
Lepidoptera							
P. oleae (Adults)	495	3.81 ± 0.78	382	2.94 ± 0.91	15.27	**	9
Indeterminate							
Acari	848	6.52 ± 1.15	70	0.54 ± 0.10	60.48	***	10
Coleoptera	878	6.75 ± 0.47	585	4.50 ± 0.33	25.48	***	12
Diptera	2185	16.81 ± 2.53	1204	9.26 ± 1.47	80.89	***	7
Total	25279	195.08 ± 12.45	7088	55.26 ± 4.93	764.26	***	22
Trophic Guild							
Phytophagous	1221	10.02 ± 1.24	675	5.93 ± 0.81	37.41	***	12
Predators	6098	46.91 ± 3.05	1921	14.78 ± 1.01	553.28	***	18
Parasitoids	645	4.96 ± 0.38	584	4.49 ± 0.45	14.49	**	10
Detritivores	12847	98.82 ± 10.96	1604	12.34 ± 2.45	932.29	***	24
Omnivorous	557	4.28 ± 0.82	445	3.42 ± 0.97	8.83	*	5
Indeterminate	3911	30.08 ± 3.17	1859	14.30 ± 1.62	150.50	***	15

n = total number of samples; [1] ns- non significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$; [2] Number of sample dates (out of 26 total) on which the F-value was significant $p<0.05$.

Phytophagous were much more abundant in 2003 than in 2002 and it happened that in both years, sprays were done after the peak value of abundance has been reached.

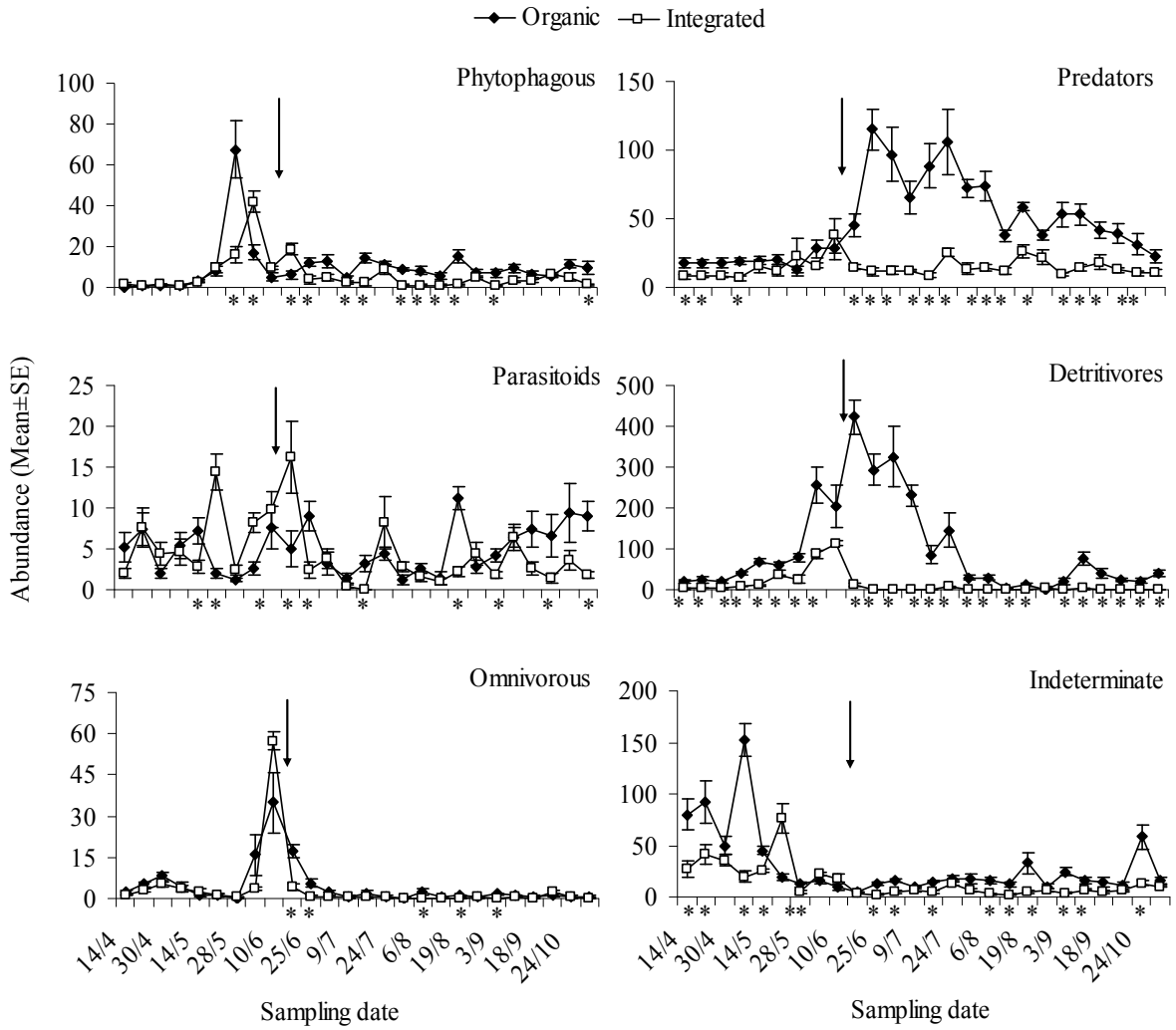

Figure 2.2. Abundance (mean ± SE) of different trophic groups in each sampling date during 2003 (n = 5). Asterisks denote dates on which significant differences were observed ($p<0.05$) between olive groves. Arrows indicate spray date in integrated protection grove. Different axis scales were used.

PRC diagram concisely shows changes through the two years in the variability of canopy arthropod community composition. Of the total variance, 43% is explained by sampling date and 29% by management regime. The Monte Carlo permutation test showed a significant difference between groves and 63% of that difference is displayed by the first PRC axis (Fig. 2.3). On the sampling dates immediately before each insecticide application, PRC shows small differences between the integrated and organic curves indicating that captures were similar. After that, PRC shows large differences and the negative values of C_{dt} indicate that captures were lower in the treatment than in reference; the greatest differences in catch between groves are clearly evident in two periods: 19 June 2002 and 17 June 2003 which coincides with sprays against *P. oleae*. After insecticide applications the arthropod community recovers very slowly. Psocoptera had the highest positive weight (b_k) followed by important predators groups such as Miridae, Coccinellidae (adults) and Formicidae that decreased in abundance after insecticide application. Taxa with weights between -0.5 and 0.5, like *P. oleae* (larvae), had a weak contribution to the overall community response. Expression (1) was applied to the two groups of predators most affected by the spraying. Thus, for abundance of mirids on 25 June 2002, the PRC diagram predicts that at integrated grove it would be exp(1.6*-0.75)=0.30 times the abundance at organic grove. For coccinellids, the fitted relative difference in abundance between organic and integrated groves on 25 June 2002 is exp(1.47*-0.75)=0.33. This agrees well with the actual data, in which the geometric mean counts on 25 June 2002 for mirids were 0.8 and 2.5, respectively, for integrated and organic groves (relative abundance 0.32) and for coccinellids were 1.1 and 3.0 (relative abundance 0.37). Similarly, on 25 June 2003, the PRC diagram predicts that the abundance of Mirids in integrated grove would be exp(1.60*-1.75)=0.06 times that observed in organic grove. For coccinellids the difference in abundance between both olive groves is exp(1.47*-1.75)=0.076. Again, in this example, the fitted relative abundance is close to the observed relative abundance of 0.057 for mirids (geometric mean counts were 2.4 and 41.9, respectively, for integrated and organic grove) and 0.074 for coccinellids (geometric mean counts were 2.2 and 29.4, respectively, for integrated and organic grove).

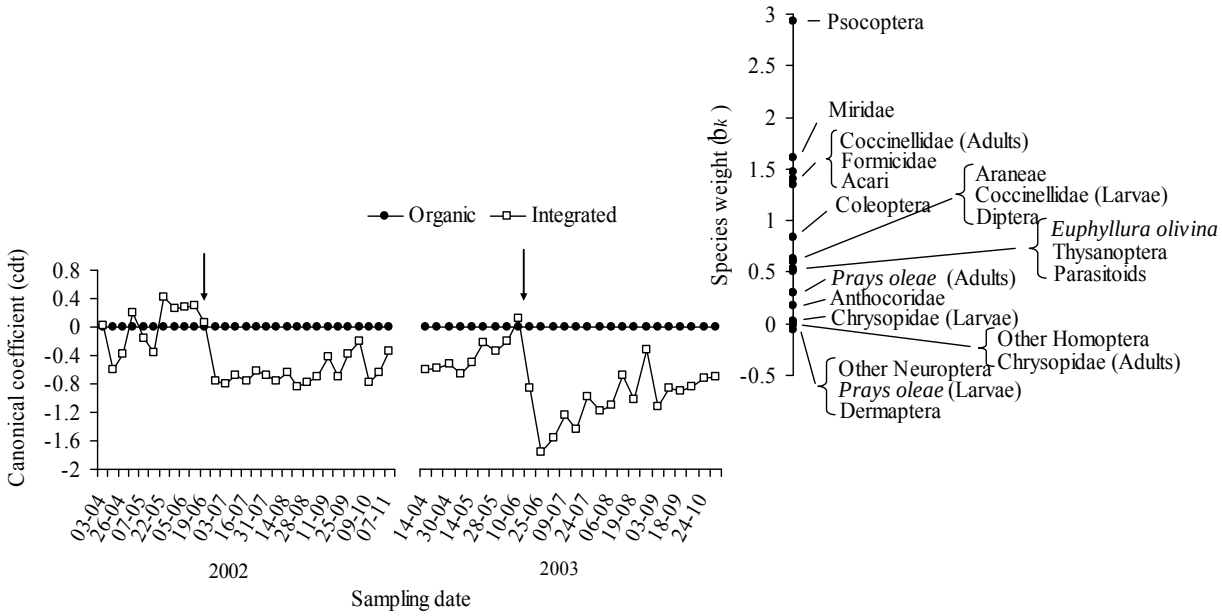

Figure 2.3. PRC diagram and species weights for sampled arthropods, showing variation in taxa abundance during two years of study. Arrows indicate spray date in integrated protection grove.

2.4. Discussion

In this study, the most representative orders were Psocoptera, Hymenoptera, Diptera and Coleoptera varying in proportion between groves and years. These groups have been reported in the literature (Petacchi and Minnocci, 1994; Belcari and Dagnino, 1995; Ruano *et al.*, 2001) as typically abundant groups in olive groves. Psocoptera is considered an unimportant economically order (Chinery, 1993) whereas the other three orders include key-groups of predators, parasitoids and phytophagous. Ants and wasps (O. Hymenoptera) and coccinellids (O. Coleoptera), for instance, play an important role in the biological control of olive pests like *P. oleae* and *Saissetia oleae* (Olivier) (Obrycki and Kring, 1998; Iperti, 1999; Morris *et al.*, 1999; Morris *et al.*, 2004).

In the two years of study, total abundance was higher in organic than in integrated olive grove. However, Ruano *et al.* (2004) found a higher total abundance of arthropods in integrated than in organic grove as a consequence of the number of Homoptera, namely due to the presence of the olive pest *E. olivina*.

Disturbance caused by the use of dimethoate to control anthophagous generation of *P. oleae* in integrated grove had a strong and dramatic effect on the abundance of different trophic groups. Predators and detritivores, in particular, showed a long recovery period. Petacchi and Minnocci (1994) using chromotropic sticky traps and Belcari and Dagnino (1995) using Malaise traps also observed an abrupt reduction of entomofauna in Italian olive groves after dimethoate application.

PRC showed that the target species, *P. oleae*, was not strongly affected by insecticide treatments, probably because these were not correctly timed as in both years they were done slightly after the abundance of anthophagous generation has reached its peak. As a consequence of successive applications of dimethoate, is likely that the pest population has developed insecticide resistance, similarly to what was demonstrated for another pest, the olive fruit fly, *Bactrocera oleae* (Gmelin) (Hawkes *et al.*, 2005).

Mirids, coccinellids and ants were the predators more affected by the insecticide, which in part can be explained by the fact that they were abundant groups when sprays were done. The effect of insecticide was even more pronounced in 2003 because the abundance of those groups reached higher values than in 2002. Rodríguez *et al.* (2003) also found a strong toxicity of the deltamethrin towards those families. Fauvel (1999) stated that mirids are rather susceptible to insecticides and are eliminated from commercial groves because of low number of generations per year. Comparatively, anthocorids are more tolerant to pesticide application being better able to maintain themselves in the presence of chemical treatments in part due to the occurrence of a greater number of generations per year and because they are more mobiles having great ability to search for protection (Fauvel, 1999). However, anthocorids are typically found in minor number than mirids in olive cultivation and in this study they were found in more abundance in the organic grove.

Iperti (1999) verified that coccinellids are, in general, highly vulnerable toward chemical treatments and dimethoate is considered a very toxic insecticide to this predaceous group. In laboratory studies, Ba M'hamed and Chemseddine (2002) concluded that *Pullus mediterraneus* is a very sensitive species to dimethoate. These authors observed high mortality rates of adults within few hours after dimethoate exposition at

recommended doses. *P. mediterraneus* was a common species in both organic and integrated olive groves (Santos *et al.*, unpublished).

Although ants are considered a key-group in olive grove (Morris *et al.*, 1999; Pereira *et al.*, 2004) namely as bioindicators of disturbances, the information concerning the effect of pesticides over this group is scarce. In olive grove, ant species nest in soil or in old olive trunks and climb to the canopy to eat insects and liquid sugars (Redolfi *et al.*, 1999). Two ideas can be addressed to justify the decrease of ants in integrated grove. On the one hand, ants could be a susceptible group and were directly affected by the spray with dimethoate. On the other hand, prey depletion on the canopy justifies the consequently decrease of these predators that stop to climb the tree staying in the soil.

Spiders are abundant predators in olive groves, but their role is relatively unknown. They were more abundant in organic than in integrated grove indicating some susceptibility to chemicals. This result is in agreement with those obtained by Ruano *et al.* (2004) and Cárdenas *et al.* (2006). In tomato fields, Yardim and Edwards (1998) also observed a severe decrease of spider populations after treatments with carbaryl, endosulfan and esfenvalerate insecticides although different species respond in different ways when exposed to pesticide residues.

Parasitoids, showed a good recovery rate after spraying probably because at the time of treatment they were protected inside the insect host being less exposed to the insecticide. However, considering the individuals affected, it is difficult to make the distinction whether parasitoids larvae died directly or because their hosts were killed by the insecticide.

Chrysopids showed to be the more tolerant predators to insecticide since their species weight in PRC diagram is next to zero. In addition, several authors reported the tolerance of chrysopids to certain insecticides. In the study of Rodríguez *et al.* (2003), chrysopids, namely the most abundant species in olive grove, *Chrysoperla carnea* Stephens, showed some resistance to deltamethrin. Furthermore, Ruano *et al.* (2001) using the beating technique and Corrales and Campos (2004) using McPhail traps caught more chrysopids in integrated groves treated with dimethoate than in organic olive groves, as occurred in our study in 2002. Stelzl and Devetak (1999) considered that, due to their voracity and tolerance to many insecticides, chrysopids (especially *C. carnea*) seems to

show all the biological and ecological attributes for mass production. Therefore, mass rearing and mass release of chrysopids should become standard methods of biological pest control.

For less abundant groups such as Dermaptera and other Neuroptera, the PRC output should be regarded with caution since abundance of those groups was very low, the impact of insecticide application may be misleading. Although according to PRC diagram they can be classified as tolerant groups more studies are needed to support this idea.

In sum, PRC method showed to be a valuable tool for evaluating effects of management regime at the community level although for groups with few sampled individuals, results must be carefully considered; otherwise the conclusions can be biased.

2.5. Conclusions

The olive management regime influenced the patterns of abundance of arthropod community. The abundance of different trophic groups was reduced after the application of dimethoate to control *P. oleae* in integrated grove. The impact of the pesticide was more pronounced on predaceous mirids, coccinellids and ants. Besides the intrinsic susceptibility of each group, the pattern of the life cycle and the number of generations per year are also ecological characteristics that influence the effect of the insecticide and the capacity to recover.

This study suggested that a timely application will be more effective in the control of the pest. Therefore, some attempt must be done in order to choose the most suitable occasion to do the application allowing also the reduction of the dose of pesticide applied. A more effective control of olive pests can be achieved by increasing the cooperation between farmers, technicians and researchers. The idea is to apply the best management practices in order to reduce negative impacts on biodiversity that provides ecological services such as biological pest control. These services promote the sustainability of agro-ecosystems and enhance nutrient cycling, and water and soil conservation.

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Chapter 3

3. HOW DOES THE TYPE OF AGRICULTURAL MANAGEMENT REGIME USED TO CONTROL PESTS IN THE OLIVE GROVE AFFECT THE COCCINELLID COMMUNITY STRUCTURE AND BIODIVERSITY?

Sónia A. P. Santos, José A. Pereira, Armando Raimundo,
Laura M. Torres & António J. A. Nogueira

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ABSTRACT

In this work we assessed the impacts of two different agricultural management regimes, organic farming (OF) and integrated pest management (IPM), on the coccinellid community structure and biodiversity in two olive groves. The field work was carried out at a weekly basis, in two different olive groves, from April to November of 2002 and 2003 and captured coccinellids were identified to species level. Abundance, species richness, Simpson's diversity index and evenness were used to study the effect of the management regime on the coccinellid community structure. A total of 23 species were identified in the two olive groves. Nine species occurred in both management regimes and in the two years of the study and the five most common were: *Scymnus interruptus*, *Chilocorus bipustulatus*, *Rhyzobius chrysomeloides*, *Pullus subvillosus* and *Mimopullus mediterraneus*. *S. interruptus* was the dominant species in the OF grove with 46.4% of the total Coccinellidae recovered while in the IPM grove *R. chrysomeloides* represented 35.7% of the total captures. Principal response curves (PRC) method was used to analyse the effect of the management regime on the abundance of coccinellid species and showed that *S. interruptus* and *C. bipustulatus* were the most affected species while *M. mediterraneus* slightly increased in abundance in the IPM compared with the OF grove. Richness and Simpson's index were higher in the OF whereas evenness was higher in the IPM grove. Differences in abundance, diversity and species composition reflect a stronger impact of IPM on the coccinellid community structure. The main effect of the management regime was a significant reduction of the abundance of the most common species of the coccinellid community in the IPM grove, which can also have implications on the

preservation of ecological functions associated with coccinellids, namely their role as control agents of olive pests.

Key-words: biodiversity, Coccinellidae, integrated pest management, organic farming, principal response curves, species abundance, species richness.

3.1. Introduction

Generalist insect predators are common in agro-ecosystems. In the olive tree canopy, coccinellids are among the most abundant groups of predators where they can have a potential function on the natural control of pests which often cause several economic losses in the crop yield (Morris *et al.*, 1999; Soares *et al.*, 2005; Santos *et al.*, 2007).

The disturbance of the agricultural systems by several factors can change the abundance and the diversity of coccinellids influencing their ability to suppress the development of pest populations (Altieri, 1999; Obrycki and Kring, 1998). Among the most frequent disturbances occurring in an agro-ecosystem is the use of synthetic and unspecific pesticides to control pests or diseases that can also be toxic to beneficial arthropods (Rodríguez *et al.*, 2003; Ruano *et al.*, 2004; Cardenas *et al.*, 2006; Santos *et al.*, 2007). After the application of a pesticide, the natural control exerted by predators over the pests is broken up either by direct pesticide-induced mortality (Walker *et al.*, 1996) or by lowering the number of preys (Obrycki and Kring 1998).

The toxicity of insecticides such as carbaryl, endosulfan and esfenvalerate (Yardim and Edwards 1998), deltamethrin (Rodríguez *et al.*, 2003) and dimethoate (Santos *et al.*, 2007) to the total abundance of coccinellids has been reported, showing a high susceptibility of this taxon to chemical products. But, in a community, different species have different sensitivities and respond differently to stress in that concern, for instance, the likeliness of exposure and the capacity of recovery (Walker *et al.*, 1996). Thus, the vulnerability of each coccinellid species to insecticides might be a serious constrain to the successful conservation of the coccinellid biodiversity that can help to promote the maintenance of ecological functions like the regulation of pests providing a long-term stability in the agro-ecosystems (Altieri, 1999; Philpott and Armbrecht 2006).

The study of the factors driving the variations in the coccinellid community structure in terms of species composition, relative abundance of each species and population dynamics is essential for the development of rational strategies to protect the olive grove. It is necessary to increase knowledge about the effect of the grove management on the coccinellid community structure. Moreover, comparable measurements of abundance and diversity from different places or times are used to study the effect of

pollutants on communities and can help us to identify when the ecosystem stability is endangered. The aim of this study was to evaluate the effect of two management regimes, organic farming and integrated pest management, on the structure of the coccinellid community in the olive tree canopy.

3.2. Material and Methods

3.2.1. Study sites

Field studies were conducted in 2002 and 2003 at two olive groves near Mirandela (Portugal): Paradela and Valbom-dos-Figos groves. Both olive groves occupied an area of 3 ha, were 10 km from one another, located at similar altitude and with the same environmental conditions.

Valbom-dos-Figos grove (41º 33' 4'' N, 7º 8' 43'' W) has followed the organic growing guidelines since 1991, no phytosanitary treatments were done and the soil was fertilized with organic nutrients two to four times a year.

Paradela grove (41º 32' 38" N, 7º 7' 29" W) has followed the Integrated Pest Management (IPM) guidelines since 2001 and according to farmer's information, a dimethoate spray (150 ml hl^{-1} of the formulation at 42.8% (W/V)) was applied each year against the anthophagous generation of the olive moth, *Prays oleae* (Bernard) on 13 June 2002 and on 16 June 2003; soil was fertilized with organic and mineral nutrients two to four times a year.

The planting density was 10 × 10 meters in Valbom-dos-Figos and 9 × 9 meters in Paradela. For both olive groves, soil was ploughed superficially with a scarifier two to four times a year to control weeds and was not irrigated.

Hereafter, olive groves will be named according their management regime, i.e., Valbom-dos-Figos will be the organic grove (OF) and Paradela the integrated pest management grove (IPM).

3.2.2. Survey of coccinellids

Between April and November of 2002 and 2003, samples of the coccinellid community were taken at approximately a weekly basis. Coccinellids were collected by the beating technique. In each sampling period, five samples per olive grove were collected. Each sample has the beatings of ten branches. All captured individuals were frozen, sorted, identified and counted under binoculars to species level. Species identification was based on external characters but extraction and observation of genitals of some species was needed to confirm the morphological identification. Coccinellid species were identified according Raimundo & Alves (1986) and Raimundo (1992).

3.2.3. Data analysis

Abundance data were evaluated for normality and homogeneity of variances with Kolmogorov-Smirnov test and Bartlett's test, respectively and when necessary, the transformation $\log_{10}(x + 1)$ was used to normalise the data. Total abundance of coccinellids, richness, Simpson's diversity index, evenness and the abundance of the five most common coccinellid species were compared using the following nested design: management regime + year + management regime × year + week (year), using the General Linear Model module of Minitab Statistical Software, release 14 (Minitab Inc. 2003). Thus, we have two management regimes (OF and IPM), two years (2002 and 2003), the interaction between management regime and year, and also the 26 sampling dates each year (nested within years) as source of variation. The main purpose was to detect differences associated with each one of the two factors (management regime and year) while taking in consideration the variability associated with the temporal cycles (obtained by nesting the random factor, sampling date, within year). Significance levels for all analyses were set at $P \leq 0.05$.

The effect of the management regime on the community of coccinellids was evaluated by the principal response curves (PRC) method using Canoco for Windows, Version 4.5 (Ter Braak & Šmilauer 2002). The PRC analysis is derived from the multivariate ordination technique redundancy analysis (RDA) (Van den Brink & Ter Braak 1999; Van den Brink, Van den Brink & Ter Braak 2003). The result of a PRC analysis is a graph that summarizes the effect of the insecticide application on the community (*y*-axis)

over time (*x*-axis) in which, in this case, the IPM grove (treatment) is related to the OF grove (reference). By definition, the reference (OF grove) is zero in every date and the effect of the management regime is quantified, in the *y*-axis, by the basic response pattern or C_{dt} at each sampling period *t*. Thus, the deviation of the treatment (IPM grove) curve *d* compared to the reference (OF grove) is proportional to the effect of insecticide spraying. The C_{dt} graph is combined with the species weight (b_k) that represents the affinity of individual species *k* with the overall-community response (C_{dt}). In this case, a positive species weight value indicates a reduced abundance of the species in the IPM grove, compared to the OF grove, while a species with a negative weight is expected to increase in abundance, relative to OF grove. Species with weights between 0.5 and -0.5 responded differently to the insecticide application, either by showing no response or by showing a response unrelated to the general pattern. The PRC analysis was followed by a Monte Carlo permutation test to test whether the PRC diagram shows a significant part of the variance explained by the application of the insecticide (Van den Brink & Ter Braak 1999). Only those species which were caught in both olive groves and occurred in both years were used in the PRC analysis.

Community composition was investigated by plotting the rank-abundance curves of each management regime, with the relative abundance of each species as its index of abundance. Thus, the relative abundance for the most common species is plotted first, then the next most common, and so on until the array is completed by the rarest species of all (Magurran 2004). The Kolmogorov-Smirnov two-sample test was used to test differences between rank-abundance curves of the two olive groves. It provides a convenient and simple method of comparing two rank-abundance curves.

Coccinellid richness (S), Simpson's diversity index (D) and evenness ($E_{1/D}$) were calculated for each sample. Richness is the number of species present in a sample. Simpson's diversity index, was calculated as 1/D, using the formula

$$1/D = 1/\sum_{i=1}^{s} p_i^2 \qquad \text{eqn 1}$$

where p_i^2 is the proportion of individuals of the *i*th species and S the total number of species. The minimum value of 1/D is 1 which is reached when the community has only a single species and the maximum is S, which is reached when a community has all species

with equal abundance. This index takes into account the number of species present as well as the abundance of each species and provides a good estimate of diversity at relatively small sample sizes and ranks assemblages consistently, i.e., an increase in the index will result in an increase in diversity. Evenness expresses the dominance and was calculated as

$$E_{1/D} = (1/D)/S \qquad \text{eqn 2}$$

$E_{1/D}$ is defined between 0 and 1, where 1 represents a community with perfect evenness, and decreases to zero as the relative abundances of the species diverge from evenness (Magurran 2004).

3.3. Results

A total of 2946 coccinellid individuals belonging to 23 species were collected after the survey of the OF and IPM olive groves in 2002 and 2003 (Table 3.1). Nine species occurred in both management regimes and in the two years of the study: *Chilocorus bipustulatus* (L.), *Exochomus (Exochomus) nigromaculatus* (Goeze), *Scymnus (Pullus) subvillosus* (Goeze), *Scymnus (Mimopullus) mediterraneus* Iablokoff-Khnzorian, *Scymnus (Scymnus) interruptus* (Goeze), *Scymnus (Scymnus) apetzi* Mulsant, *Nephus (Bipunctatus) bisignatus* (Boheman), *Rhyzobius litura* (Fabricius) and *Rhyzobius chrysomeloides* (Herbst). These species accounted for 99.2% and 92.6% of all coccinellids recorded, respectively in the OF and in the IPM groves. The remaining species accounted, respectively, for 0.8% and 7.4% of the total abundance. Four species were found only in the OF grove: *Scymnus (Scymnus) rufipes* (Fabricius), *Rhyzobius lophantae* (Blaisdell), *Coccinella (Coccinella) setempunctata* L. and *Propylaea quatuordecimpunctata* (L.), and five species were collected only in the IPM grove: *Scymnus (Scymnus)* coenosp. *apetzoides* Capra/ Fürsch, *Nephus (Sidis) semirufus* Weise, *Nephus (Sidis) hiekei* Fürsch, *Oenopia doublieri* (Mulsant) and *Subcoccinella vigintiquatuorpunctata* (L.). Except for *S. vigintiquatuorpunctata* all the coccinellid species captured were predators.

Table 3.1. Total abundance of each coccinellid species captured in the organic farming (OF) and in the integrated pest management (IPM) olive groves during 2002 and 2003, n = 130.

Species	OF grove		IPM grove	
	2002	2003	2002	2003
*Platynaspis luteorubra (*Goeze)	4	1	4	
Chilocorus bipustulatus (L.)	298	203	10	9
Exochomus quadripustulatus (L.)	4	1	1	
Exochomus (Exochomus) nigromaculatus (Goeze)	1	13	2	7
Stethorus punctillum Weise		3	22	
Scymnus (Pullus) subvillosus (Goeze)	114	106	34	28
Scymnus (Mimopullus) mediterraneus Iablokoff-Khnzorian	76	36	57	58
Scymnus (Scymnus) interruptus (Goeze)	206	908	21	48
Scymnus (Scymnus) rufipes (Fabricius)		1		
Scymnus (Scymnus) apetzi Mulsant	9	17	7	17
Scymnus (Scymnus) coenosp. *apetzoides* Capra/Fürsch				2
Nephus (Bipunctatus) bisignatus (Boheman)	6	4	2	2
Nephus (Sidis) semirufus Weise			3	
Nephus (Sidis) hiekei Fürsch				2
Nephus (Sidis) helgae Fürsch		1		1
Rhyzobius lophantae (Blaisdell)		1		
Rhyzobius litura Fabricius	9	5	7	1
Rhyzobius chrysomeloides (Herbst)	234	137	135	59
Adalia (Adalia) decempunctata (L.)	1		1	2
Coccinella (Coccinella) setempunctata (L.)	1	1		
Oenopia doublieri (Mulsant)				1
Propylaea quatuordecimpunctacta (L.)		1		
Subcoccinella vigintiquatuorpunctata (L.)			1	
Total abundance	963	1439	307	237
Richness	13	17	15	14

The rank abundance curves showed that the coccinellid community of the IPM grove is dominated by four species and after about the fifth most abundant species, the rank abundance curve lied above the curve for the OF grove (Fig. 3.1). Therefore, the IPM grove showed higher species evenness than the OF grove, where the community is essentially dominated by five species. After the two years, a total of eighteen species were collected in the OF and nineteen in the IPM grove. The species composition of the coccinellid community varied with the management regime and year. In 2002, *C. bipustulatus* dominated the community in the OF grove representing 30.9% of total abundance, followed by *R. chrysomeloides* (24.3%) and *S. interruptus* (21.4%). In the IPM

grove, the community was dominated by *R. chrysomeloides* (44.0%) followed by *M. mediterraneus* (18.6%) and *P. subvillosus* (11.1%). In 2003, *S. interruptus* largely dominated the community in the OF grove representing 63.0% of total abundance, followed by *C. bipustulatus* (14.1%) and *R. chrysomeloides* (9.5%). In the IPM grove, three species with similar abundances dominated the community: *R. chrysomeloides* (24.9%), *M. mediterraneus* (24.5%) and *S. interruptus* (20.3%).

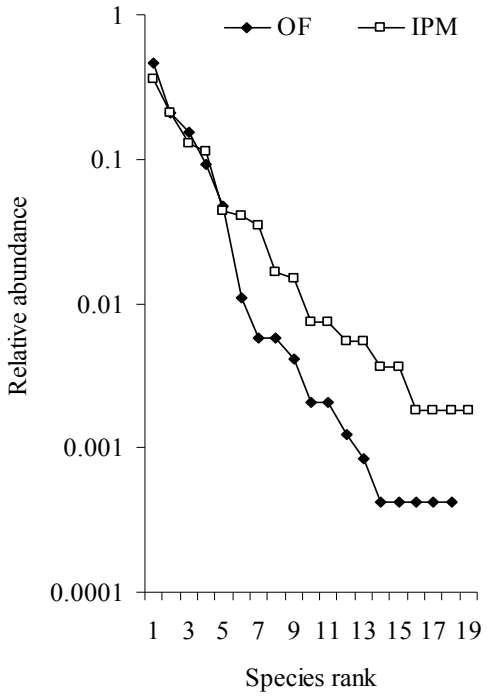

Figure 3.1. Rank abundance curves for the organic farming (OF) and integrated pest management (IPM) olive groves.

The overall coccinellid abundance was significantly higher in the OF, with 2402 individuals, than in the IPM grove, with 544 individuals captured (Table 3.2). PRC diagram shows changes through the two years of study in the variability of coccinellid community. Of the total variance, 29% is explained by the sampling date and 32% by the management regime. The Monte Carlo permutation test showed a significant difference between management regimes and 73% of that difference is displayed by the first PRC axis

(Fig. 3.2). PRC diagram shows small differences between the OF and the IPM groves curves on the sampling dates immediately before each spray. After that, the negative values of C_{dt} for the IPM indicate that the total of captures were lower in this grove than in the OF grove. The effect was even more evident in 2003. Only in October of both years, the values of C_{dt} for the IPM approached the OF grove. *S. interruptus* and *C. bipustulatus* obtained high species weights in the diagram, indicating a reduced abundance in the IPM grove. On the contrary, *M. mediterraneus* obtained a lower negative value, indicating a slightly increase in abundance in the IPM compared with the OF grove.

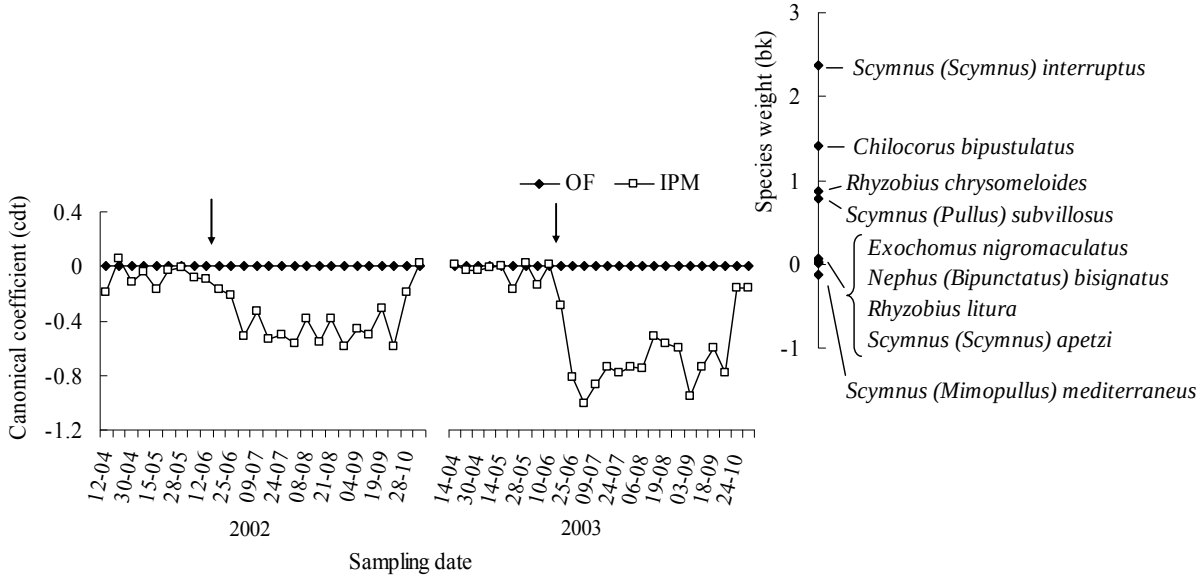

Figure 3.2. Principal response curves diagram and species weight for the most common coccinellid species, showing variation in abundance during 2002 and 2003. Arrows indicate spray dates in the IPM grove.

The changes in abundance of the five most abundant species are shown in Fig. 3.3 and the results of the correspondent nested ANOVAs are summarized in Table 3.2. In general, abundance increased from July till the end of sampling period in the OF grove. However, in the IPM grove, only the abundance of *M. mediterraneus* increased after the spray with dimethoate. Moreover, during summer months of 2003, the IPM grove reached higher abundances than the OF grove. The abundance of *C. bipustulatus*, *P. subvillosus*, *S. interruptus* and *R. chrysomeloides* over the two years was significantly higher in the OF than in the IPM grove, while for *M. mediterraneus* there were no significant differences

between management regimes in any year. Only for *S. interruptus* a significant interaction between management regime and years was observed.

Table 3.2. Statistical output for the nested analysis of variance of the total abundance of coccinellids, Simpson's Index, richness, evenness and of the five species considered to be the most important in the PRC analysis.

	Management regime	Year	Management regime × Year	Week (Year)
Total Abundance	$F_{1, 466} = 234.29$ $P < 0.001$	$F_{1, 466} = 0.46$ $P = 0.500$	$F_{1, 466} = 6.84$ $P = 0.009$	$F_{50, 466} = 5.18$ $P < 0.001$
Chilocorus bipustulatus	$F_{1, 466} = 268.36$ $P < 0.001$	$F_{1, 466} = 1.28$ $P = 0.263$	$F_{1, 466} = 2.74$ $P = 0.098$	$F_{50, 466} = 2.27$ $P < 0.001$
Pullus subvillosus	$F_{1, 466} = 38.58$ $P < 0.001$	$F_{1, 466} = 1.02$ $P = 0.318$	$F_{1, 466} = 3.09$ $P = 0.079$	$F_{50, 466} = 2.62$ $P < 0.001$
Mimopullus mediterraneus	$F_{1, 466} = 0.78$ $P = 0.379$	$F_{1, 466} = 0.07$ $P = 0.785$	$F_{1, 466} = 2.80$ $P = 0.095$	$F_{50, 466} = 4.08$ $P < 0.001$
Scymnus interruptus	$F_{1, 466} = 170.61$ $P < 0.001$	$F_{1, 466} = 12.93$ $P = 0.001$	$F_{1, 466} = 41.65$ $P < 0.001$	$F_{50, 466} = 5.97$ $P < 0.001$
Rhyzobius chrysomeloides	$F_{1, 466} = 34.60$ $P < 0.001$	$F_{1, 466} = 11.54$ $P = 0.001$	$F_{1, 466} = 0.26$ $P = 0.609$	$F_{50, 466} = 1.93$ $P < 0.001$
Richness	$F_{1,466} = 162.49$ $P < 0.001$	$F_{1, 466} = 0.06$ $P = 0.810$	$F_{1, 466} = 0.12$ $P = 0.729$	$F_{50, 466} = 5.68$ $P < 0.001$
Simpson's Index	$F_{1, 466} = 65.90$ $P < 0.001$	$F_{1, 466} = 0.52$ $P = 0.473$	$F_{1, 466} = 3.66$ $P = 0.056$	$F_{50, 466} = 4.36$ $P < 0.001$
Evenness	$F_{1, 466} = 2.51$ $P = 0.114$	$F_{1, 466} = 2.59$ $P = 0.114$	$F_{1, 466} = 2.29$ $P = 0.131$	$F_{50, 466} = 1.20$ $P = 0.175$

In the OF grove, higher values of species richness were registered in summer of both years, namely during the months of July, August and September. However, in the IPM grove, the first peak of species richness was reached in April and again in August of 2002 and in August and September of 2003 (Fig. 3.4). Species richness was significantly higher in the OF (overall mean ± standard error of the mean - SE: 2.55 ± 0.13 in 2002 and 2.64 ± 0.15 in 2003) than in the IPM grove (1.36 ± 0.09 in 2002 and 1.33 ± 0.10 in 2003) (Table 3.2) and that there were no differences between years in species richness.

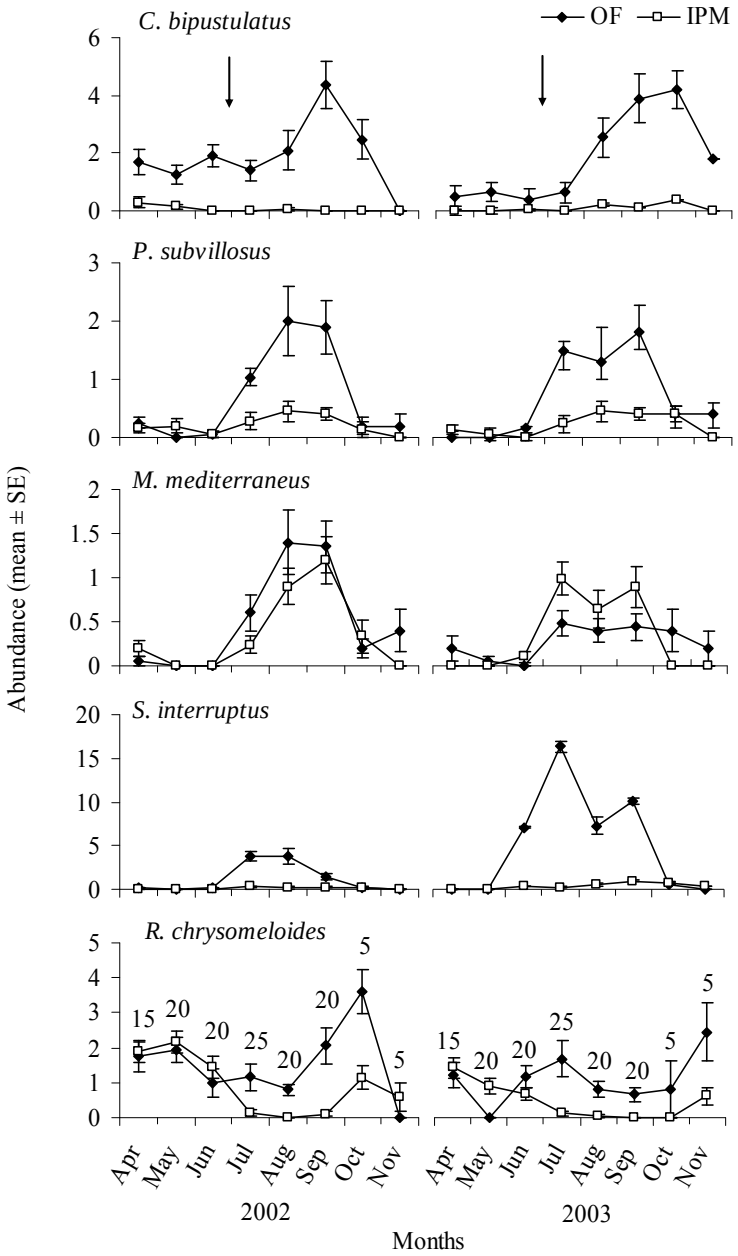

Figure 3.3. Monthly mean abundance (± standard error of the mean - SE) of the species considered to be the most important in the PRC analysis from April to November of 2002 and 2003, in the OF and IPM groves. The numbers above bars represent the number of samples (n) on which the mean is based. Arrows indicate spray dates in the IPM grove. Note different scale of y-axis

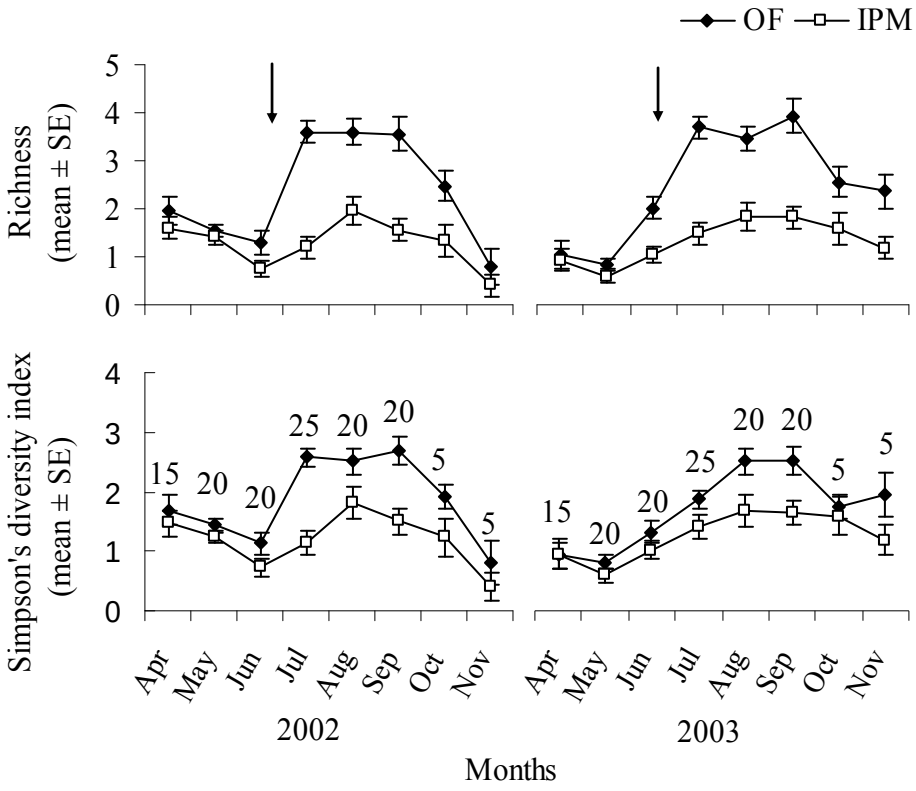

Figure 3.4. Monthly mean Simpson's diversity index (±SE) and richness (±SE) of coccinellid community in the OF and IPM groves throughout 2002 and 2003. The numbers above bars represent the number of samples (n) on which the mean is based. Arrows indicate spray dates in the IPM grove.

The fluctuations of Simpson's diversity index were similar to those obtained for richness. Although, the differences between management regimes observed for Simpson's index were smaller than for richness, which was even more evident in July of 2003 (Fig. 3.4). Simpson's index was significantly higher in OF (2.00 ± 0.09 in 2002 and 1.73 ± 0.09 in 2003) than in IPM grove (1.27 ± 0.08 in 2002 and 1.25 ± 0.09 in 2003) (Table 3.2).

Evenness was higher in the IPM (0.75 ± 0.02) compared with the OF grove (0.74 ± 0.03) in 2002 and in 2003 (0.74 ± 0.04 and 0.65 ± 0.03 respectively). However, no differences were found between management regimes in both years and between years for the same grove (Table 3.2).

3.4. Discussion

The differences found between management regimes indicated that the insecticide application in the IPM grove had a strong impact on the abundance, species composition and diversity of coccinellid community.

Among the 23 coccinellid species identified at the OF and IPM groves, nine species were represented in both olive groves in the two years. From these, *C. bipustulatus*, *P. subvillosus*, *M. mediterraneus*, *S. interruptus* and *R. chrysomeloides* were in general, the most abundant species completing several life cycles and developing important functions in this agro-ecosystem, namely as natural predators of olive pests. Argyriou & Katsoyannos (1977) referred *C. bipustulatus* as an abundant and widely distributed species in Greek olive groves, in a study where *Exochomus quadripustulatus* (L), *P. subvillosus* and *S. apetzi* were also found. Ba M'hamed & Chemseddine (2002) reported *M. mediterraneus* as a common species in the olive groves of the Mediterranean region.

Coccinellid species represented by less than a total of ten individuals appeared mostly in summer causing an increase in the species richness. These species can be considered sporadic and probably do not use the olive tree as a typical habitat. Their appearance may be attributed to the migration from the surrounding field vegetation (weeds and shrubs) and due to their lower abundance they don't have a significant impact in the control of olive pests but are determinant to the species richness.

The community structure apparently changed as a consequence of the management regime, suggesting different susceptibilities of the coccinellid species to the insecticide. After the two years study and considering only the five most abundant species, with the exception of *M. mediterraneus*, all the other species were less abundant in the IPM than in the OF grove. *S. interruptus* and *C. bipustulatus* were the most affected species, followed by *R. chrysomeloides* and *P. subvillosus*. The significant reduction of these species, as it was shown by PRC analysis, can be due to direct toxic effects like the intrinsic susceptibility to the insecticide caused by different detoxifying capacities (Walker *et al.* 1996). In addition, insecticides can affect species indirectly via the depletion of the prey population. This, associated with differences in the mobility of each predator to search for new habitats may influence fecundity and longevity (Obrycki & Kring 1998). Also, different feedings strategies like prey stage selection and the voracity of each species may

induce various exposures to the insecticide (Singh *et al.* 2004). As a consequence, large-sized species (e.g. *C. bipustulatus*) will potentially ingest more preys and hence more insecticide than smaller species. In the IPM grove, the community was reduced to four dominant species, against five in the OF grove, due to the significant decline of *C. bipustulatus*, which is one of the most referred predators of *Saissetia oleae* (Olivier) either in the olive or citrus groves (Limón *et al.* 1976; Argyriou & Katsoyannos 1977). Therefore, the significant reduction of this species from the IPM grove can have negative consequences for the natural control of olive pests, which can increase rapidly resulting in pest outbreaks.

The decrease in the abundance of common species, leaving a niche for other species, can explain the increase of *M. mediterraneus* abundance, which was probably an indirect effect. *M. mediterraneus* is a small-sized species that can be easily predated by other arthropods, including coccinellids, influencing the outcome of interactions between the competitors (Rosenheim *et al.* 1995). Eggs and young larvae, for instance, may be particularly vulnerable to predation. This interaction was already observed among the aphidophagous coccinellid species *Adalia bipunctata* (L.), *A. decempunctata* (L.), *Coccinella septempunctata* L. and *C. undecimpunctata* L. in a laboratory study (Agarwala & Dixon 1992).

Thus, it seems that in the OF grove, *M. mediterraneus* was affected by predation and interspecific competition which repressed the growth of the population. In the IPM grove, the decline in the abundance of potential predators that occurred after the insecticide application may have favoured the increase of *M. mediterraneus* in the community. However, the presence of *M. mediterraneus* in the IPM grove does not automatically mean that this species was insensitive to the insecticide. In fact, Ba M'hamed & Chemseddine (2002) observed a high mortality rate of adults within few hours after dimethoate exposition at recommended doses. Probably, this species had a fast recovery rate either by immigration from the surrounding environment or due to its life history strategy.

The influence of the management regime in the remaining species showed in the PRC diagram is inconclusive because of the few numbers of individuals collected.

In sum, changes in the overall abundance observed in the IPM grove were determined by changes of the most abundant species. The regular disturbances occurring in

this grove may have changed the natural balance between species and direct or indirect effects of the insecticide determined the species composition of the community. Consequently, more sensitive species will be progressively eliminated and the community will be dominated by resilient species. Contrarily, in the OF grove where disturbances were minimal, species composition was determined by the species that were the most effective competitors.

The fluctuation of species richness and Simpson's diversity index throughout sampling dates exhibited several peaks, being the community more diverse during summer in both olive groves. The management regime apparently influenced both richness and diversity since significantly more species and higher diversity indexes at each sampling period were obtained in the OF compared to the IPM grove. However, differences between groves observed for richness were greater than that observed for Simpson's index. This was even more evident in 2003 and in particular in July due to the greatest abundance of *S. interruptus* in the OF grove. In this year, evenness was significantly higher in the IPM grove that showed a more even distribution of individuals among species due to a significant decline of *S. interruptus* and *C. bipustulatus*.

The significant effects observed after the insecticide application on the coccinellid community structure, especially in the abundance of the most common species, can compromise ecological functions like the control of olive pests. Therefore, it is necessary to implement or enhance plant protection practices that encourage the conservation of these natural enemies, which can be achieved by avoiding or strongly reducing insecticide use. In integrated pest management, pesticide applications must be scheduled according to the life history of the beneficial species (coccinellids) and the pest they intend to control. Thus, the conjugation of biological and chemical pest control will reduce harmful effects while pest control will be more effective.

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Chapter 4

4. SEASONAL SYNCHRONY BETWEEN THE PREDATORY COCCINELLID COMMUNITY AND THE PEST SAISSETIA OLEAE *(HEMIPTERA: COCCIDAE) IN TWO OLIVE GROVES UNDER DIFFERENT MANAGEMENT REGIMES*

Sónia A. P. Santos, José A. Pereira, Armando Raimundo,
Laura M. Torres & António J.A. Nogueira

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ABSTRACT

The black scale, *Saissetia oleae*, is one of the main olive pests and coccinellids are among the principal predators that feed scales. The relationship between the temporal abundance of five common coccinellid species (*Chilocorus bipustulatus*, *Scymnus (Pullus) subvillosus*, *Scymnus (Mimopullus) mediterraneus*, *Scymnus (Scymnus) interruptus*, *Rhyzobius chrysomeloides*) in the olive grove and the different phenological stages of *S. oleae* was investigated in two olive groves conducted under different management systems (Integrated Pest Management and Organic Farming) during 2002 and 2003. Coccinellids and black scale were randomly sampled at a fortnightly basis and correlation analyses between the abundance of coccinellid species and the different stages of the pest were carried out. Results showed that the management system influenced the abundance and the fluctuations of the predators' species. In both years and groves, the greatest abundance of coccinellids occurred between June and November, corresponding also with the period of greatest abundance of first and second instar nymphs of *S. oleae*. Significant positive correlations were obtained between the second instar nymph and four out of five coccinellid species, being potentially the most predated stage of the pest. On the contrary, no significant positive correlations were found between third and fourth instar nymphs and coccinellid species. *P. subvillosus* and *S. interruptus* were the coccinellid species that showed a higher number of significant positive correlations with the different stages of the pest indicating their potential as biological agents of *S. oleae*.

Key-words: Coccinellids, *Saissetia oleae***,** predator-prey interactions, natural control, organic farming, integrated pest management.

4.1. Introduction

The black scale, *Saissetia oleae* (Olivier, 1791) (Hemiptera: Coccidae), is a cosmopolitan plant-sucking pest that damages branches and leaves of the olive tree (*Olea europaea*, L.) and causes high losses in yield (Civantos, 1999). In Portugal, the black scale is considered one of the main pests of the olive grove (Pereira, 2004).

Native enemies can have an important role in the natural control of the black scale and their survey and identification are primary steps to recognize their potential for the regulation of the pest. In the olive grove, parasitoids and coccinellids are among the most referred natural enemies of *S. oleae* (Argyriou and Katsoyannos, 1977; Velimirovic, 1994; Ba M'Hamed and Chemseddine, 2001; Ba M'Hamed and Chemseddine, 2002). Parasitoids have been largely studied and inclusively used to control *S. oleae* in successful projects (Orphanides, 1993). However, few studies are known concerning coccinellids. Species like *Chilocorus bipustulatus* L., 1758, *Scymnus* (*Mimopullus) mediterraneus* Iablokoff-Khnzorian, 1972, *Scymnus* (*Pullus) subvillosus* (Goeze, 1777) and *Scymnus* (*Scymnus) interruptus* (Goeze, 1777) are common in olive and citrus groves of the Mediterranean region and have been referred as potential predators of coccids, in particular *S. oleae* (Argyriou and Katsoyannos, 1977; Uygun and Elekçioğlu, 1998; Magro and Hempetinne, 1999; Ba M'Hamed and Chemseddine, 2001; Ba M'Hamed and Chemseddine, 2002). In Portuguese olive groves, coccinellid community showed a high abundance and diversity with more than 20 species identified (Santos, unpublished data) being a potential group of predators to use against *S. oleae*.

Field surveys are useful methods to study the relationships between natural enemies and their preys (Powell *et al*., 1996). In general, predators are in contact with their prey for only a short time and few or any remains are left, what makes difficult to determine predator-prey interactions (Mills, 1997). Therefore, a small number of surveys have been performed to identify predatory coccinellids of *S. oleae*. To overcome this difficulty, a valuable study of the role of predators can be made by statistically correlating their numbers against those of the pest (Kidd and Jervis, 1996). This indirect approach provides an idea about the interaction between the community of coccinellids and the pest, and consequently about which species are potential predators of S. *oleae*. Thus, the aim of this investigation was to study the synchrony between the abundance of five coccinellid species

(*C. bipustulatus*, *M. mediterraneus*, *S. interruptus P. subvillosus* and *Rhyzobius chrysomeloides* (Herbst., 1792)) and the abundance of each phenological stage of *S. oleae* in two olive groves under different management systems, organic farming and integrated pest management, during two consecutive years, 2002 and 2003. The choice of these five species was based on their representativity in the olive grove where they are very frequent and abundant, representing 84-97% of the overall community of coccinellids (Santos, unpublished data).

4.2. Material and Methods

4.2.1. Study areas

Field studies were carried out in two olive groves near Mirandela (Portugal). One grove, Valbom-dos-Figos (41º 33' 4'' N, 7º 8' 43'' W), followed the organic growing guidelines since 1991, no phytosanitary treatments were done and soil was fertilized with organic nutrients two to four times a year. The other grove, Paradela (41º 32' 38" N, 7º 7' 29" W), followed the integrated pest management (IPM) guidelines since 2001. According to farmer's information, a dimethoate spray (150 ml hl^{-1} of the formulation at 42.8% (W/V)) against the anthophagous generation of *Prays oleae* (Bernard) was applied annually in June: in 2002 treatment was applied on 13 June and in 2003 on 16 June; soil was fertilized with organic and mineral nutrients two to four times a year.

The planting density was 10 × 10 meters in Valbom-dos-Figos and 9 × 9 meters in Paradela. Both olive groves occupied an area of 3 ha and were 10 km from one another, soil was ploughed superficially with a scarifier two to four times a year and was not irrigated and trees were pruned every two or three years.

Hereafter, olive groves will be referred according to its management system as organic grove and IPM grove.

4.2.2. Sampling procedure

Sampling was carried out at a fortnightly basis, between April and November of two consecutive years: 2002 and 2003.

For *S. oleae* sampling, five samples were randomly collected in both olive groves. Each sample derived from ten randomly selected trees where four branches (one branch per orientation) with four leaves were sampled giving a total of 160 leaves per sample. All phenological stages (first, second, third and fourth instar nymphs and mature females) were sorted and counted under binoculars.

Coccinellids were collected by the beating technique. In each sampling period, five samples per olive grove were collected. Each sample has the beatings of ten branches randomly selected. Both adults and larval stages captured at each sampling period were sorted and counted under binoculars. Adult coccinellid species were identified according Raimundo and Alves (1986) and Raimundo (1992).

4.2.3. Data analysis

Univariate statistical analyses were performed using the Statistica Statistical package, version 7.0 (StatSoft, 2004). Data were evaluated for normality and homogeneity of variances with Kolmogorov-Smirnov test and Bartlett's test, respectively and the transformation $\log_{10}(x + 1)$ was used to normalise the data. The abundances of *S. oleae* and of adults and larvae of coccinellids caught in both olive groves over different times were compared by repeated measures analysis of variance (Zar, 1996). One-way ANOVA was used to compare mean abundances between years in each olive grove.

Pearson's correlation coefficients between the abundance of coccinellid species and the abundance of phenological stages of *S. oleae* were determined in order to investigate the interaction between predators and pest fluctuations. This was carried out for each olive grove on the total of the two years studied.

Correspondence analyses (CA) were performed with Canoco for Windows, Version 4.5 (ter Braak and Šmilauer 2002). CA is an unconstrained multivariate method adapted to species abundance data (Legendre and Legendre, 1998) and was used to look for similarity in distributions of relative abundance of the species, measured by their Chi-square distance. The species' point in the CA diagram is at the centroid of the samples where it occurs and, consequently points in proximity correspond to species often occurring together.

4.3. Results

4.3.1. Fluctuations of S. oleae *population*

A total of 52851 and 54645 specimens of the different phenological stages of *S. oleae* were collected, respectively, in the organic and IPM grove during the two years of study. Repeated measures ANOVA showed that the total abundance of *S. oleae* was similar in both olive groves in 2002 ($F_{1, 8} = 1.25$, $P > 0.05$) and in 2003 ($F_{1, 8} = 0.02$, $P > 0.05$). Considering year-to-year data, one way-ANOVA showed that there were no significant differences within the organic ($F_{1, 143} = 1.01$, $P > 0.05$) and the IPM grove ($F_{1, 138} = 0.37$, $P > 0.05$).

The patterns of abundance of the different phenological stages of *S. oleae* were similar in both olive groves. The first instar nymphs were abundant from early July to middle of August, reaching the peak of abundance at the end of July of both years. The second instar nymphs occurred mainly from the end of July to the end of the sampling period. The peak of abundance was reached at the end of August in both years. In 2002, the third instar nymphs were abundant mainly in April and again in November, in the organic grove. In the IPM grove, abundance peaked essentially in November. In 2003, the pattern of abundance of this stage was slightly different. In the organic grove, it peaked in the middle of April and then at the end of July. In the IPM grove, it peaked at the end of July 2003. In 2002, fourth instar nymphs occurred during the spring and at the end of October and early November, reaching its maximum abundance in November, in both groves. In 2003, this stage was abundant in April and May. In 2002, mature females reached its maximum abundance in the middle of June in both groves. In 2003, the peak was reached in the middle of May also in both groves that was earlier than in 2002 (Fig. 4.1).

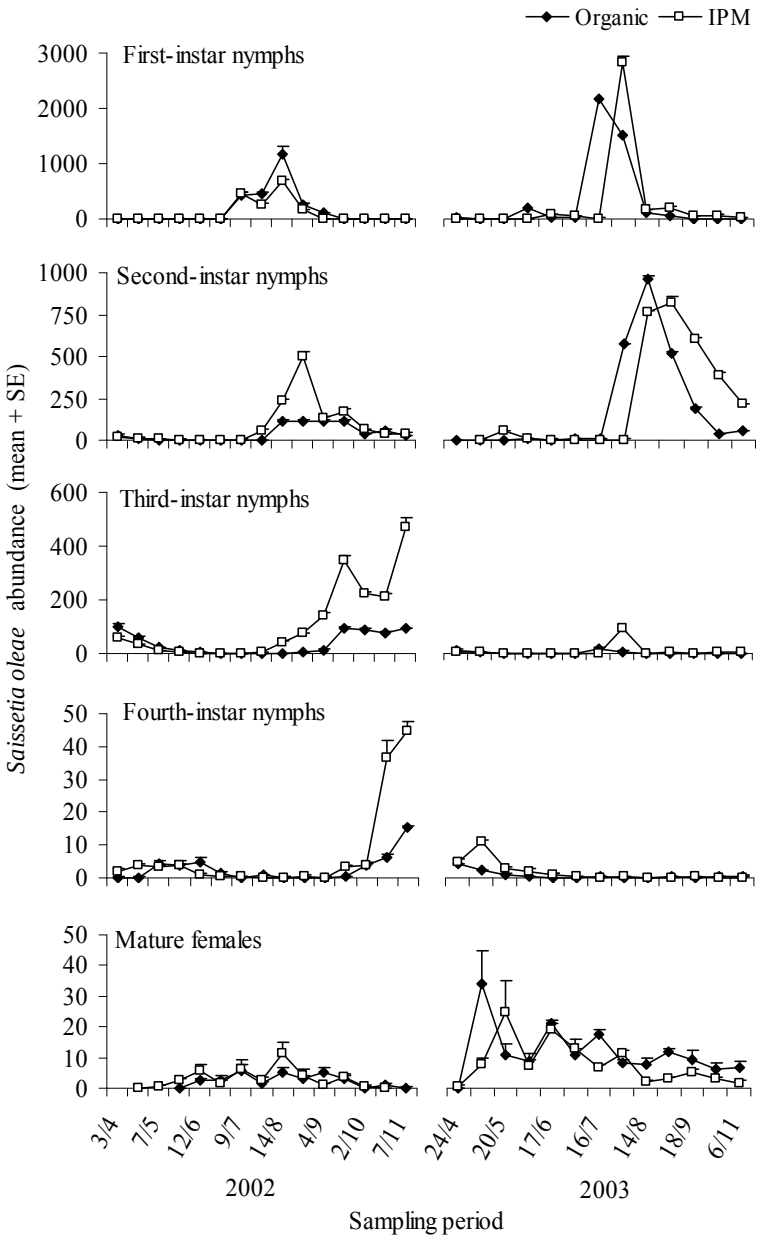

Figure 4.1. Fluctuations (mean + SE) of the different phenological stages of *Saissetia oleae* during 2002 and 2003, in the organic and IPM groves (n = 5). Note different scale of y-axis.

4.3.2. Fluctuations of coccinellid species

A total of 1302 adult coccinellids and 269 larvae were collected in the organic grove and 223 adults and 125 larvae in the IPM grove during the two years of study. Significant differences between groves were found for adults ($F_{1, 8}$ = 76.86, $P < 0.001$ in 2002 and $F_{1, 8}$ = 178.71, $P < 0.001$ in 2003) and larvae ($F_{1, 8}$ = 8.42, $P < 0.05$ in 2002 and

$F_{1, 8} = 35.2$, $P < 0.001$ in 2003). But, there were no significant differences between years in the organic ($F_{1, 143} = 0.79$, $P > 0.05$ for adults and $F_{1, 143} = 2.56$, $P > 0.05$ for larvae) and in the IPM grove ($F_{1, 138} = 0.23$, $P > 0.05$ for adults and $F_{1, 138} = 0.03$, $P > 0.05$ for larvae).

In the organic grove, the total of adults peaked from the end of August to October of 2002, but in 2003 the peaked abundance was reached earlier, from the middle of June to the end of July. Larvae had two main periods of abundance, the first occurred in April and the second occurred during August and September of both years (Fig. 4.2). In the IPM, the first peak of abundance of adult coccinellids was reached in April and May 2002. However, after the insecticide application in June, a decrease in the abundance was observed and only from the middle of August to the end of sampling period a slight recovery occurred, although abundance remained low in comparison with the organic grove. Larvae abundance was high in April and May 2002, but after that, it decreased and no recovery was observed. During 2003, four small peaks of abundance were observed. The first occurred in April, the second in June, the third in August and the last, in September.

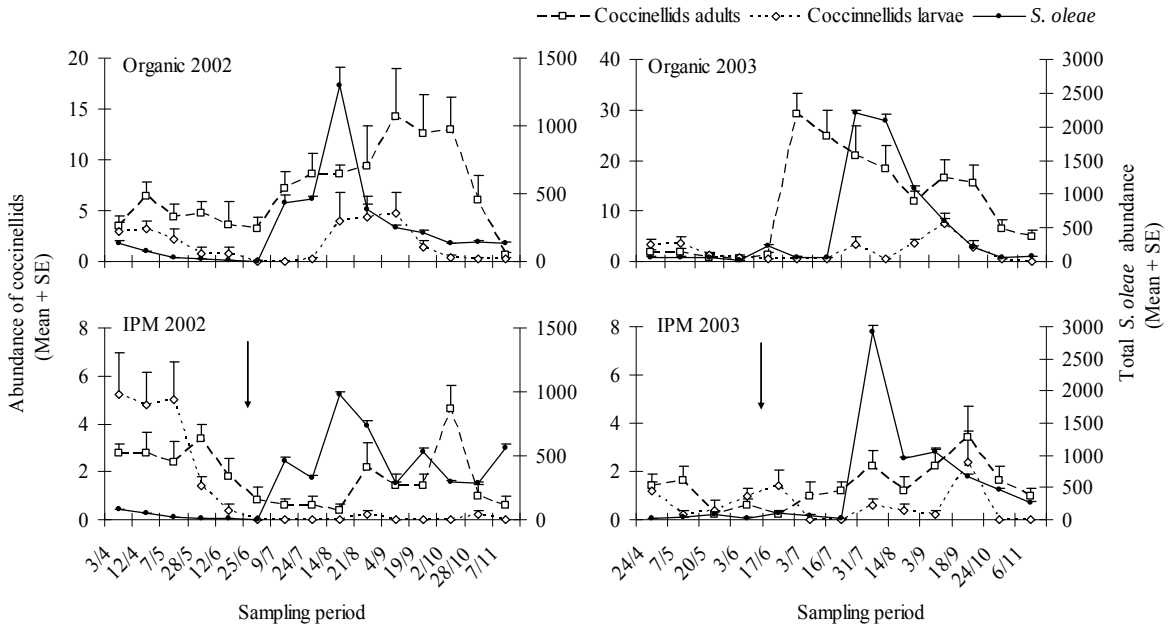

Figure 4.2. Abundance (mean + SE) of total specimens of *Saissetia oleae* and coccinellids (larvae and adult) collected in each sampling date in the organic and IPM olive groves during 2002 and 2003 (n = 5). Arrows indicate the date of the spray application in the IPM grove. Note different scale of y-axis.

The patterns of abundance of coccinellid species were slightly different in the groves studied. In the organic grove, three main peaks of abundance were registered during the year, with a variable dominance of each species in the community (Fig. 4.3). The first peak occurred from April to the end of May and *R. chrysomeloides* dominated the community followed by *C. bipustulatus*. In this period the other species were less abundant. The second peak occurred from the middle of June to the middle of August with the dominance of *S. interruptus*. The last period was dominated by *C. bipustulatus* and occurred from the end of August to the end of October.

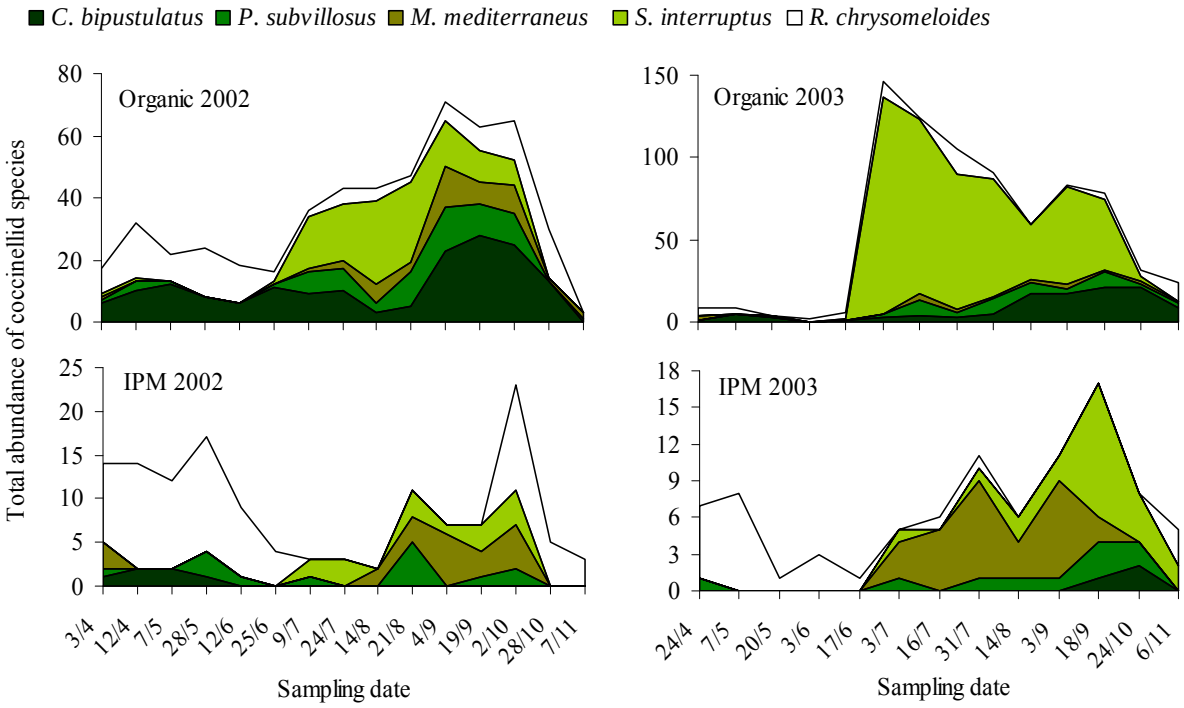

Figure 4.3. Total abundance of coccinellids species during 2002 and 2003, in the organic and IPM olive groves. Note different scale of y-axis.

In the IPM grove, the dominance and the fluctuations of the coccinellid species were different when compared with the organic grove. *R. chrysomeloides* dominated the community in spring and in autumn. *C. bipustulatus* showed only one annual peak of abundance which occurred from April to middle of May in 2002 and from middle of September to the end of October in 2003. The other three species were more abundant from early July to the middle of October of both years and apparently, presented three

short consecutive generations during summer when *M. mediterraneus* dominated the community (Fig. 4.3).

4.3.3. Interactions between coccinellids and S. oleae

In the organic grove, the increase of the abundance of total coccinellids at the end of June coincided with the increase in abundance of the first instar nymphs (Fig. 4.2). Significant positive correlations were found between *P. subvillosus* and *S. interruptus* and the first instar nymphs of *S. oleae*. In early September, the abundance of coccinellids reached a new peak, which coincided with the increase of the number of the second instar nymphs of the phytophagous. Significant positive correlations were observed between four species of coccinellids (which were in decreasing order of significance *P. subvillosus*, *C. bipustulatus*, *M. mediterraneus* and *S. interruptus*) and the second instar nymphs of *S. oleae*. A significant positive correlation was also found between coccinellid larvae and the second instar nymphs of the black scale. On the other hand, no significant positive correlations were found between coccinellid and the third and fourth instar nymphs of the pest (Table 4.1).

Table 4.1. Values for Pearson's correlations (*r*) between coccinellid species and different phenological stages of *S. oleae* in the organic grove during 2002 and 2003 (n=29).

	First-instar	Second-instar	Third-instar	Fourth-instar	Mature females
C. bipustulatus	-0.112 $^{n.s.}$	0.454 *	-0.023 $^{n.s.}$	-0.309 $^{n.s.}$	0.013 $^{n.s.}$
P. subvillosus	0.475 **	0.695 ***	-0.056 $^{n.s.}$	-0.558 **	0.263 $^{n.s.}$
M. mediterraneus	0.330 $^{n.s.}$	0.595 ***	0.199 $^{n.s.}$	-0.275 $^{n.s.}$	0.048 $^{n.s.}$
S. interruptus	0.708 ***	0.397 *	-0.359 $^{n.s.}$	-0.604 ***	0.610 ***
R. chrysomeloides	-0.063 $^{n.s.}$	-0.088 $^{n.s.}$	0.325 $^{n.s.}$	0.040 $^{n.s.}$	-0.241 $^{n.s.}$
Total coccinellids	0.487 **	0.600 **	-0.169 $^{n.s.}$	-0.574 **	0.400 *
Larvae	0.274 $^{n.s.}$	0.403 *	0.172 $^{n.s.}$	-0.154 $^{n.s.}$	0.111 $^{n.s.}$

n.s.- non significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

In the IPM grove, the peak of abundance of both total coccinellid adults and larvae occurred in April, when the third and fourth instar nymphs of *S. oleae* were the dominant stages and significant positive correlations were found between *R. chrysomeloides* and the fourth instar nymph of the black scale (Table 4.2). A second peak of coccinellids abundance was reached in September and October and coincided with the occurrence of the second instar nymphs of *S. oleae* and significant positive correlations were observed between three coccinellid species and that stage of the pest, respectively by decreasing order of significance, *S. interruptus*, *P. subvillosus* and *M. mediterraneus*. Similarly to organic grove, also the second instar nymphs of *S. oleae* was the stage that presented more significant positive correlation with three out of five coccinellid species (Table 4.2).

Table 4.2. Values for Pearson's correlations (r) between coccinellid species and different phenological stages of *S. oleae* in the IPM grove during 2002 and 2003 (n=28).

	First-instar	Second-instar	Third-instar	Fourth-instar	Mature females
C. bipustulatus	-0.031 $^{n.s.}$	0.248 $^{n.s.}$	-0.049 $^{n.s.}$	-0.111 $^{n.s.}$	-0.202 $^{n.s.}$
P. subvillosus	0.354 $^{n.s.}$	0.543 **	0.268 $^{n.s.}$	-0.299 $^{n.s.}$	0.118 $^{n.s.}$
M. mediterraneus	0.433 *	0.439 *	0.304 $^{n.s.}$	-0.400 *	0.173 $^{n.s.}$
S. interruptus	0.487 **	0.578 **	-0.158 $^{n.s.}$	-0.459 *	0.175 $^{n.s.}$
R. chrysomeloides	-0.625 ***	-0.636 ***	-0.082 $^{n.s.}$	0.407 *	-0.239 $^{n.s.}$
Total coccinellids	0.017 $^{n.s.}$	0.097 $^{n.s.}$	0.084 $^{n.s.}$	-0.018 $^{n.s.}$	0.009 $^{n.s.}$
Larvae	-0.332 $^{n.s.}$	-0.334 $^{n.s.}$	-0.214 $^{n.s.}$	0.068 $^{n.s.}$	0.023 $^{n.s.}$

n.s. non significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

The associations between coccinellid species and nymphal stages of *S. oleae* are shown in the ordination diagram (Fig. 4.4). For both olive groves, the first two axes of the CA explained 60% of the variance within species. The proximity among points displayed by the ordination diagram suggests an association between *S. interruptus*, *P. subvillosus*, *M. mediterraneus* and the first and second instars nymphs of *S. oleae* while *C. bipustulatus* showed a closer association between the second and the third instars nymphs in the organic grove than in the IPM grove.

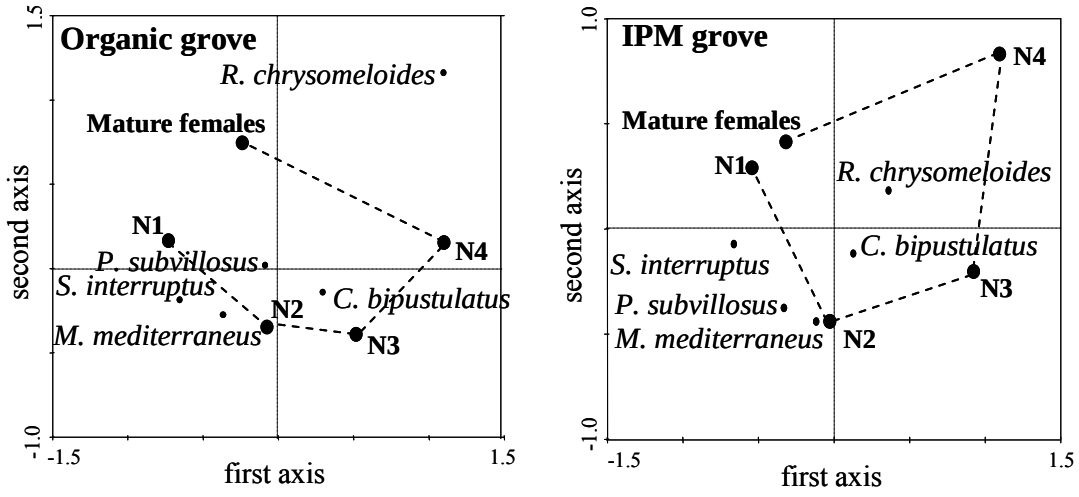

Figure 4.4. Correspondence analysis (CA) ordination diagram for coccinellids species and different phenological stages of *Saissetia oleae* in organic grove (Total inertia = 0.759, axis 1 eigenvalue = 0.313, axis 2 eigenvalue = 0.137) and in the IPM grove (Total inertia = 0.904, axis 1 eigenvalue = 0.342, axis 2 eigenvalue = 0.197).

4.4. Discussion

From this study, several significant positive correlations were found between some coccinellid species and *S. oleae*, indicating the existence of seasonal synchrony between predators and pest. Thus, *S. interruptus* presented the larger number of positive correlations with the black scale namely, with the first and second instar nymphs and the mature females. However, the correlation between *S. interruptus* and mature females of *S. oleae* does not reflect a clear functional association between them because the predation pressure on mature females with eggs is unlikely to occur due to the hardness of the female integument that protect the eggs. Instead, some predation might be exerted on recently emerged nymphs. The period of higher abundance of *P. subvillosus* coincided with that of the first and second instar nymphs. Furthermore, *C. bipustulatus* abundance correlated well with the number of the second instar nymphs of *S. oleae* and appears clearly associated with stages N1 and N2 in both groves. Also, our data suggest that coccinellid larvae can be important predators principally of the second instar nymphs of the pest. Apparently, immature stages of *S. oleae*, with predominance of the first and second nymph, were potentially the most predated. The prevalence of the second instar nymphs during the end of summer is of great interest for the natural control of this pest because (1) this stage is

very sensitive to predators action (Passos-Carvalho *et al*., 2003) and (2) at this time the abundance and diversity of coccinellids is high, making the suppression of *S. oleae* more efficient (Snyder *et al*., 2006). On the contrary, the third and the fourth instar nymphs were probably the least predated stages in part because their peak abundances coincide with the winter period when the abundance of the predators is reduced.

The significant decline in the abundance of the first and second instars nymphs of *S. oleae* which occurred from August to the end of sampling period, in both olive groves and years can be attributed to the action of factors like high temperatures that normally occur during summer (Stratopoulou and Kapatos, 1990; Pucci *et al*., 1986; Fernandez *et al*., 1979), parasitism (Stratopoulou and Kapatos, 1998; Pereira, 2004) and predation (Argyriou and Katsoyannos, 1977). The predators' action is the less studied aspect and little information is known about the potential predation exerted by coccinellids on *S. oleae*. Argyriou and Katsoyannos (1977) referred that *C. bipustulatus* and *P. subvillosus* were species commonly found in Greek olive groves and both were successfully bred on *S. oleae*, in the laboratory. Also, Ba M'Hamed and Chemseddine (2001) showed that *M. mediterraneus* was able to complete its life cycle consuming a large amount of *S. oleae* eggs.

The management practices carried out in the studied groves has apparently influenced in different ways the pest and the predators. It seems that *S. oleae* was not influenced by the management regime probably because mature females, the dominant stage in June, were especially tolerant to insecticide sprays (Passos-Carvalho *et al*., 2003). In addition, the fluctuations of the pest population were similar in both olive groves. However, it seems that the abundance of coccinellid species was influenced by the management regime and shorter generations apparently occurred during both years in the IPM than in the organic grove, which can be seen as an ability of different populations to rapidly increase in abundance and respond to a disturbance (Begon *et al*., 1996). Also, the synchrony between the predators and the pest seems to be affected since the number of positive correlations obtained in the IPM grove was lower than in organic grove. The application of insecticides causes the disruption of the population of coccinellids and consequently, diminishes their potential for controlling the black-scale populations.

The efficiency of predators will increase if seasonal synchrony with the pest exists. However, a significant positive correlation between their populations does not mean necessarily that the predators have impact on the control of the pest. It may only indicate that the predators rapidly respond to the variation of the pest numbers and that a great coexistence between predators and pest exists, but the predation rate can be low and consequently, inefficient to control the pest; on the other hand, significant negative correlations may indicate that the predators slowly respond to the variation of pest population and consequently, an asynchronism between predators and pest exists, but predation rate can be high. Alternatively, the two populations may show no correlation in time, but this not necessarily means that predators had no impact because the combination of positive and negative associations between guilds of predators and their prey may result in the appearance of no correlation at all (Kidd and Jervis, 1996).

4.5. Conclusions

This study showed that the patterns of abundance of the pest were similar between groves. However, the patterns of abundance of coccinellids vary between groves. The two groves were dominated by different species of coccinellids, in particular after pesticide application in the IPM regime.

In both olive groves the coexistence between coccinellids and *S. oleae* is likely to reflect predator-prey relationships, already referred by other researchers. This relationship is fairly consistent between groves: *S. interruptus*, *P. subvillosus*, *P. mediterraneus* are associated with stages N1 and N2 of *S. oleae*, while *C. bipustulatus* is associated with stages N2 and N3. However, further studies need to be carried out, under controlled conditions, to assess if the synchrony between the predator species and the different phenological stages of the prey implies predation or is merely coincidental.

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Chapter 5

5. IDENTIFICATION OF PREDATOR-PREY RELATIONSHIPS BETWEEN COCCINELLIDS AND SAISSETIA OLEAE (HEMIPTERA: COCCIDAE), IN OLIVE GROVES, USING AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Sónia A.P. Santos, José A. Pereira, Maria da Conceição Rodrigues,
Laura M. Torres, Ana Maria N. Pereira & António J.A. Nogueira

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ABSTRACT

A polyclonal antiserum was developed and characterized for the detection of immature stages of the black-scale, *Saissetia oleae*, in whole body homogenized field-collected coccinellid species, using an indirect enzyme-linked immunosorbent assay (ELISA). The indirect ELISA showed to be sensitive to the *S. oleae* antiserum, detecting a protein content between 0.118 and 0.0374 μg mL^{-1}. The specificity of the ELISA was tested by assaying a range of sympatric predators and alternative preys with the *S. oleae* antiserum. Coccinellid larvae obtained the highest cross-reaction and a positive-negative threshold was established at 0.674 μg mL^{-1} protein. A total of 1322 coccinellids were field-collected in three olive groves located in Trás-os-Montes (north-east of Portugal) by the beating technique and were analyzed to detect *S. oleae* proteins in their guts. Field collected coccinellids which attained a *S. oleae* protein concentration equivalent higher than the threshold were considered as a positive reaction. In the overall collected coccinellids, 21.2% reacted positively with the *S. oleae* antiserum. *Chilocorus bipustulatus* and coccinellid larvae obtained the highest percentages of positives with 43.4 and 40.8%, respectively. The greatest frequency of positive responses occurred in the beginning of July, in the mid-August and in the mid-October coinciding with the occurrence of the first, second and third instar nymphs of *S. oleae*, respectively. Thus, in this study, the role of coccinellids as natural control agents of *S. oleae* was highlighted by the number of individuals and species that tested positives for *S. oleae* antiserum.

Key-words: *Saissetia oleae*; *Chilocorus bipustulatus*; *Scymnus (Pullus) subvillosus*; *Scymnus (Scymnus) interruptus*; *Scymnus (Mimopullus) mediterraneus*; *Rhyzobius*

chrysomeloides Coccinellids, ELISA, Gut content analysis, Polyclonal antiserum, Predation.

5.1. Introduction

Saissetia oleae (Olivier, 1791) (Hemiptera: Coccidae) is an important pest of olive groves in Trás-os-Montes region (north-east of Portugal) where it often causes significant economic losses in the crop yield (Pereira, 2004). Presently, the control of the pest is based exclusively on the use of chemical insecticides. However, is desirable to reduce the use of these products due to their environmental and human health risks (Boller *et al.*, 2004) and also due to the probability of development of resistance to insecticides within the pest population (Hawkes *et al.*, 2005). This goal can be achieved by improving the use of natural enemies. Predator-prey interactions are central processes controlling changes in pest populations in an agroecosystem like the olive grove (Naranjo and Hagler, 2001; Symondson, 2002). 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 *S. oleae* (Argyriou and Katsoyannos, 1977; Santos *et al.*, unpublished data).

Unlike parasitism or disease, insect predation is usually difficult to observe directly in the field. Consequently, analytical methods must often be used to assess prey consumption (Sopp *et al.*, 1992). Serological arthropod gut analyses, such as the enzyme-linked immunosorbent assay (ELISA), have been widely used to investigate predation on invertebrates offering a rapid identification of the remains of specific prey in the guts of predators (Fichter and Stephen, 1981; Sunderland *et al.*, 1987; Sopp *et al.*, 1992; McIver and Tempelis, 1993; Symondson and Liddell, 1993; Hagler *et al.*, 1997; Hagler and Naranjo, 1997; Symondson *et al.*, 1996; Morris *et al.*, 1999). Serological tests were successfully used in olive groves to study the natural enemies of the olive moth, *Prays oleae* (Bernard), whose main predators were found to be Formicidae, Heteroptera and Coleoptera (Morris *et al.*, 1999; Lozano *et al.*, 2002).

Serological methods are commonly used in ecological research because of their sensitivity, specificity and also because they are rapid enough to facilitate the test of a large number of predators in a short period (Naranjo and Hagler, 2001; Symondson and

Hemingway, 2002). A polyclonal antiserum can be prepared with the selected prey species and reacted against a variety of field-caught predators, to test whether those predators feed naturally on the pest species and to determine what proportion of predator individuals contain prey remains.

Previous studies have shown that some predatory coccinellid species (*Chilocorus bipustulatus* (L.), *Scymnus (Pullus) subvillosus* (Goeze), *Scymnus (Mimopullus) mediterraneus* Iablokoff-Khnzorian, *Scymnus (Scymnus) interruptus* (Goeze), and *Rhyzobius chrysomeloides* (Herbst)) were seasonally correlated with different nymphal stages of the black-scale (Santos *et al.*, unpublished data). In olive groves this coexistence between coccinellids and *S. oleae* is likely to reflect predator-prey relationships. Thus, it is necessary to assess if the synchrony between the predator species and the different phenological stages of the potential prey implies predation or is merely coincidental.

The main purposes of this work are: (1) develop and characterize a polyclonal antiserum against the black-scale, *S. oleae*, (2) identify the most important coccinellid species that predates this pest in the field, using an indirect – ELISA.

5.2. Materials and Methods

5.2.1. Antiserum production

The immunogen was prepared by homogenizing 0.85 g of immature stages (N1, N2, N3 and N4) of *S. oleae*, collected from the olive tree (*Olea europaea* L.), in 8.5 mL of ½ phosphate buffered saline (PBS, pH 7.4). The homogenate was centrifuged at 4000 rpm for 15 min and the supernatant transferred to a clean centrifuge tube and centrifuged at 10000 rpm for 15 min. A final protein concentration of 37.4 mg mL^{-1} was measured by the Lowry direct procedure (BioRad DC Protein Assay, BioRad Laboratories Gmbh, Munich, Germany). The immunogen was stored in aliquots at –20 ºC until immunization. One week before the first immunization, a six month old rabbit was bled to take the pre-immunization serum (NRS - non-reactive rabbit serum). The first immunization was applied with one intramuscular and three subcutaneous injections with a mixture of 0.5 mL antigen solution (0.1 mL immunogen homogenate and 0.4 mL of ½ PBS) and 0.5 mL Freund's Complete Adjuvant. Additional injections were given 14, 28, and 126 days after the first, using

Freund's incomplete adjuvant. Antiserum (AS) was firstly collected 42 days after the first immunization and then weekly till 154 days after the initial injection. After clotting, the serum was separated by centrifugation at 4 000 rpm for 15 min and then at 10 000 rpm for 15 min. A bulk of antiserum was prepared and stored in aliquots at –20ºC for subsequent use.

Optimum concentrations of antigen, NRS and AS were established, by preliminary checkerboard titrations of reagents, at 1:20000, 1:1000 and 1:1000 respectively.

5.2.2. Antiserum characterization

The detection of *S. oleae* antigens was performed by indirect enzyme-linked immunosorbent assay (ELISA) following the general protocol described by Symondson and Liddell (1993).

The detection limit was established relative to total proteins in the *S. oleae* standard dilutions. All indirect ELISAs were performed in disposable flat-bottomed 96-well microassay plastic plates (Sarstedt Inc, Newton, USA). Two ELISA plates were coated with a × 3.16 dilution series of nine *S. oleae* standards, from $1{:}10^4$ to $1{:}10^8$ resulting in antigen concentrations from 3.74 μg mL^{-1} to 0.374 ng mL^{-1}, as well as two negative controls with PBS. Plates were left to incubated overnight at 4ºC to allow the antigen binding and then washed three times with PBS-Tween 20 (0.05%) (PBST). A 250 μL aliquot of non-fat milk in PBST (4% w/v) was added to each well for 30 min and incubated at 37ºC to block any unoccupied antigenic sites. The blocking solution was emptied and 200 μL of NRS was added to the odd rows of each ELISA plate. In parallel, 200 μL of AS was added to the even rows of each ELISA plate. Plates were incubated for 2 h and then washed three times with PBST and 200 μL of goat anti-rabbit IgG conjugated with the enzyme horseradish peroxidase (Sigma-Aldrich, Chemie Gmbh, Steinheim, Germany) diluted 1:5000 in PBST was applied to each well and incubated at room temperature for 2 h. The plates were washed three times with PBST and 200 μL of the enzyme substrate, orthophenylenediamine (OPD) (Sigma-Aldrich, Chemie Gmbh, Steinheim, Germany) in citrate-phosphate buffer was applied to each well. After 30 min in the dark, the reaction was stopped with 50 μL of sulphuric acid 2.5 M and the absorbance of each well was measured in a microplate reader Multiskan Ex (Labsystems, Finland) at 492 nm. The

detection limit was conventionally established at AS ≥ 2 × NRS (Symondson and Liddell, 1993).

In order to detect cross-reactivity, the antiserum was ELISA tested as described above, against a large range of starved arthropods, including non-target prey and predators, at a final dilution of 1:20000 (w/v) in PBS. Arthropods were captured in the field and starved for 7 days at 22ºC under a 16:8 L:D regime to clean gut and then frozen as negative predator controls (antigen-free). Subtracting the NRS absorbance to the AS absorbance of each sample the background effect was eliminated.

In addition, ten specimens of the five commonest coccinellid species in the olive grove, namely *C. bipustulatus*, *P. subvillosus*, *S. interruptus*, *M. mediterraneus*, and *R. chrysomeloides*, and ten coccinellid larvae were placed in individual Petri dishes (9 cm diameter x 2 cm height) with moistened filter paper covering the bottom of the box and fed *ad libitum* on *S. oleae* nymphs after starving seven days. Coccinellids were monitored and after a feeding period were immediately frozen at -20ºC, to be used as positive predator controls.

5.2.3. Detection within field caught predators

Coccinellids species were collected in three different olive groves near Mirandela (north-east of Portugal): Valbom-dos-Figos (41º 33' 4'' N, 7º 8' 43'' W) that followed the organic farming guidelines, Paradela I (41º 32' 38" N, 7º 7' 29" W) and Paradela II (41º 32' 45" N, 7º 7' 19" W) that were conducted under integrated pest management (IPM). All the olive groves were considered heavily infested by *S. oleae,* because an average of more than four adult females per branch was found – the economic threshold established for *S. oleae* (Gomes and Cavaco, 2003) – after an inspection, in the beginning of the sampling period, of a random sample of 100 branches with 40 cm per olive grove. The information about the occurrence of the different phenological stages of *S. oleae*, showed in Figure 3, was based on previous studies (Pereira, 2004; Santos *et al.*, unpublished data).

Sampling was carried out weekly, between July and October of 2005. This period comprises the period of maximum abundance of both coccinellid species and *S. oleae* nymphs (Pereira, 2004; Santos *et al.*, 2007). Adults and larvae of coccinellids were

collected by the beating technique. In every sampling period, fifty branches per olive grove were randomly beaten and coccinellids collected were immediately transferred to Eppendorf tubes on ice to diminish the activity. In the laboratory, specimens were identified to species according Raimundo and Alves (1986) and Raimundo (1992) and then frozen at – 20ºC prior to analysis.

To prepare coccinellids for the indirect-ELISA, each specimen was washed for approximately 10 s in a PBS bath in order to remove small particles from the body surface, dried, weight and ground with PBS at the standard dilution of 1:20000. The homogenate was centrifuged for 15 min at 10000 rpm and used in duplicate wells to coat an ELISA microplate following the same procedure described above. All ELISA plates incorporated a dilution series of *S. oleae* protein standards.

5.2.4. Establishment of positive-negative thresholds

The concentration of *S. oleae* proteins for an unknown sample (e.g. starved arthropods or field collected predators) can be calculated using the regression equation derived from the dilution series of known standards on each ELISA plate converted to Ln, according equation (1).

$$\text{Ln}\,(AS_{absorbance} - NRS_{absorbance}) = a + b \times \text{Ln}\ S.\ oleae \text{ protein concentration} \quad (1)$$

where a is the Y-axis intercept and b is the regression coefficient. The *S. oleae* protein concentration equivalents (CE) of unknown samples can be calculated entering the values obtained for absorbance into the regression equation (Symondson and Liddell, 1993), as showed in equation (2).

$$CE\,(\mu g.mL^{-1}) = e^{\left(\frac{\ln(AS_{absorbance} - NRS_{absorbance}) - a}{b}\right)} \quad (2)$$

The threshold for the detection of positives was set using two different approaches. A threshold value was set at the mean plus 2.5 × standard deviation (SD) of the calculated CE recorded for the arthropod species giving the strongest cross-reaction, according Symondson and Liddell (1993).

In parallel with this method, a linear discriminant analysis (LDA) was used as tool to classify the predictor variable – the CEs estimated – in two groups: those that consumed

and those that did not consume on black-scale using Minitab Statistical Software, release 14 (Minitab Inc., 2003). Thus, in the first stage of LDA, discriminant functions were calculated using CEs obtained from laboratory starved and fed coccinellids. The convergence point of the discriminant functions was used to assess the quality of threshold level previously defined. In a second stage, the discriminant functions calculated with the known groups were also used to classify the state of the field collected coccinellids and thus, predict which consumed or not on black-scale. Values of CEs measured in field collected coccinellids and positioned above the threshold level correspond to the group of coccinellids that consumed black scale, and values below the threshold correspond to the group of coccinellids that did not consume on black-scale for both analyses.

5.3. Results

5.3.1. Antiserum characterization

The effectiveness of the *S. oleae* antiserum is presented in Fig. 5.1, where the limit of detection was conventionally said to occur when AS ≥ 2 × NRS. Furthermore, the limit of detection for the bulk of antiserum prepared was found between dilutions of 1:316000 and 1:1000000, corresponding to a *S. oleae* protein content of 0.118 and 0.0374 μg mL^{-1}, respectively.

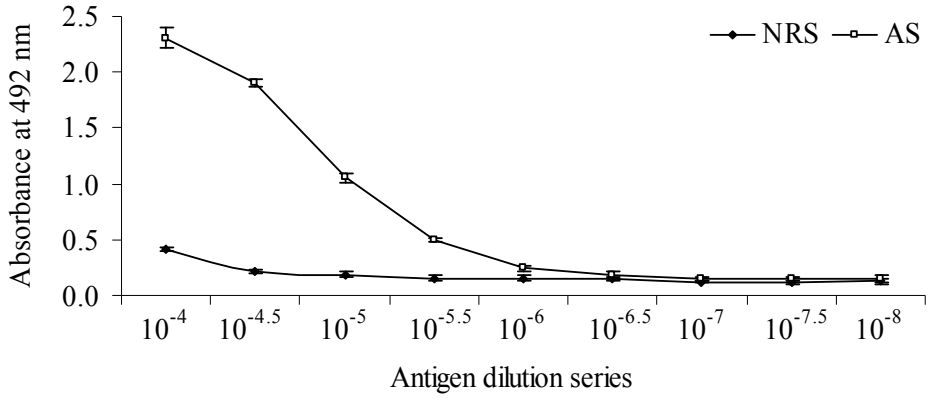

Figure 5.1: Detection limit for the bulk of antiserum (AS) (mean ± standard error of the mean - SE; n = 8) tested against a dilution series of *S. oleae* antigen. AS and NRS was used at 1:1000 and the concentrations of the antigen ranged from 3.74 μg mL^{-1} to 0.374 ng mL^{-1}.

Results of *S. oleae* cross-reactions with 17 sympatric arthropods including various predators and alternative preys are shown in Table 5.1. The polyclonal antiserum cross-reacted mostly with coccinellid larvae presenting a mean CE of 0.439 μg mL^{-1} (s = 0.094). Moreover, preys like *P. oleae*, *Euphyllura olivina* (Costa) and Psocoptera showed lower cross-reactivity with *S. oleae* antiserum than coccinellid larvae.

The CEs of coccinellids that had fed on *S. oleae* in the laboratory were highly variable, but were clearly positioned above the CEs of unfed coccinellids (Fig. 5.2). One specimen of *M. mediterraneus*, and another of *R. chrysomeloides* would have been declared negative, even though they had just fed on *S. oleae*.

Table 5.1. Cross-reactivity between the *S. oleae* polyclonal antiserum and a range of starved insect predators and potential alternative preys.

Species	CE (μg mL^{-1}) ± SE
Euphyllura olivina (Costa) (Hemiptera: Psyllidae)	0.342 ± 0.021
Deraeocoris lutescens (Schilling) (Heteroptera: Miridae)	0.172 ± 0.008
Chysoperla carnea (Stephens) (Neuroptera: Chrysopidae)	0.321 ± 0.050
Crematogaster scutellaris (Olivier) (Hymenoptera: Formicidae)	0.216 ± 0.043
Cataglyphis iberica (Emery) (Hymenoptera: Formicidae)	0.135 ± 0.010
Cataglyphis hispanica (Emery) (Hymenoptera: Formicidae)	0.131 ± 0.008
Lasius niger (L.) (Hymenoptera: Formicidae)	0.146 ± 0.038
Coccinella setempunctata L. (Coleoptera: Coccinellidae)	0.186 ± 0.039
Scymnus (Mimopullus) mediterraneus Iablokoff-Khnzorian (Coleoptera: Coccinellida	0.109 ± 0.009
Rhyzobius chrysomeloides (Herbst.) (Coleoptera: Coccinellidae)	0.108 ± 0.008
Scymnus (Pullus) subvillosus (Goeze) (Coleoptera: Coccinellidae)	0.198 ± 0.006
Chilocorus bipustulatus L. (Coleoptera: Coccinellidae)	0.193 ± 0.034
Scymnus (Scymnus) interruptus (Goeze) (Coleoptera: Coccinellidae)	0.143 ± 0.021
Nephus (Bipunctatus) bisignatus Boheman (Coleoptera: Coccinellidae)	0.157 ± 0.014
Larval instars (Coleoptera: Coccinellidae)	0.439 ± 0.030
Prays oleae larvae (Bernard) (Lepidoptera: Yponomeutidae)	0.050 ± 0.001
Psocoptera	0.124 ± 0.008

CE - *S. oleae* protein concentration equivalents; SE - standard error of the mean; n = 10.

The significance level above which a sample is deemed to contain *S. oleae* proteins was established either by calculating the mean + 2.5 SD (Symondson and Liddell, 1993) for ten coccinellid larvae, or by using the discriminant analysis, giving a cross-reaction

threshold level of 0.674 µg mL^{-1} and 0.679 µg mL^{-1}, respectively. These values can be considered equivalent since they are numerically close and no observation occurred between them. For the first method, any CE that was above this threshold was considered as a positive outcome for the presence of *S. oleae* in the gut of coccinellid species. The discriminant function predicted 97% of true positives, i.e. individuals fed on black-scale, while both thresholds led to 100% prediction of true negatives.

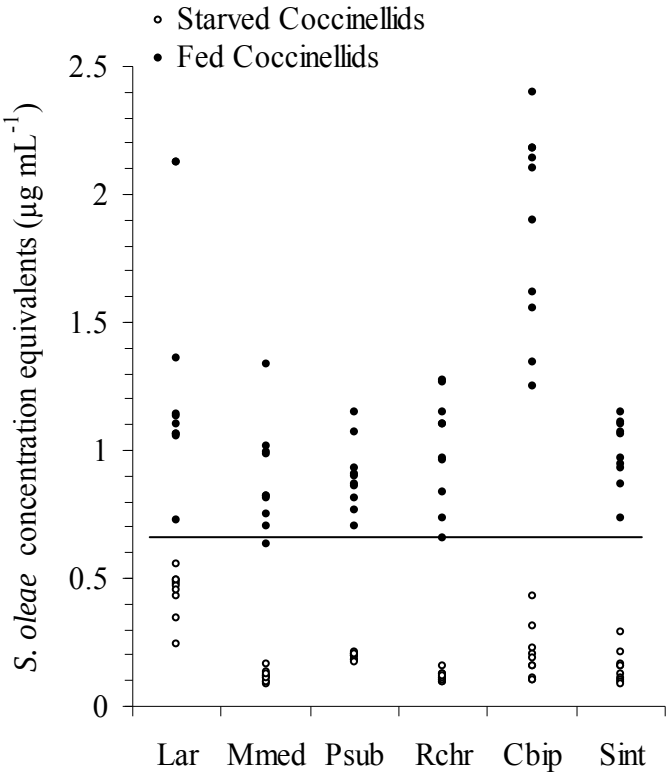

Figure 5.2. ELISA results for starved coccinellids and coccinellids that fed on *S. oleae*. Horizontal line indicates the threshold for *S. oleae* protein concentration equivalents. Lar = Coccinellid larvae, Mmed = *Mimopullus mediterraneus*, Psub = *Pullus subvillosus*, Rchr = *Rhyzobius chrysomeloides*, Cbip = *Chilocorus bipustulatus*, Sint = *Scymnus interruptus*.

5.3.2. Detection within field caught predators

A total of 1165 adult coccinellids belonging to ten species and 157 coccinellid larvae were collected in three olive groves of Trás-os-Montes region and analyzed by indirect ELISA to detect *S. oleae* remains in their guts. Positive results based on the method used by Symondson and Liddell (1993) were coincident with those obtained using

discriminant functions. Among the captured coccinellids, 280 (21.2%) tested positive for the presence of *S. oleae* and *C. bipustulatus* and coccinellid larvae obtained the highest percentages of positives (Table 5.2). *C. bipustulatus* reached 41.3 and 46.9% of positives and larvae reached 34.7 and 63.6%, respectively in the organic and in the IPM groves. From the eight species that reacted positively for the presence of the black-scale, *Exochomus quadripustulatus* (L.), *Scymnus (Scymnus) apetzi* Mulsant and *Nephus (Bipunctatus) bisignatus* (Boheman) showed similar percentages of positives but few individuals were captured. On the other hand, 147 specimens of *R. chrysomeloides* were captured but a low percentage of positives (7.7% in the organic grove and 0.0% in the IPM groves) was obtained.

M. mediterraneus and *S. interruptus* were more abundant from the beginning of July till the end of August, registering high percentage of positives in the majority of the sampling dates (Table 5.2). This period coincides with the occurrence of the first-instar nymphs of *S. oleae* (Fig. 5.3). *P. subvillosus* was more abundant from the beginning of August till the end of September but the highest percentages of positives occurred in the middle of July with 66.7% of the individuals captured in the IPM groves reacting positive. *C. bipustulatus* was more abundant from the middle of August till the end of the sampling time and the highest percentage of positives was registered from the middle of September till October. Over this period, both second and third-instar nymphs of the pest co-existed in the field (Fig. 5.3).

Figure 5.3: Occurrence of the different phenological stages of *Saissetia oleae* (Pereira, 2004; Santos *et al.*, unpublished data) in the olive grove from July to October.

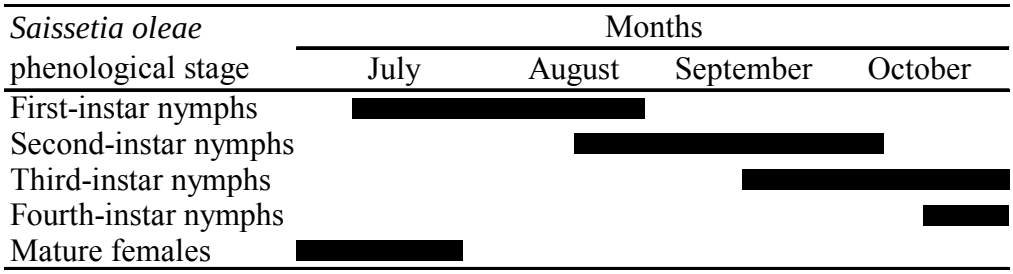

Table 5.2. Number of individuals assayed and percentage of ELISA positives for coccinellid species collected in organic and IPM olive groves from Trás-os-Montes region (Portugal).

Date	Cbip		Pmed		Psub		Sapt		Sint		Rchr		Spun		Bbis		Equa		Enig		Larv		Total	
	No. assayed	% positives	No. assayed	% positives	No. assayed	% positives	No. assayed	% positives	No. assayed	% positives	No. assayed	% positives	No. assayed	% positives	No. assayed	% positives	No. assayed	% positives	No. assayed	% positives	No. assayed	% positives	No. assayed	% positives
Organic grove																								
01/07	8	37.5			7	14.3			11	9.1	2	0.0											28	17.9
07/07	6	33.3	6	33.3	2	0.0			13	23.1	4	25.0									1	100.0	32	28.1
14/07	8	25.0	5	0.0	5	40.0			15	26.7	4	0.0									1	0.0	38	21.1
21/07	4	25.0	3	33.3	7	42.9			20	10.0	3	0.0									9	33.3	46	2.2
28/07	1	0.0	2	0.0	3	0.0			25	20.0	4	25.0									16	31.3	51	21.6
05/08	1	0.0	8	0.0	5	20.0	1	0.0	18	33.3											23	34.8	56	26.8
12/08	5	20.0			11	27.3	3	0.0	19	26.3											18	38.9	56	28.6
19/08	8	50.0	2	0.0	8	12.5			16	18.8	5	0.0									22	40.9	61	27.9
01/09	12	41.7	6	0.0	14	14.3			8	12.5	3	0.0									17	35.3	60	23.3
08/09	21	42.9	6	0.0	6	0.0			10	20.0	4	0.0									5	20.0	52	23.1
15/09	16	12.5	7	0.0	10	10.0			3	0.0	10	20.0	2	0.0							6	33.3	54	13.0
21/09	24	37.5	9	0.0	8	25.0	1	0.0	8	25.0	12	8.3									1	0.0	63	22.2
29/09	12	66.7	3	0.0	10	20.0			2	0.0	16	12.5									1	0.0	44	27.3
06/10	9	77.8	2	0.0	1	0.0			2	0.0	22	4.5									3	33.3	39	23.1
13/10	8	75.0	1	0.0							15	0.0									1	0.0	25	24.0
Total	143	41.3	60	5.0	97	18.6	5	0.0	170	20.0	104	7.7	2	0.0	0	0.0	0	0.0	0	0.0	124	34.7	705	23.4
IPM groves																								
01/07	9	44.4							10	0.0	9	0.0					7	28.6	1	0.0	3	0.0	39	15.4
07/07	1	0.0	37	40.5	3	66.7	2	50.0	8	25.0	3	0.0	1	0.0	1	0.0					2	100.0	58	37.9
14/07	1	0.0	24	8.3	3	66.7	2	50.0	12	41.7	11	0.0	1	0.0	1	0.0					4	100.0	59	23.7
21/07	1	100.0	41	14.6	11	0.0			15	20.0	6	0.0					1	0.0	1	0.0	1	100.0	77	14.3
28/07	1	100.0	28	14.3	6	0.0			10	10.0					1	100.0	2	0.0	1	0.0	3	33.3	52	15.4
05/08			33	9.1	5	0.0	1	0.0	13	7.7	2	0.0									4	75.0	58	12.1
12/08			35	17.1	7	0.0	2	0.0	5	0.0	2	0.0					1	0.0			2	50.0	54	13.0
19/08	4	25.0	26	3.8	4	50.0			5	20.0											9	77.8	48	25.0
01/09			20	5.0	13	7.7			2	0.0	1	0.0					1	0.0			2	50.0	39	7.7
08/09			14	7.1	12	8.3			1	0.0	1	0.0					1	0.0			2	50.0	31	9.7
15/09			10	0.0	9	44.4			1	0.0	1	0.0											21	19.0
21/09	7	85.7	6	0.0	6	0.0									1	0.0					1	0.0	21	28.6
29/09			14	21.4	10	20.0					3	0.0			2	0.0							29	17.2
06/10	4	50.0	11	0.0	2	0.0					4	0.0											21	9.5
13/10	4	50.0							6	50.0													10	50.0
Total	32	46.9	299	14.0	91	15.4	7	28.6	88	14.8	43	0.0	2	0.0	6	16.7	13	15.4	3	0.0	33	63.6	617	17.8

Cbip = *Chilocorus bipustulatus*, Enig = *Exochomus nigromaculatus*, Equa = *Exochomus quadripustulatus*, Mmed = *Mimopullus mediterraneus*, Psub = *Pullus subvillosus*, Sapt = *Scymnus apetzi*, Sint = *Scymnus interruptus*, Nbis = *Nephus bisignatus*, Spun = *Stethorus punctillum*, Rchr = *Rhyzobius chrysomeloides*, Lar = Coccinellid larvae.

5.4. Discussion

The microplate procedure is widely used and provides a useful method for establishing trophic links in the field that could not be detected using population survey alone (Sunderland, 1996). Furthermore, is particularly suited to large scale testing of field samples, such as might be required, for example, in surveys of predation that remains one of the most difficult to study ecological processes but one that is critical to understand if predators are efficient in the agricultural pest control (Naranjo and Hagler, 2001).

In general, the indirect ELISA is considered a very sensitive method (Hagler, 1998) and the *S. oleae* antiserum showed to be sufficiently sensitive and accurate to be used in predation studies with field-collected coccinellids. However, the efficiency of indirect ELISA can depend on the predator: prey protein ratio, as shown by Hagler *et al.* (1997). Thus, when a predator ingested on a small amount of prey, the indirect ELISA can be less efficient at detecting prey proteins in the predator; that can be even more difficult in larger predators due to a high predator: prey protein ratio (Hagler *et al.*, 1997). Nevertheless, in the laboratory fed coccinellids, it was the largest specie *C. bipustulatus*, that presented the highest CE probably because it ingested more *S. oleae* during the feeding period than the smallest species of the test, *M. mediterraneus*. The variability in the CE values of coccinellids fed with *S. oleae* was greater than starved coccinellids reflecting the amount of preys actually ingested in the laboratory experiment. This pattern was also observed by authors like Fichter and Stephen (1981) and Symondson and Liddell (1993).

The CE calculated for coccinellids based on the regression equation derived from the standard dilution series on each ELISA plate insured the independence of the day-by-day variation in conditions, reagents and plate-binding properties. Moreover, both negative-positive thresholds established were sufficiently conservatives to prevent against false positives. But, the trade-off for this benefit was that the likelihood of underestimate predation increased because the probability of a false negative has been increased, especially when the quantity of *S. oleae* ingested was small.

The outcome of the gut content immunoassay in field-collected coccinellids can be influenced by differences between species or individuals that in turn can be attributable to such factors as (1) the amount of prey consumed, (2) time since feeding or (3) the rate of digestion (Sunderland *et al.* 1987; Hagler and Naranjo, 1997; Hardwood *et al.*, 2004).

Since the amount of prey consumed in the field is not known, a high value of absorbance could represent a large prey item, a prey item that had been extensively preyed upon, several small prey items, a very recent feeding, or any combination thereof (Greenstone, 1996). Time since feeding and digestion rate of each coccinellid species have a great influence on the proportion of that species containing detectable prey antigens at any given time in the field (Sunderland *et al.*, 1987). Time elapsed from prey ingestion to predator analysis can also contribute to increase false negatives due to antigen decay as predator digestion proceeds (Fichter and Stephen, 1981; Hagler and Naranjo, 1997). Given these limitations, although the strength of the ELISA reaction is unlikely to be a good quantitative indicator of *S. oleae* predation, it is a good qualitative indicator of which coccinellid species predate *S. oleae*.

Several field-collected coccinellid species tested positive for *S. oleae* antigen. *C. bipustulatus*, *M. mediterraneus*, *P. subvillosus* and *S. interruptus* were the species that showed simultaneously high abundance and high number of positives over the sampling period. In contrast, *R. chrysomeloides* showed high abundance but low number of positives. In general, the percentage of coccinellids testing positive was high in mid-July agreeing with the occurrence of the first instar nymphs of *S. oleae*, in mid-August when both first and second instar nymphs were present, and from the middle of September to the end of the sampling period which coincided with the presence of both second and third instar nymphs. The predation of *S. oleae* by *M. mediterraneus*, *P. subvillosus* and *S. interruptus* tested positive mainly when the most vulnerable stages of the pest (i.e., the first and second instar nymphs) were abundant whereas *C. bipustulatus* tested positive mainly when the second and third instar nymphs of *S. oleae* were present. This agreement between the percentage of positives, the abundance of the pest and the abundance of coccinellid species is an indication that predation would probably occur over those nymphal stages. Previously, Argyriou and Katsoyannos (1977) in Greek olive groves and Limón *et al.* (1976) in Spanish citrus groves considered *C. bipustulatus* and *E. quadripustulatus* as potential predators of *S. oleae*.

The occurrence of different coccinellid species in the olive grove may have complementary effects concerning *S. oleae* natural control, i.e. consuming different nymphal stages, coccinellids can contribute for a more effective suppression of the pest. Less common species like *E. quadripustulatus*, *S. apetzi* and *N. bisignatus* seems to have

an important complementary action in the predation of the pest, but more studies are needed to confirm this hypothesis.

In summary, predation of *S. oleae* by coccinellids can be detected by ELISA and both *C. bipustulatus* and larval stages are likely predators of *S. oleae* due to the high percentage of positives found over the sampling period (43.4% and 41%, respectively). Moreover, 19% of the individuals of *S. interruptus* tested positive and this was also one of the most abundant species. This result, in association with the dynamics of the communities of the coccinellids and the pest (*S. oleae*) suggest that they might have a important role in the control of this pest. Therefore, the protection of the abundance and species richness of coccinellids should be favoured as a practice of pest population control in olive groves.

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Chapter 6

6. VORACITY OF COCCINELLID SPECIES ON DIFFERENT PHENOLOGICAL STAGES OF THE OLIVE PEST SAISSETIA OLEAE *(HEMIPTERA, COCCIDAE)*

Sónia A.P. Santos, José A. Pereira, Laura M. Torres & António J.A. Nogueira

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ABSTRACT

Coccinellidae are well known predators in agroecosystems. In olive groves they may exert control against scales, such as the black scale, *Saissetia oleae* (Olivier, 1791). Laboratory studies on the consumption of three phenological stages (eggs, first and second instar nymphs) of *S. oleae* by four coccinellid species (*Chilocorus bipustulatus*, *Scymnus (Pullus) subvillosus*, *Scymnus (Mimopullus) mediterraneus* and *Scymnus (Scymnus) interruptus*) were carried out. *C. bipustulatus* presented a significantly high consumption of eggs, first and second instar nymphs compared with the other species. All coccinellids consumed eggs and first instar nymphs; however the second instar nymphs were only consumed by *S. interruptus* and *C. bipustulatus*. In a second experiment, larval stages of *C. bipustulatus* were reared on different phenological stages of *S. oleae*. Coccinellid larvae fed with eggs or first instar nymphs completed their life cycle, contrarily to those that were fed with second instar nymphs. The apparent voracity of *C. bipustulatus* on the different phenological stages of *S. oleae* is an aspect that suggests the possible use of this coccinellid species in biological control programs against this pest in olive groves.

Key-words: Coccinellids, predation, *Chilocorus bipustulatus*, *Scymnus (Mimopullus) mediterraneus*, *Scymnus (Pullus) subvillosus*, *Scymnus (Scymnus) interruptus*, natural control, black scale.

6.1. Introduction

In the Mediterranean countries, the black scale, *Saissetia oleae* (Olivier, 1791), is an important pest in the olive grove and natural enemies belonging to the Coccinellidae family are known to be associated with this pest (Argyriou and Katsoyannos, 1977; Magro and Hemptinne, 1999; Ba M'hamed and Chemseddine, 2001; Ba M'hamed and Chemseddine, 2002).

Voracity studies intend to investigate the potential of a natural enemy to consume a specific prey. This elucidation about the predatory characteristics of coccinellids is the basis for developing management strategies to successfully combat black scale infestations. Such information would be useful for determining which developmental stage of *S. oleae* is the most predated and will facilitate further laboratory rearing of these insects, which is a prime objective in a biological control program (Sahayaraj and Paulraj, 2001). Successful natural control of pests depends on the fact that a predator kills or consumes a sufficient number of pest individuals to maintain its density at a low level (Sengonca *et al.*, 2005).

Coccinellid species like *Chilocorus bipustulatus* (L.), *Scymnus (Pullus) subvillosus* (Goeze), *Scymnus (Mimopullus) mediterraneus* Iablokoff-Khnzorian and *Scymnus (Scymnus) interruptus* (Goeze) are common in the olive grove of the Mediterranean region and both adults and larvae are predaceous stages (Argyriou and Katsoyannos, 1977; Ba M'hamed and Chemseddine, 2002). Field studies showed that the most predated phenological stages of *S. oleae* by coccinellids were potentially the first and second instar nymphs (Santos *et al.*, unpublished data). Moreover, *S. oleae* eggs have been used to feed different coccinellid species in laboratory cultures (Argyriou and Katsoyannos, 1977; Ba M'hamed and Chemseddine, 2001; Ba M'hamed and Chemseddine, 2002). Therefore, it is important to gain insight about the effective predated stages of *S. oleae* by coccinellids. This information will help on further studies concerning the natural control of *S. oleae* by coccinellids in field and also the maintenance of laboratory cultures of coccinellids reared in *S. oleae*.

The objective of this study was to investigate, under controlled conditions, the ability of selected coccinellid species to use different phenological stages of *S. oleae* as food item.

6.2. Material and Methods

To study the consumption of different phenological stages of *S. oleae* by coccinellid species, two experiments were carried out: (1) screening of coccinellids voracity using adult stages, and (2) assessment of feeding rates, along the life-cycle of the most voracious coccinellid species, using eggs, first instar and second instar nymphs of *S. oleae* as food items.

To perform the first experiment, specimens belonging to the four most common coccinellid species namely, *M. mediterraneus*, *P. subvillosus*, *S. interruptus* and *C. bipustulatus*, were captured by the beating technique in an organic olive grove - Valbom-dos-Figos (41º 33' 4'' N, 7º 8' 43'' W) - located near Mirandela, Trás-os-Montes (north-east of Portugal). The three different phenological stages of *S. oleae* (eggs, first and second instar nymphs) were collected with the leaf.

In the laboratory, 30 coccinellid specimens of each species were placed in individual Petri dishes (9 cm diameter x 2 cm height) with moistened filter paper covering the bottom of the box and starved during 24 hours.

Each species was separated in three groups of ten specimens. Each group was fed daily with a different phenological stage of *S. oleae* during five days: group 1 was fed with 100 eggs/day, group 2 with 10 first instar nymphs/day and the third group with 10 second instar nymphs/day. During five days, specimens were allowed to forage for 24 h after which the food was renewed and the total number of prey eggs, N1 nymphs or N2 nymphs was recorded.

Based on the results of the first experiment, *C. bipustulatus* was selected to perform the second experiment where the consumption of eggs and first and second instar nymphs of *S. oleae* by larval stages and adults of *C. bipustulatus* was studied.

C. bipustulatus adult specimens were captured in the same grove mentioned above. In the laboratory, they were coupled in Petri dishes (9 cm diameter x 2 cm height) with moistened filter paper covering the bottom of the box and fed with *S. oleae* eggs. Newly laid eggs were transferred individually to a clean Petri dish with a soft brush and daily observed till hatching. Forty-five recently emerged L_1 larvae were divided in three groups with fifteen specimens. Group 1 was supplied with 100 eggs/day, group 2 with 10 first

instar nymphs/day and group 3 with 10 second instar nymphs/day. Each Petri dish was examined daily to record consumption. Moulting marked the differentiation between larval stages. Freshly emerged adults were used to make the same set of experiments as in larval stages.

All experiments were conducted at a temperature of 25±2º C and under a 16L: 8D h regime.

Univariate statistical analyses were performed using the Statistica Statistical package, version 7.0 (StatSoft, 2004). Data were evaluated for normality and homogeneity of variances with Kolmogorov-Smirnov test and Bartlett's test, respectively. One-way ANOVA was used to compare the consumption of *S. oleae* phenological stages by each coccinellid species. A significant level of 0.05 was used for all statistical tests.

6.3. Results and Discussion

In the first experiment, the food intake by the four coccinellid species tested was maintained constant during the five days, except in the case of the consumption of the first instar nymphs by *S. interruptus* that varied significantly (Table 6.1). *C. bipustulatus* showed a significantly high consumption of all phenological stages of the black scale when compared with the other species tested (Fig. 6.1). Thus, an average of 381.3 ± 15.3 (mean ± standard error of the mean - SE, n = 10) eggs followed by 8.6 ± 0.7 first instar nymphs and 15.3 ± 1.9 second instar nymphs were consumed after the five days of the experiment. *S. interruptus* consumed a total mean of 160.1 ± 6.9 eggs, 5.2 ± 0.7 first-instar nymphs and 0.3 ± 0.2 second-instar nymphs and *P. subvillosus* consumed 150.4 ± 20.5 eggs and 4.5 ± 1.2 first-instar nymphs. On the other hand, *M. mediterraneus* showed a significantly low consumption of eggs and the lowest consumption of the first instar nymphs, with a mean total of respectively 52.9 ± 4.5 and 2.8 ± 0.4. Second instar nymphs were not consumed by *M. mediterraneus* and *P. subvillosus*.

In laboratory studies, Argyriou and Katsoyannos (1977) showed that was possible to obtained consecutive generation of *C. bipustulatus* fed with *S. oleae* (phenological stage was not specified) but unsuccessful attempts were obtained for *P. subvillosus*. Also, Ba M'Hamed and Chemseddine (2001) showed that *M. mediterraneus* completed its life cycle consuming a large amount of eggs of *S. oleae*.

Table 6.1. Consumption by coccinellids species (n = 10) of three phenological stages of *Saissetia oleae* (mean ± standard error of the mean) during five days of experiment.

Coccinellid species	Food item	Days					$F_{4, 45}$	P
		1	2	3	4	5		
Chilocorus bipustulatus	eggs	81.6±3.75	76.3±3.04	77.9±3.51	75.4±3.04	70.1±6.27	1.04	n.s.
	N1	2.6±0.40	1.8±0.44	1.5±0.27	1.2±0.25	1.5±0.34	2.37	n.s.
	N2	4.1±0.71	3.5±0.50	2.9±0.28	2.6±0.50	2.2±0.36	2.34	n.s.
Scymnus (Mimopullus) mediterraneus	eggs	10.5±1.89 ^{ab}	12.9±1.38 ^{ab}	13.9±1.55 ^{a}	7.5±1.30 ^{b}	8.1±1.49 ^{ab}	3.40	*
	N1	0.9±0.18	0.8±0.25	0.5±0.17	0.4±0.22	0.2±0.13	2.20	n.s.
	N2	0	0	0	0	0	-	-
Scymnus (Pullus) subvillosus	eggs	40.7±6.49	32.8±5.36	27.9±5.57	27±4.32	22±3.39	1.90	n.s.
	N1	1.5±0.31	0.9±0.28	0.7±0.33	0.7±0.37	0.7±0.40	1.04	n.s.
	N2	0	0	0	0	0	-	-
Scymnus (Scymnus) interruptus	eggs	30.7±5.34	36.6±3.63	33±3.16	34.6±3.56	25.2±3.91	1.21	n.s.
	N1	2±0.37 ^{a}	1.6±0.40 ^{ab}	0.8±0.20 ^{bc}	0.6±0.22 ^{bc}	0.2±0.13 ^{c}	6.85	***
	N2	0.2±0.13	0	0	0.1±0.10	0	1.44	n.s.

Means sharing the same letter within rows are not significantly different at p>0.05. ns- non significant.

Under field conditions, the consumption of *S. oleae* eggs by the majority of coccinellid species will be difficult due to the hard integument of the egg-bearing females. Eventually, only *C. bipustulatus* can predate egg-bearing females because it possesses highly modified mandibles that are unique to the genus - they are acutely angled with a single tooth at their apex, which can be inserted between the scale and the substrate (Honda and Luck, 1995). The other tested coccinellid species probably consume first and second instar nymphs of *S. oleae* that are abundant stages during summer and autumn in olive groves (Pereira, 2004; Santos *et al.*, unpublished data). Nonetheless, due to its high nutritive value, eggs can be used as food for rearing laboratory cultures since they were successfully fed by all coccinellid species.

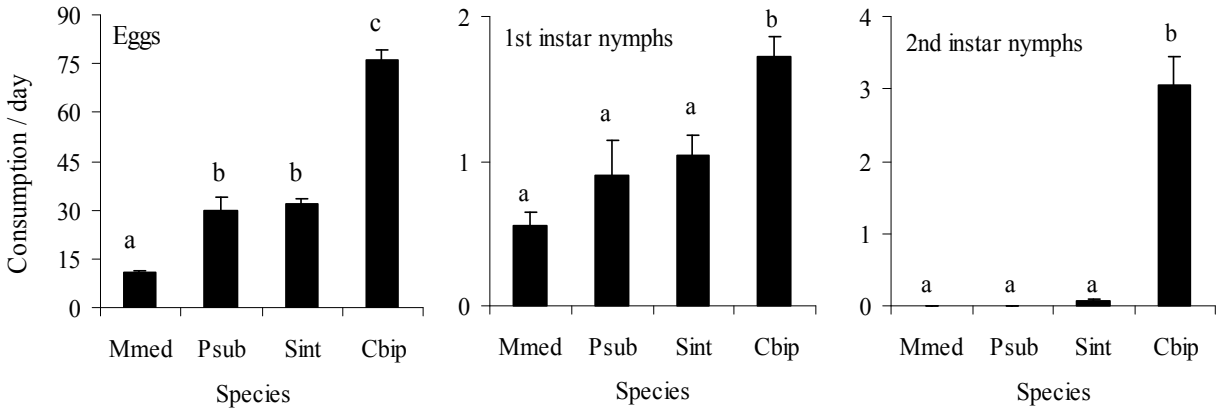

Figure 6.1. Consumption (mean ± standard error of the mean) of the three phenological stages of *Saissetia oleae* by the four coccinellid species tested: Mmed - *Mimopullus mediterraneus*, Psub - *Pullus subvillosus*, Sint - *Scymnus interruptus*, Cbip - *Chilocorus bipustulatus*. n=10. Different axis scales were used. Bars sharing the same letter are not significantly different at $P>0.05$.

The consumption of the different nymphal stages of *S. oleae* depends mostly on the characteristics of the tegument of the prey (Honda and Luck, 1995), the relation between the sizes of predator and prey and also the nutritional quality of the prey (Roger et al, 2000). Easily penetrated covers are more likely to be suppressed than thick covers (Honda and Luck, 1995). In this way, it was probable that first instar nymphs are softer and easier to handle by smaller coccinellid species (e. g. *M. mediterraneus*) than the second instar nymphs. On the other hand, because of their large size, adult *C. bipustulatus* can easily chew through the covers of greater nymphs that provide higher energetic gain than smaller nymphs (Provost *et al.*, 2006). Thus, the predation of each phenological stage of *S. oleae* by coccinellids can be considered as a trade-off between the energetic value and the morphological characteristic of the integument associated to each stage.

In the second experiment, the consumption of *S. oleae* eggs and first instar nymphs by the successive larval stages of *C. bipustulatus*, whose life cycle is represented in Fig. 6.2, increased with their development. The general pattern of food consumption by larval stages showed a gradual increase immediately after each moult but in third and fourth larval stages, a small decline occurred as larvae approached the ecdysis (Fig. 6.3). No differences were found between the developmental period of *C. bipustulatus* specimens fed

with eggs and those fed with first nymphal nymphs. Ponsonby and Copland (2000) observed a similar pattern in all larval stages of *Chilocorus nigritus* (F.) reared on the cyanophyllum scale *Abgrallaspis cyanophylli* (Signoret). A total mean of 976 ± 11.22 eggs of *S. oleae* were consumed by each larva of *C. bipustulatus* during its larval stage. First instar larvae consumed 3.6%, second instars 9.5%, third instars 27.3% and fourth instars 59.6% of the total eggs intake. Considering the intake of first instar nymphs of *S. oleae*, a mean total number of 86 ± 6.1 was consumed by each larva of *C. bipustulatus*, where first instar larvae consumed 10.3%, second instar 12.2%, third instar 26.5% and fourth instar 51.0% of total intake. Compared with *C. nigritus*, the consumption by first instar larvae of *C. bipustulatus* was generally high and the fourth instar was very low - 3 and 73% respectively (Ponsonby and Copland, 2000).

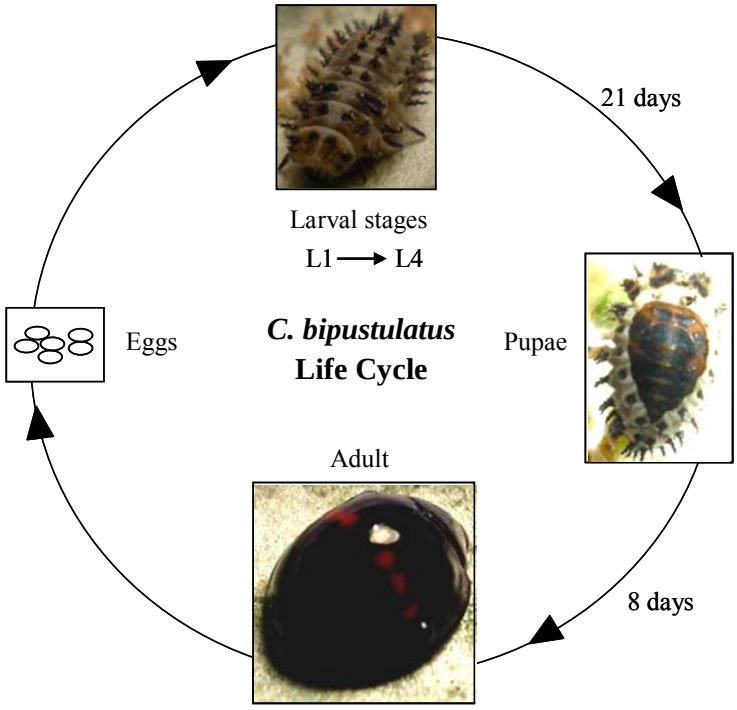

Figure 6.2. *Chilocorus bipustulatus* life cycle scheme.

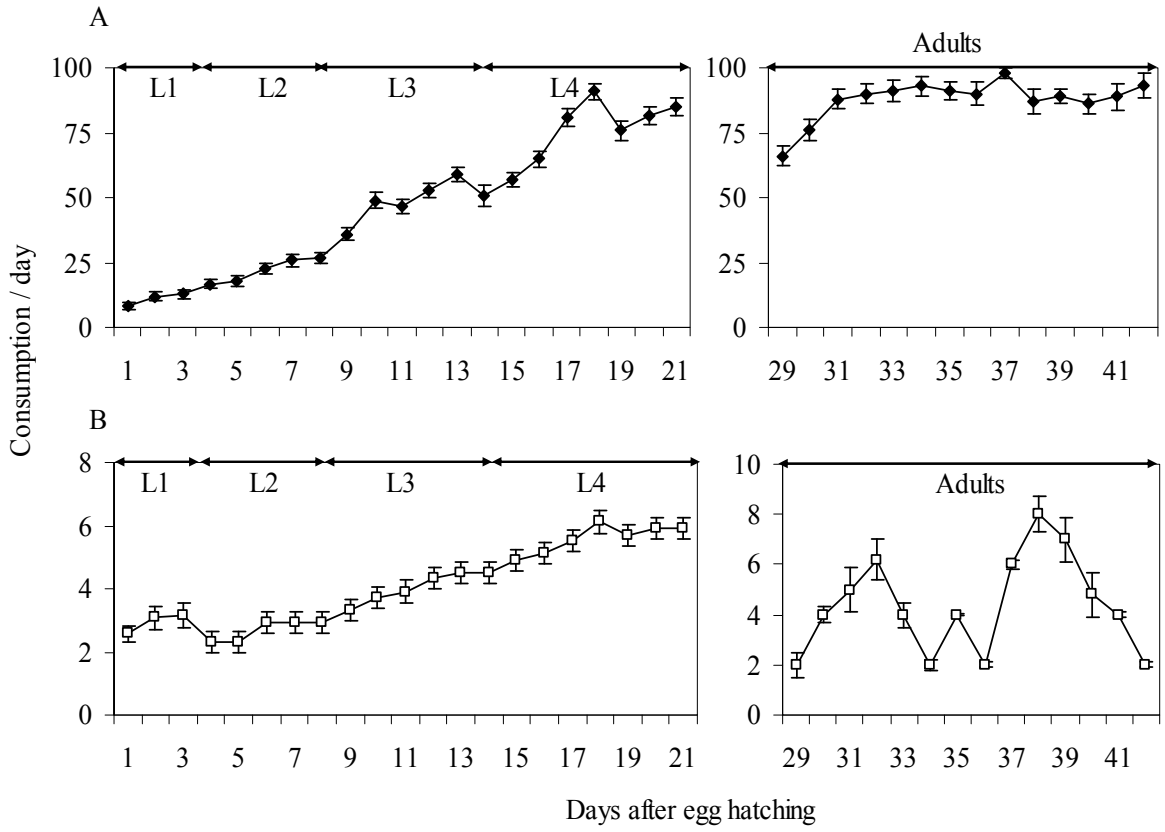

Figure 6.3. Consumption of eggs (A) and first instar nymphs (B) (mean ± SE) of *Saissetia oleae* by *Chilocorus bipustulatus* larvae and adult stages. Different axis scales were used. n=15.

The egg consumption by adults increased in the first six days after eclosion and then it remained constant till the end of the experiment (Fig. 6.3). According Ponsonby and Copland (2000), adult food intake in coccidophagous coccinellids is known to be low after eclosion, increasing gradually to a peak at the end of the first week. Each adult consumed a mean of 1227 ± 51.98 eggs after 14 days of the experiment. The consumption of the first instar nymphs increased in the first four days and a mean of 61 ± 11.2 first instar nymphs of *S. oleae* was consumed by each adult specimen after 14 days of experiment.

None of the second instar nymphs supplied were totally consumed by the first larval specimens of *C. bipustulatus*, which only reached the second instar and consequently, the life cycle was not completed. As a result, alternative preys should be essential items in the first larval stages of this coccinellid species to successfully complete its life cycle. Despite their polyphagy, coccinellid adults tend to feed more certain types of

food (Iperti, 1999) and the beneficial effect that food has on individual predators leads to increased rates of growth, development and fertility, and decreased rates of mortality (Begon *at al.*, 1996).

This study provides a better understanding of the consumption of different phenological stages of *S. oleae* by coccinellid species and the capacity of both larval and adult stages of *C. bipustulatus* to feed on this pest and complete their life-cycles. The apparent voracity of *C. bipustulatus* on the different phenological stages of *S. oleae* is an attribute that should make it an important predator contributing for the natural biological control of the pest in the olive grove. Moreover, the larva is the most voracious stages of coccinellids (Stathas, 2000), requiring great amounts of food to grow up rapidly. This aspect supports the likely employ of this coccinellid species in biological control programs against *S. oleae*. Thus, the mass release of larval specimens, particularly the most resistant third instar larvae, can be done in order to maximize the predaceous action of *C. bipustulatus* in the olive grove.

Acknowledgments

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Chapter 7

7. INTEGRATION OF SOOTY MOLD EFFECTS ON PHOTOSYNTHESIS AND GAS EXCHANGES OF OLIVE TREE, **OLEA EUROPAEA** *CV. COBRANÇOSA*

Sónia A. P. Santos, Conceição V. Santos, Sónia Silva, Glória Pinto,
Laura M. Torres & António J. A. Nogueira

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ABSTRACT

The effect of sooty mold in field collected olive leaves, *Olea europaea* cv. Cobrançosa, was studied by comparing different biochemical parameters such as water content, osmolality, chlorophyll *a*, *b*, and *a*/*b* contents, fluorescence, carbon dioxide fluxes and lipid peroxidation. These parameters were measured in healthy leaves and in leaves covered with sooty mold and these data were integrated both by analyzing microscopic histological slides in healthy and covered leaves and by analyzing a model constructed in order to describe the effect of sooty mold in olive photosynthesis and gas exchange. Sooty mold covered leaves showed significantly lower levels of water content and of basal (F_0), maximal (F_m) and variable fluorescence (F_v). However, for osmolality, chlorophyll *a*, *b* and *a*/*b* contents, F_v/F_m ratio and lipid peroxidation, statistical differences were not detected between healthy leaves and sooty mold covered leaves. Histological analysis revealed a complex fungi hyphae proliferation in both leaf surfaces of covered leaves that predominated in the abaxial surface. This fungi proliferation resulted in a decrease of foliar light absorbance, mainly due to fungi coverage of the adaxial surface and also a decrease of foliar free gas exchanges, mainly due to fungi proliferation on the abaxial surface. Since these mechanisms are essential for photosynthesis and respiration processes, plant's normal physiological metabolism involving light absorption and CO_2 exchange would be severely affected.

Key-words: sooty mold, *Olea europaea*, fluorescence, chlorophyll, CO_2 flux, lipid peroxidation

7.1. Introduction

The cultivation of *Olea europaea* L. is a traditional part of European agriculture and has both socio-economic and environmental importance, in particular in Mediterranean Basin (Loumou and Giourga, 2003; Nuberg and Yunusa, 2003). Despite its importance, olive faces several diseases with severe impact in tree production, one of which is sooty mold. It is consensual that sooty mold is a complex of dark-pigmented fungi of several genera (e.g. *Capnodium*, *Cladosporium* and *Fumago*), which have been described as nonparasitic, saprophytic, and superficial on plants (Panis, 1977a; Reynolds, 1999; Jouraevaa et al, 2006). This fungi complex covers both leaf surfaces and small branches giving a black aspect to the olive tree (e.g. Reynolds, 1999).

The development of the sooty mold in olive trees is typically a consequence of heavy infestations caused by the black scale, *Saissetia oleae* (Olivier) (Hemiptera: Coccidae), a parthenogenetic insect pest that sucks plant sap. Much of the water and sugars in the sap pass through the black scale gut, being excreted and producing abundant honeydew that covers the olive leaves and supports the proliferation of the sooty mold (Passos-Carvalho *et al.*, 2003; Jouraevaa et al, 2006). Strategies to control this disease are still almost restricted to spraying the olive tree with cupper or pruning the trees, which reduce the population of *S. oleae* (e.g. Passos-Carvalho *et al.*, 2003). Attempts to use *Fusarium larvarum* in the biocontrol of the black scale proliferation were proposed (Cozzi *et al.*, 2002), though reliable transfer of this strategy to large scale field trials is still needed.

The coverage of the leaves by the sooty mold complex can have several consequences for the olive tree. It has been proposed that one of the main problems associated to fungus leaf coverage is the decrease of photosynthesis, and thus a consequent alteration of plant‘s normal metabolism/physiology, and ultimately growth. For example, Panis (1977b) and Passos-Carvalho *et al.* (2003) mentioned negative effects of the sooty mold on parameters such as photosynthesis, chlorophyll, and respiration to the olive tree but no scientific evidences were provided. Studies on other species also showed interference of sooty mold on leaf performance and photosynthesis (e.g. Sparks *et al.*, 1991, Wood *et al.*, 1988). Nevertheless, and despite the great economic importance of

olive in Mediterranean countries, few studies were done showing scientific data on sooty mold effect in olive tree photosynthesis, gas exchanges and leaf anatomy.

Plant responses to biotic stress can be assessed using biochemical indicators such as: a) water content/osmolality (e.g. Oliveira *et al.*, 2007), b) lipid peroxidation, like malondyaldehyde production (MDA) that often presents good correlation with membrane degradation (e.g. Oliveira *et al.*, 2007), c) decrease of both chlorophyll contents and fluorescence (e.g. Guo *et al.*, 2005, Santos *et al.*, 2005, Synková *et al.*, 2006) or d) carbon dioxide (CO_2) flux (e.g. Shtienberg, 1992, Guo *et al.*, 2005).

Understanding if the fungus has either a direct or indirect effect in photosynthesis, and at what level (e.g. photophosphorilation or Calvin cycle) gives further knowledge on the interactions between host-fungus and contribute for the development of alternative strategies to control this disease. Therefore, the objective of this work was to study the effect of sooty mold on gas exchanges and related processes in covered olive leaves, in particular photosynthesis, by analyzing photosynthetic parameters (chlorophyll content and fluorescence) and the enrichment/depletion of atmospheric CO_2. These quantitative data were complemented by histo-anatomical analyses in healthy and in covered leaves.

7.2. Material and Methods

7.2.1. Study site and plant material

The study site was located in Paradela (Mirandela - north-east of Portugal) (41º 32' 38" N, 7º 7' 29" W), a 3 ha olive tree cv. Cobrançosa field with a planting density of 9 × 9 meters that has followed the Integrated Pest Management guidelines for plant protection since 2001.

In November 2006, ten randomly selected olive trees from Cobrançosa cultivar were sampled. In each tree, five current-season branch segments with visible sooty mold coverage and five healthy branch segments were collected. Branches were carried out to a growth chamber at 22 ± 2ºC, placed in a container with water and left overnight to acclimatize.

7.2.2. Histological analysis

For light microscopy analysis, leaf samples were treated according to Pinto (2007). Briefly, leaves with and without sooty mold were fixed in 2.5% glutaraldehyde in 1.25% (w/v) piperazine-N,N'-bis(2-ethane sulfonic acid) (PIPES) buffer (pH 7.4) for 3 h and washed in PIPES. Tissues were transferred to 1.0% (w/v) osmium tetroxide in PIPES buffer, rinsed, dehydrated through a graded ethanol series and embedded in a graded low-viscosity epoxi resin. Semi-thin sections were stained with toluidine blue. Samples were analyzed in a Nikon Eclipse 80i light microscope (Nikon Co, Japan). For scanning electron microscopy (SEM) analysis, material preparation was performed according to Pinto *et al.* (2002). Briefly, leaf samples were fixed with 2.0% (v/v) glutaraldehyde in PIPES buffer. Dehydration was achieved by successive immersions in aqueous ethanol solutions of increasing concentration (30% - 100% v/v), in acetone solutions of increasing concentration (30% - 100% v/v) and finally in a critical point device (Baltec CPD 030) using CO_2 as transition agent. Samples were fixed on steel supports and coated with gold using a JEOL metalizer (FFC-1100) at 1100-1200 V, 5 mA. Samples were observed in a scanning electron microscope (Hitachi, S4100, Japan) at 20 kV.

7.2.3. Biochemical parameters

7.2.3.1. Osmolality and leaf water content

For osmolality analysis, fifty leaves covered with sooty mold and fifty healthy leaves were submitted to freeze/unfreeze cycles to assure membrane rupture and centrifuged at 13 000 g for 10 min (Santos *et al.*, 2001). The osmolality of the leaf extracts was measured in an automatic osmometer (Knawer, Berlin, Germany) and expressed in mmol kg^{-1}.

Water content was determined in fifty leaves covered with sooty mold and fifty healthy leaves calculating the difference between fresh and dry weights after drying leaves in the oven for 48 h at 60ºC.

7.2.3.2. Chlorophyll concentration and fluorescence parameters

Chlorophyll fluorescence was monitored using a Plant Efficiency Analyser (Hansatech Instruments, Norfolk, UK). Healthy leaves and leaves covered with sooty mold were dark adapted for 30 min prior to measurement and illuminated with a peak wavelength 650 nm and a saturating light intensity of 3000 μmol m^{-2} s^{-1}. The maximum quantum yield of PSII [$(F_m-F_0)/F_m$] was determined after the estimation of the basal non-variable chlorophyll fluorescence (F_0) and the maximal fluorescence induction (F_m) with all PSII reaction centers open (Maxwell and Johnson, 2000). The concentrations of chlorophylls *a*, *b* and *a/b* ratio were determined, according to Arnon (1949), in the same leaf used for the fluorescence measurements. The petiole and central vein were removed from the leaves and each sample included one leave.

7.2.3.3. CO_2-exchanges

The effect of sooty mold on leaf CO_2 fluxes was measured using an infrared CO_2 gas analyzer (IRGA), model ADC 225-MK3 (Analytical Development Co., LTD, Hoddesdon, England), coupled to a thermal flow meter and using air collected outside the lab as reference air (Heinemeyer *et al.*, 1989). This apparatus has 12 independent lines, passing through sample holding chambers, and one reference line (Fig. 7.1). One olive branch segment with eight leaves attached was introduced in each sample tube and CO_2 was measured during a period of 14 h at 20ºC. Six of the tubes contained branch segments with healthy leaves and the other six tubes contained branch segments with leaves with sooty mold. Furthermore, half of the tubes with healthy leaves were placed in the dark and the others were illuminated with a light intensity of 2000 Lux (200 μmol m^{-2} s^{-1}). The same procedure was done with sooty mold leaves. A flow rate of 205 ± 10 mL min^{-1} (mean ± standard error of the mean) was maintained in both reference and sample tubes throughout the experiment. The CO_2 measurements were given by the differential between the CO_2 concentrations of the air leaving the sample compared to that of the reference. Based on preliminary experiments, seven independent trials were used to generate a suitable number of replicates. In each trial, treatments were randomly assigned to each channel. After the measurements, the total leaf area of each sample, healthy area (HA) and covered area with

sooty mold (CA) were quantified using the image analysis software SigmaScan Pro 5.0 (SPSS, 1999).

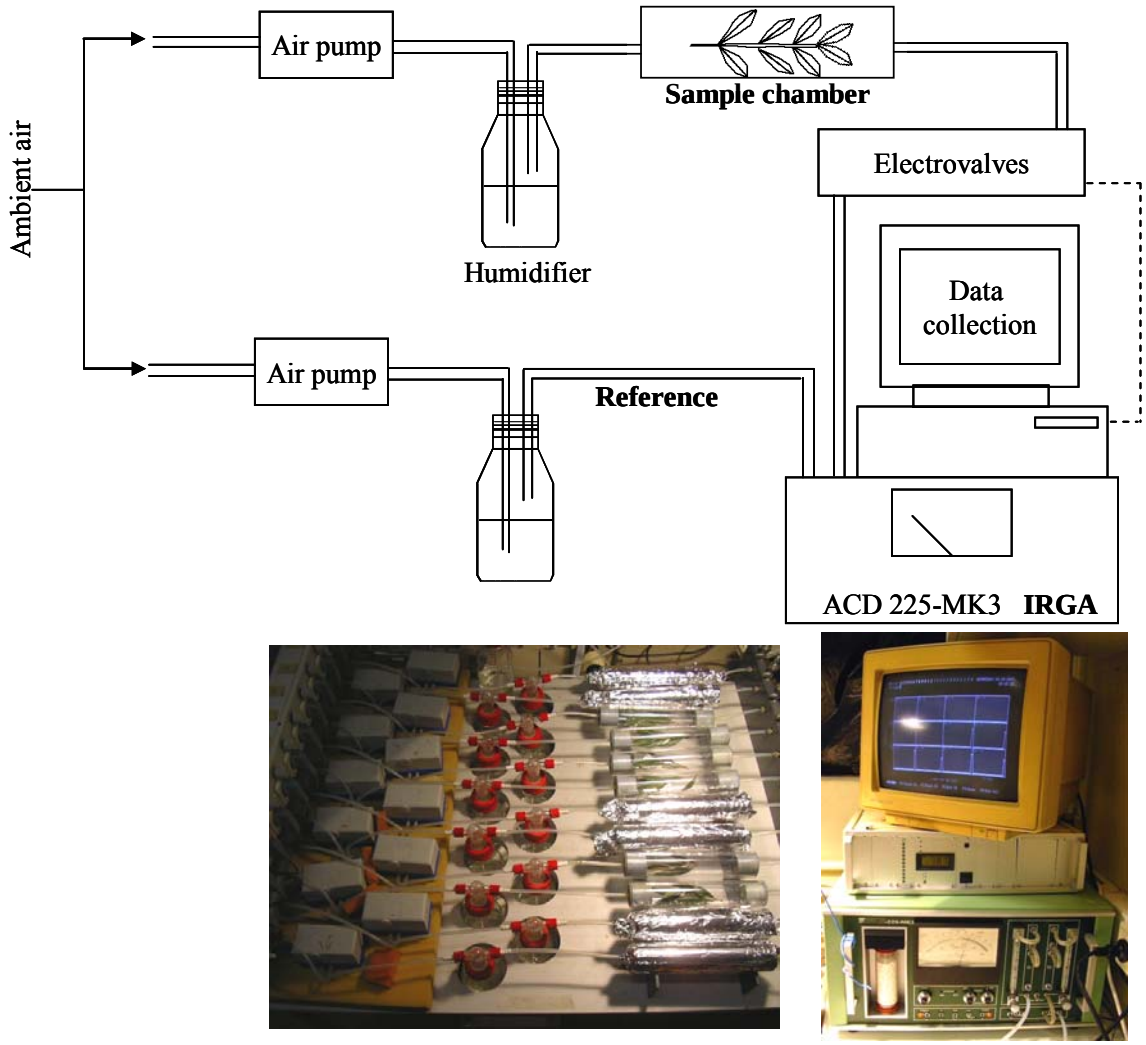

Figure 7.1. Scheme of infrared CO_2 gas analyzer (IRGA) setup and gas-flow apparatus used in respiration measurements.

7.2.3.4. Estimation of Lipid Peroxidation

Malondialdehyde (MDA) level is routinely used as an index of lipid peroxidation and was estimated according to Dhindsa and Matowe (1981). For measurements, 0.25 g of fresh leaves was deep frozen in liquid nitrogen and ground in 5 mL of 0.5% trichloroacetic acid (TCA). After centrifuging at 10 000 g for 10 min, 1 mL of extract was taken and 4 mL

0.5% thiobarbituric acid (TBA) in 20% TCA was added. The mixture was heated for 30 min at 95ºC, immediately cooled in an ice bath and centrifuged again at 10 000 g for 10 min. The specific absorbance of products and nonspecific background-absorbance were read at 532 and 600 nm, respectively. The concentration of MDA was determined in its unit equivalent using a molar extinction coefficient 155×10^5 mM^{-1} cm^{-1} and was expressed as nmol g^{-1} fresh weight.

7.2.4. Data analysis

Principal Component Analysis (PCA) was applied to the results of biochemical parameters. PCA was chosen due to the expected linear response model relating biochemical parameters and the presence of sooty mold in leaves (Van den Brink *et al.*, 2003). The analysis was performed with Canoco for Windows, Version 4.5 (ter Braak and Šmilauer, 2002) using fluorescence and biochemical parameters to play the role of species data, and a binary matrix with healthy/sooty mold covered leaves was used to play the role of supplementary environmental data. Species data were centred and standardized within Canoco for Windows. Biochemical parameters, except CO_2 fluxes measurements, were compared using a one-way ANOVA.

Differential of CO_2 concentrations (ΔCO_2) were compared with a factorial design using the following model: "presence of sooty mold + light + presence of sooty mold * light". The main purpose was to detect differences in the differential of CO_2 concentrations associated with the presence of the sooty mold on the leaves and the influence of the light. General Linear Model module of Minitab Statistical Software, release 14 (Minitab Inc., 2003) was used for this analysis.

A model was developed to describe changes in ΔCO_2 that were assumed as reflecting the CO_2 exchanges associated with three different physiological processes: leaf respiration (LR), sooty mold respiration (SMR) and leaf photosynthesis (LP). The underlying assumptions concerning CO_2 exchanges were:

- Leaf respiration is assumed to be proportional to non covered area, since sooty mold may interfere with stomata gas exchanges in covered leaf areas;
- Sooty mold respiration is proportional to the covered area;

- Sooty mold blocks light absorption, not allowing mesophyll to perform photosynthetic light dependent reactions in covered leaf areas;
- Total covered area (CA) is correlated with both covered abaxial and adaxial areas.

Thus, the equations to represent each of these processes (LR, SMR, LP) can be written as linear functions of the healthy area (HA) and covered area (CA):

$$LR = a + b \times HA \qquad \text{(Eq. 1)}$$

$$SMR = c + d \times CA \qquad \text{(Eq. 2)}$$

$$LP = e + f \times HA \qquad \text{(Eq. 3)}$$

where *a, c* and *e* are elevation constants and *b, d* and *f* are slopes. The arguments supporting these assumptions will be further explained in the discussion.

Generically, ΔCO_2 can be written as

$$\Delta CO_2 = LR + SMR + LP \qquad \text{(Eq. 4)}$$

where positive changes in ΔCO_2 were assumed to be associated with leaf respiration and with sooty mold respiration and negative changes in ΔCO_2 were assumed to be associated with the leaf photosynthesis. Some partial function can be equal to zero, depending on the existing conditions (light and extent of leaf coverage by sooty mold):

	Healthy leaves	Covered leaves
Light	$\Delta CO_2 = LR+LP$ (Eq. 5)	$\Delta CO_2 = LR+SMR+LP$ (Eq. 6)
Dark	$\Delta CO_2 = LR$ (Eq. 7)	$\Delta CO_2 = LR+SMR$ (Eq. 8)

Equation 4 and its coefficients were solved iteratively using the SOLVER add-in from Microsoft Excel 11 using the minimization of the residual sum of squares as target. The goodness of fit of the regression model was assessed using the adjusted coefficient of determination (r^2_{adj}). The F-statistics for the regression was determined using standard methodologies (Zar, 1996).

The relationships between the total covered area, the covered abaxial area (CAA_b) and the covered adaxial area (CAA_d) were established using linear regression. The slopes

and elevations of the resulting equations were compared by ANCOVA (Zar, 1996). The significance level used for all analyses was always 0.05.

7.3. Results

7.3.1. Histological analysis

Leaves with sooty mold showed a typical dark colour covering large areas of both abaxial and adaxial surfaces, contrasting with the bright green colour of healthy leaves (Fig. 7.2 A). Also, the xylem vessels have small dimensions. Healthy leaves showed thick waxy cuticle (mostly at the upper surface), and an abundant trichome complex, mostly at the abaxial surface that often presents sunken stomata (Fig. 7.2 B and L). At the adaxial surface, the mesophyll had overall 3-4 layers of palisade parenchyma, while a larger number of spongy layers were present and besides the main vascular strand (with secondary growth), minor small vascular strands were also present in the leaf blade, independently of the presence of fungus (Fig 7.2 B, C, F).

In covered leaves, the presence of sooty mold hyphae was visible, mostly at the abaxial surface (Fig. 7.2 C-F, H, J, K, M). Often the presence of black scale was also visible (Fig. 7.2 F-H). Both surfaces in covered leaves showed particular dark structures and hyphae proliferation covering the trichome complex (Fig 7.2 C-F, J, K, M), and large air spaces were present between this dark and compact film (formed by the matrix-fungus-trichome mesh) and the leaf epidermis (Fig 7.2 e.g. H, J). Considering inside leaf anatomy, no significant changes were observed between the mesophyll of covered and healthy leaves (Fig. 7.2 e.g. B, F).

7.3.1. Biochemical parameters

Healthy leaves had an average water content of 43.08 ± 0.40% (mean ± standard error of the mean) and an osmolality of 958.38 ± 35.34 mmol/kg. Sooty mold covered leaves, showed a significant decrease of water content to 38.96 ± 0.15% and a slightly increase of the osmolality to 969.38 ± 40.28 mmol/kg (Table 7.1).

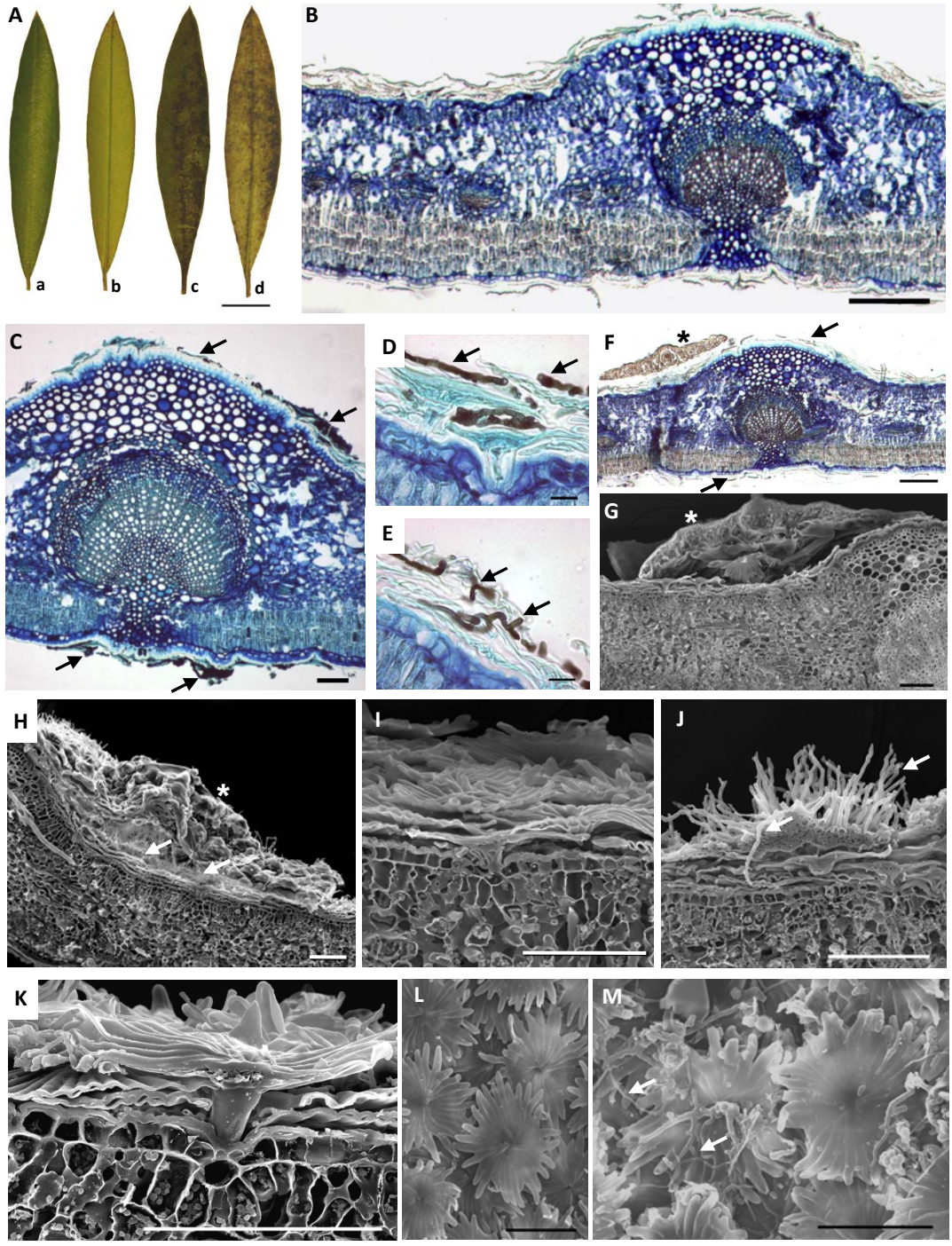

Figure 7.2. Healthy leaves and sooty mold covered leaves of *Olea europaea* cv. Cobrançosa examined by light microscope (B-F) and scanning electron microscope (G-M). Aspect of: the adaxial surface of a healthy leaf (Aa), abaxial surface of a healthy leaf (Ab), adaxial surface of a covered leaf (Ac) and abaxial surface of a covered leaf. Transversal sections of: a healthy leaf (B, I) and covered leaves (C, J, K). Details of sooty mold hyphae on trichomes (D, E). Transversal sections of covered leaves where the black scale appears on the abaxial surface (F, G, H). Frontal view of the trichomes of a healthy leaf (L) and of a covered leaf (M). Arrows indicate the sooty mold hyphae and asterisks indicate the transversal section of the black scale. Scale bars = 1 cm (A), 50 µm (B-F) and 100 µm (G-M)

The average values of chlorophyll contents of healthy leaves showed that the content of chl *b* was less than half the content of chl *a* and that chlorophyll *a* and *b* contents, as well as chl *a*/*b* ratio, were not statistically ($p>0.05$) affected by the presence of the sooty mold. Concerning the pigment fluorescence (mostly PSII photosystem), the leaves covered with sooty mold showed statistically significant lower levels of basal (F_0), maximal (F_m) and variable fluorescence (F_v) but, considering F_v/F_m ratio, no significant differences were found between healthy and sooty mold covered leaves and a mean value of 0.8 was determined (Table 7.1).

Table 7.1. Mean ± standard error of the mean (SE) of each biochemical parameter determined in healthy leaves and in leaves covered with sooty mold and statistical output for the ANOVA, n = 50.

Biochemical Parameters	Healthy leaves	Covered leaves	Statistical Output	
F_0	550,26 ± 9,50	291,20 ± 17,72	$F_{1, 80} = 57.67$	**(P<0.001)**
F_m	2874,80 ± 50,06	1608,70 ± 96,08	$F_{1, 80} = 64.00$	**(P<0.001)**
F_v	2344,96 ± 53,34	1317,90 ± 80,64	$F_{1, 80} = 59.51$	**(P<0.001)**
F_v/F_m	0,81 ± 0,00	0,82 ± 0,01	$F_{1, 80} = 2.08$	(P=0.166)
Osmolality (mmol/kg)	958,38 ± 35,34	969,38 ± 40,28	$F_{1, 80} = 0.15$	(P=0.706)
Chl *a* (mg/g FW)	0,99 ± 0,06	0,95 ± 0,05	$F_{1, 80} = 0.06$	(P=0.808)
Chl *b* (mg/g FW)	0,40 ± 0,03	0,36 ± 0,02	$F_{1, 80} = 0.37$	(P=0.548)
Chl *a/b*	2,46 ± 0,04	2,62 ± 0,04	$F_{1, 80} = 2.91$	(P=0.105)
MDA (nmol/g FW)	3,94 ± 0,10	4,15 ± 0,15	$F_{1, 80} = 1.62$	(P=0.219)
Water content (%)	43,08 ± 0,40	38,96 ± 0,15	$F_{1, 80} = 91.92$	**(P<0.001)**

The ordination diagram resulting from the PCA analysis for different biochemical parameters is shown in Figure 7.3. In general, samples corresponding to leaves covered with sooty mold were clearly separated from those samples determined from healthy leaves. The most determinant parameters for PCA distribution were those related with fluorescence (namely, F_0, F_m and F_v) and water content that were significantly higher in healthy leaves and MDA content that slightly increased in sooty mold covered leaves.

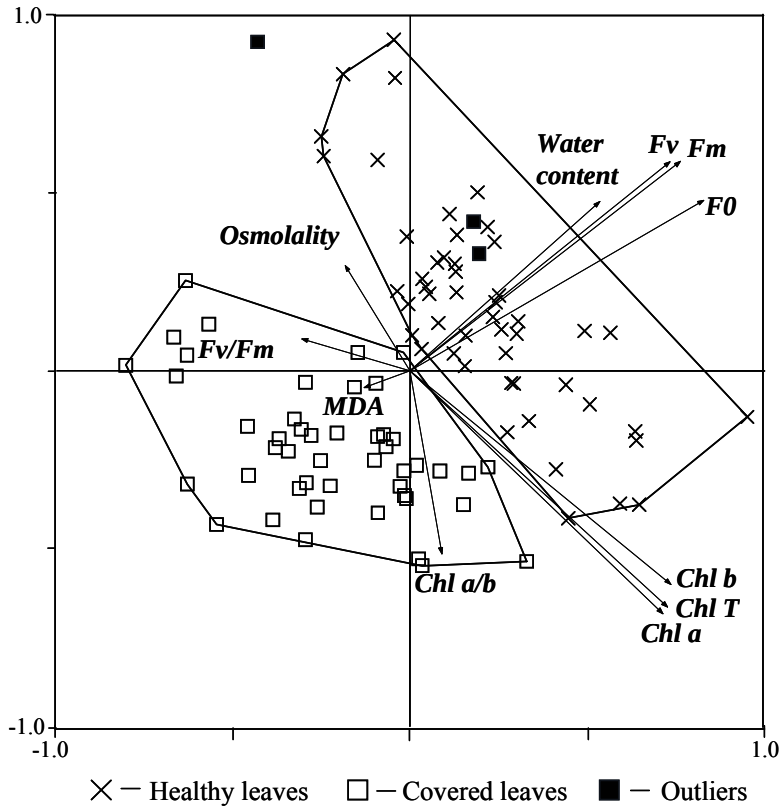

Figure 7.3. Principal Component Analysis (PCA) ordination diagram for the different biochemical parameters measured in healthy and in sooty mold covered leaves. The first and second axis of PCA explained 34.6% and 25.3% of the total variability, respectively.

The differentials of CO_2 concentrations measured during a period of 14 h are shown in Figure 7.4. The main differences were observed between illuminated segment branches where higher differentials of CO_2 concentrations were measured in sooty mold covered leaves than in healthy leaves. During the first four hours of the experiment, negative values for differential of CO_2 concentrations were measured in illuminated healthy leaves. After this, the differential of CO_2 concentrations increased reaching a value slightly above zero that was maintained constant till the end of the experiment. Illuminated sooty mold covered leaves showed mean positive values during the considered period although lower values were measured in the first two hours of the experiment. Considering segment branches placed in darkness, the highest mean values for the differential of CO_2 concentrations were measured in covered leaves, although clear differences between sooty mold covered leaves and healthy leaves were not apparent (Fig. 7.4). However, when the full data set is considered, differences between the four treatments became evident (Fig. 7.5). The interaction between sooty mold and light was not statistically significant ($F_{1,}$

$_{80}$=3.09, p=0.083) and thus this factors can be analyzed independently. Significant differences were found between differential of CO_2 concentration measured in healthy and covered leaves ($F_{1, 80}$=26.66, p<0.001) and between illuminated and darkness leaves ($F_{1, 80}$=205.76, p<0.001).

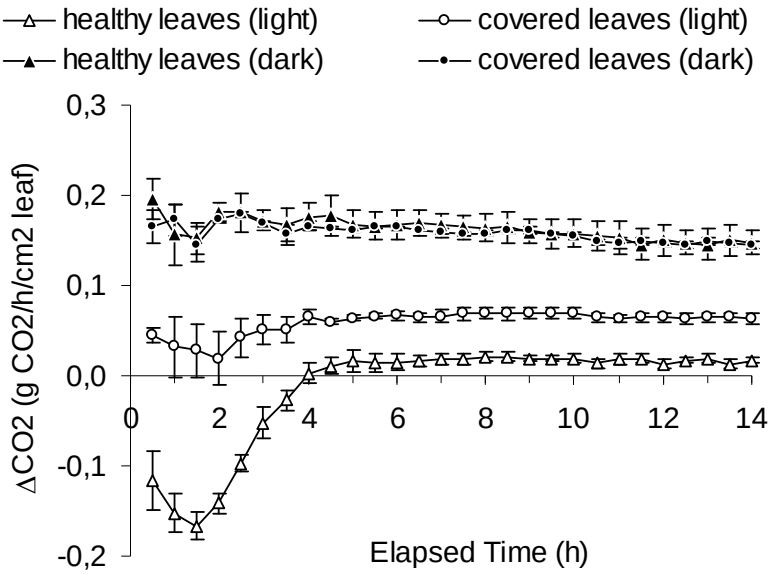

Figure 7.4. Differential concentrations of CO_2 (mean ± SE, n=3) measured during a period of 14 hours in branch segments with healthy leaves and with sooty mold covered leaves placed in the dark or in the light. Arrow indicates the compensation point.

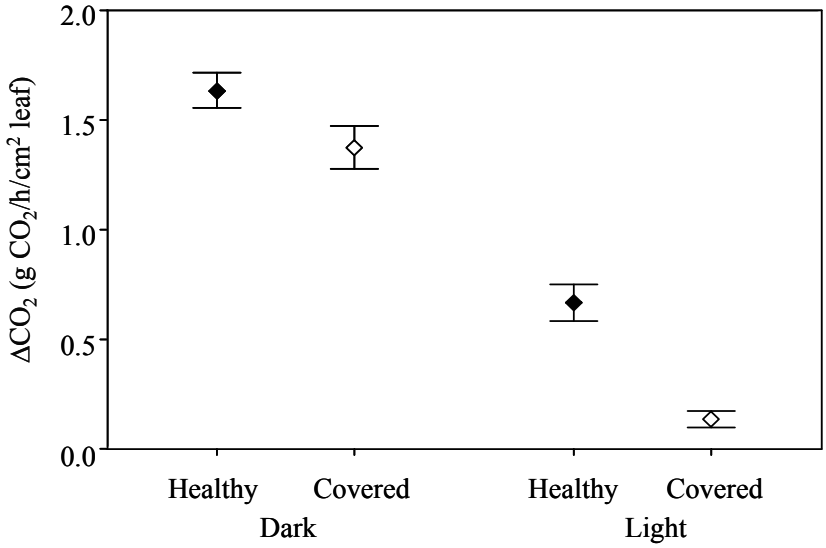

Figure 7.5. Differential concentration of CO_2 (mean ± SE, n=21) measured in olive branch segments with healthy leaves and sooty mold covered leaves placed in the dark or in the light.

The solution of equation 4, to describe the differential of CO_2 concentration, yielded the following result (where three observations were considered outliers and were eliminated):

$$\Delta CO_2 = (1.850 + 0.041 \times HA) + (1.162 + 0.029 \times CA) - (1.819 + 0.039 \times HA) \quad \text{(Light)}$$
$$\Delta CO_2 = (1.850 + 0.041 \times HA) + (1.162 + 0.029 \times CA) \quad \text{(Dark)}$$

with an $r^2_{adj} = 80.5\%$ $(n = 81)$ and $F_{1,\ 79} = 67.2$, $P<0.0001$). From these general equations we can extract partial equations associated with each of the experimental conditions tested in our experimental design.

	Healthy leaves	Covered leaves
Light	$\Delta CO_2 = (1.850 + 0.041 \times HA) - (1.819 + 0.039 \times HA)$	$\Delta CO_2 = (1.850 + 0.041 \times HA) + (1.162 + 0.029 \times CA) - (1.819 + 0.039 \times HA)$
Dark	$\Delta CO_2 = (1.850 + 0.041 \times HA)$	$\Delta CO_2 = (1.850 + 0.041 \times HA) + (1.162 + 0.029 \times CA)$

The residuals were randomly distributed and were independent of both the variations of the healthy area of the leaves (HA) (Fig. 7.6a) and of the covered area with sooty mold (CA) (Fig. 7.6b).

As shown from the scatter plots of measured versus predicted values for the differential of CO_2 concentration, the fitted values compare well with those measured, indicating that the developed model provides a good description of the data (Fig. 7.7).

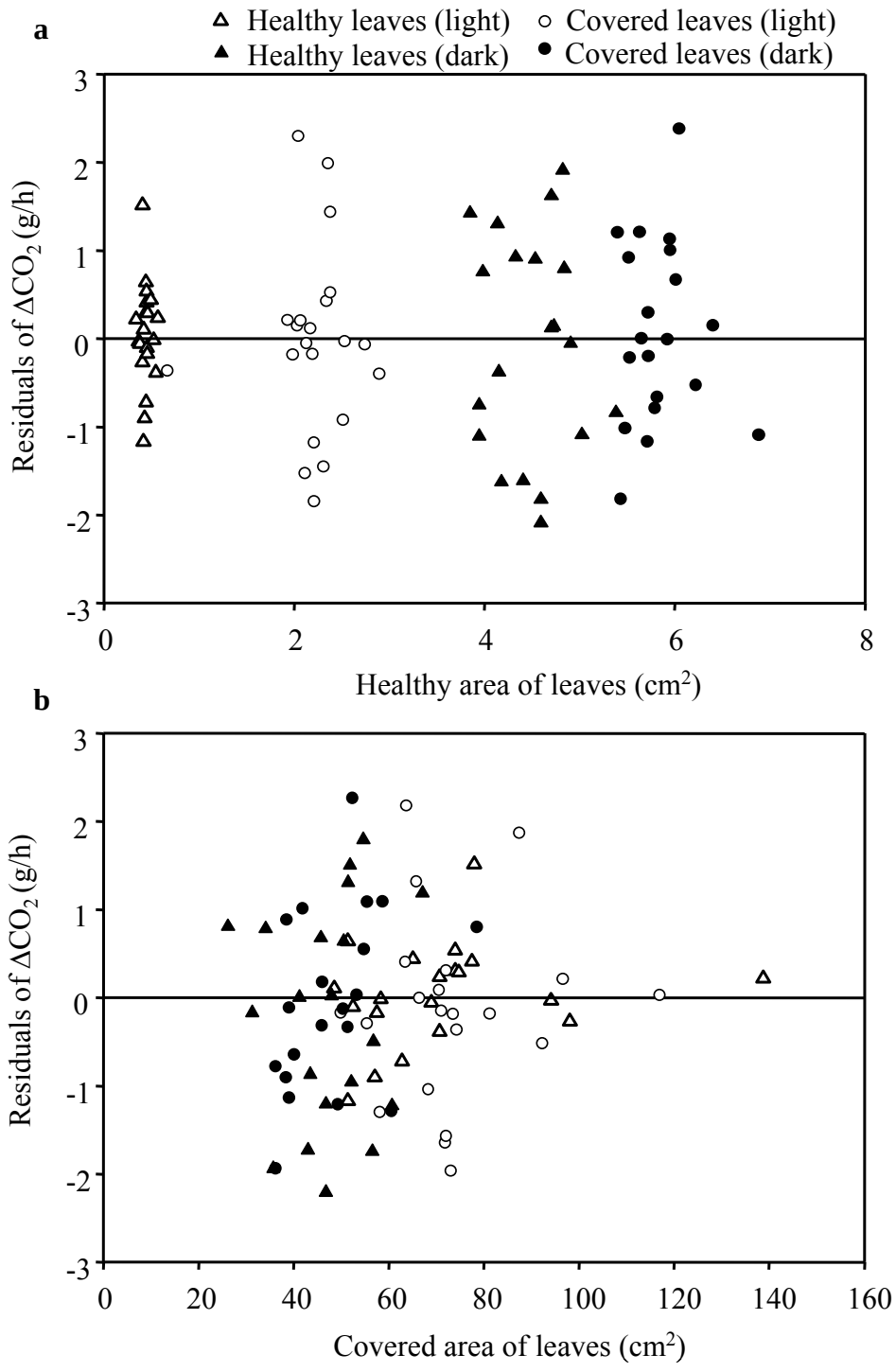

Figure 7.6. Relationship between the residuals from model predictions and each of the independent variables: a) healthy and b) covered area of the leaves.

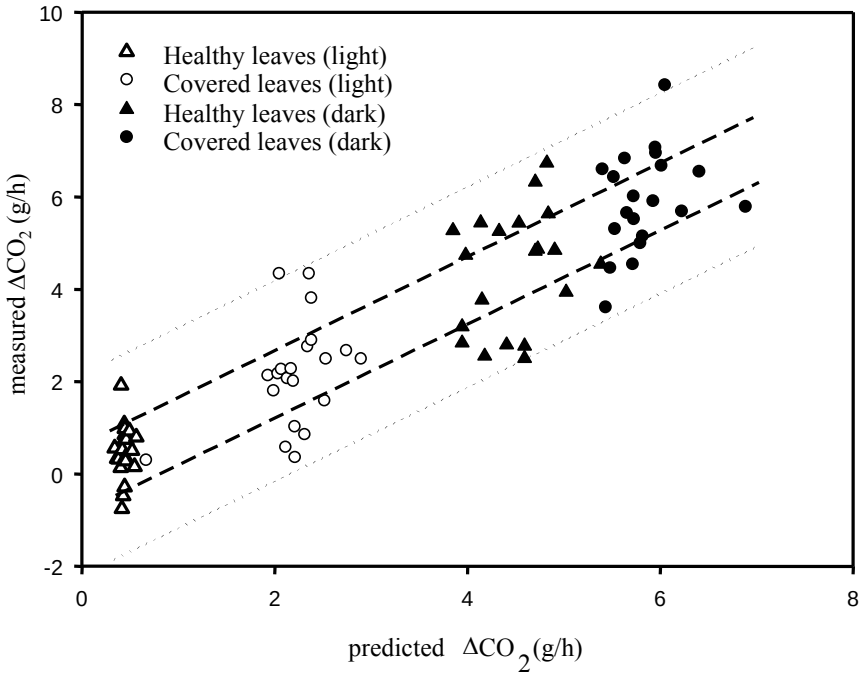

Figure 7.7 – Relationship between predicted and measured changes in CO_2. Dashed lines express the trends in 95% confidence limits and the dotted lines express the trends in the 95% prediction limits (these limits were calculated from Monte Carlo simulations).

In covered leaves, correlation analyzes showed that the area of sooty mold on the adaxial surface did not correlate with the area at the abaxial surface (r=0.113; df=40, p=0.158). However, strong correlations and dependencies were found between total covered area (CA) and:

- the covered abaxial area (CAA_b)

  $CA=12.352 + 0.905\ CAA_b$ (r= 0.732; df=40, p<0.001) (Eq. 9)

- the covered adaxial area (CAA_d)

  $CA=17.696+ 0.868\ CAA_d$ (r= 0.595; df=40, p=0.003) (Eq. 10)

These equations showed statistically identical slopes (ANCOVA: $F_{1,\ 80} = 0.028$; p=0.869), but the elevations were statistically different (ANCOVA: $F_{1,\ 81} = 9.055$; p=0.0035).

7.4. Discussion

Olive trees are adapted to dry conditions by presenting, e.g., leaves with thick waxy cuticle in adaxial surface, and abundant hairs, sunken stomata and small vessels in abaxial; characteristics that have been described for other cultivars too (e.g. Beede and Goldhamer, 1994; Nuberg and Yunusa, 2003). Also, the xylem vessels have small dimensions, a characteristic often present in xerophytic plants (e.g. Larsen *et al.*, 1989; Nuberg and Yunusa, 2003), leading to the prevention of excessive water loss under stress conditions.

Histological data confirmed that the fungi hyphae did not enter the mesophyll tissues, and may form a dense and complex fungi matrix covering both sides of leaves. Furthermore, no significant changes between healthy and covered leaves' mesophyll were found. Also Nieves-Rivera (2005) referred that *Avicennia germinans* leaves covered with sooty mold could remain robust and intact. Similar studies on sooty mold in mahogany (*Swietenia macrophylla*) also showed no penetration in leaves and no histological changes inside the leaves (Filho and Paiva, 2006). The presence of this complex fungi matrix, in particular on the adaxial surface, limits the light reaching the mesophyll cells as presumed by other authors (e.g. Panis, 1977b, Passos-Carvalho *et al.*, 2003). Filho and Paiva (2006) showed that sooty mold on adaxial surface promoted a light blockage of more than 40% of light in mahogany leaves, with severe consequences to photochemical activity, while Wood *et al.* (1988) demonstrated that these fungi complexes block light mostly at wavelengths between 400 and 700 nm.

Besides the main effects on adaxial leaf surfaces, the distribution of the fungi on the abaxial surface, may also affect stomata function and gas exchange. Histological analyses of covered olive leaves showed, in general, a higher distribution of the fungi on abaxial surface with respect to adaxial one. This data strongly supports the hypothesis that, by creating a microenvironment immediately outside the stomata (e.g. increasing the relative humidity), it conditions stomata functioning, affecting gas exchange (photosynthesis, respiration and transpiration).

The average value found for water content in healthy leaves (0.75 g/g DW) is below the average described for other cultivars of olive (e.g 1.56 g/g DW) (Nuberg and Yunusa, 2003). However, one should consider that these olive groves are not associated with an irrigation system. Furthermore, the water content also decreases under sooty mold

coverage conditions to 0.64 g/g DW indicating that plant-water relations may be affected by this disease. In olive tree, sooty mold is strongly associated with black scale that in turn, is associated with high temperatures (Noguera *et al.*, 2003). Therefore, this disease will probably cause more drastic effects during summer increasing stress brought on by limited moisture.

Considering the effects on chlorophyll contents and fluorescence, data show that sooty mold does not affect significantly chl *a* and chl *b* contents. Therefore, the action of this fungi complex on photosynthesis/photophosphorilation will involve mechanisms, other than chlorophyll content. For chlorophyll fluorescence, F_0 represents the minimal fluorescence yield, and happens when all reaction centers are in an active, "open" state. The decrease of F_0, found for covered leaves indicates that sooty mold directly or indirectly affects the PSII reaction centers. In addition, the reduction of F_m may reflect an increase of energy dissipation, and/or indicate a reduction in the saturating light intensity reaching the mesophyll due to the presence of the fungi filter. This last hypothesis is the most likely as the no significant changes observed in $(F_m-F_0)/F_m$ ratio indicating that the PSII photochemical efficiency was not apparently affected. In fact, the F_v/F_m ratio can be considered as a measure of the quantum efficiency of the electron transport in PSII (Maxwell and Johnson, 2000). Thus, the non detected differences in chlorophylls may support that these pigments in the reaction centers may not be affected and that the thick black coverage of the leaf created by the sooty mold acts as a barrier to light absorption significantly reducing the photosynthetic efficiency. Nieves-Rivera (2005) also referred non adverse effects of sooty mold on photosynthesis, though recognized that large covered leaf areas by sooty mold could prevent it. Nevertheless, the light harvesting complexes (LHCs) are also composed by other pigments (e.g. carotenoids) which contents should be analyzed in the future. Moreover, the apparent increase of MDA contents may indicate that covered leaves become to suffer senescence effects (e.g. oxidative stress) (Santos *et al.*, 2001), and the direct or indirect impact of sooty mold on this peroxidation levels deserves further investigation. A well known consequence of lipid peroxidation (and formation of MDA) is membrane degradation, and chloroplasts are well described as being one of the most sensitive organelles during this process (Lutts *et al.*, 1996; Santos *et al.*, 2005). The effect of sooty mold on oxidative stress is particularly important, once the leaves analyzed are from the current season, and therefore, the coverage process may be considered at an

initial stage. Therefore, an analysis of the different stages of sooty mold evolution with time would give valuable information on the interaction between sooty mold attack and oxidative stress as well as chloroplast degradation and eventual leaf-induced senescence.

The model used in this paper showed with high reliability that, the light is a determinant factor in the analyses of CO_2 fluxes associated to sooty mold-leaves interactions. Thus, under light conditions, the net CO_2 flux measured in healthy leaves is due to leaves' respiration and photosynthesis (the combination of Eqs. 1 and 3 gives Eq. 5, see Materials and Methods). In this case, a negative differential of CO_2 flux was measured during the first hours of the experiment in healthy leaves, demonstrating that, in short time, photosynthesis occurred at higher rates than respiration, until a compensation point has been reached. On the other hand, in covered leaves, the net CO_2 flux is due to leaf and sooty mold respirations and leaf photosynthesis (the combination of Eqs. 1-3 gives the Eq. 6). Results showed positive values for differential CO_2 fluxes, even in the first hours of the experiment, supporting that photosynthesis decreased and/or respiration increased.

Under dark conditions, in healthy leaves, only leaf respiration is considered (Eqs. 1 and 7), while in covered leaves both leaf and sooty mold respirations are present (combination of Eqs 1 and 2 gives Eq. 8). Thus, covered leaves presented higher differential CO_2 fluxes than healthy leaves, reflecting the sum of leaf and fungi complex respirations. Olive is a hypostomatic species (e.g. Baldini *et al.*, 1997; Chartzoulakis *et al.*, 1999) and gas exchanges will only take place through the lower surface. Therefore, due to the microenvironments created by the fungi matrix, CO_2 exchanges would be affected by the coverage of the abaxial surface.

Based on the given identical slopes but on different elevations, equations 9 and 10 strongly support the idea that abaxial surface is more susceptible to the establishment of sooty mold and, once it occurs, leads to higher areas of leaf coverage. This fact has never been describe but is highly predictable due to both higher occurrence of the black scale on the abaxial surface (Pereira, 2004), and the characteristics of lower surface (thinner cuticle, stomata, spongy parenchyma, and higher proximity of phloem cells) (e.g. Baldini *et al.*, 1997).

The adopted experimental design and the analyses framework outlined here have the potential to be further exploited in studies involving complex interaction between sooty

mold and host species in general and olive trees in particular. Also the validity of this model at different stages of the disease progression should be explored.

Acknowledgments

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Chapter 8

8. GENERAL DISCUSSION AND CONCLUSIONS

The olive groves sampled to study the canopy arthropod community showed a great diversity of functionally important taxa that were classified into 12 orders: Acari, Araneae, Coleoptera, Dermaptera, Diptera, Heteroptera, Homoptera, Hymenoptera, Lepidoptera, Neuroptera, Psocoptera and Thysanoptera. From those, five families (Formicidae, Coccinellidae, Miridae, Chrysopidae and Anthocoridae) and three pest species were identified, the olive moth *Prays oleae*, the black scale *Saissetia oleae* and the olive psylla *Euphyllura olivina* (Chapters 2 and 4). This diversity was already observed by other authors in different olive groves from the Mediterranean Basin (Petacchi and Minnocci, 1994; Belcari and Dagnino, 1995; Morris *et al.*, 1999; Rodríguez *et al.*, 2003; Ruano *et al.*, 2004).

The management regime used to control olive pests showed to be a critical factor of disturbance for the canopy arthropod community (Chapters 2 and 3). Both Integrated Pest Management (IPM) and Organic Farming (OF) regimes regard the protection of the environment and its biodiversity (Council Regulation (EEC) no. 2092/91 of 24 June 1991; Boller *et al.*, 2004). However, it was observed that in the IPM olive grove, the application of dimethoate to control the anthophagous generation of *P. oleae* caused immediately a significant depletion of the abundance of different functional groups of arthropods. Detritivores (e.g. Psocoptera) and predators (e.g. Miridae, Coccinellidae and Formicidae), in particular, were the most affected taxa, showing also a long recovery period after the application of the insecticide. These effects suggest that spraying insecticides usually do not avoid periods of great activity of predators. Although farmers are aware that insecticide application should occur only when the prey population reaches the economic threshold, they usually spray within a narrow time frame every year (at the end of the flowering period).

Among the most abundant predators found in the olive grove, coccinellids have been referred in the literature as potential natural enemies of *S. oleae* (Argyriou and Katsoyannos, 1977; Velimirovic, 1994; Ba M'Hamed and Chemseddine, 2001; Ba M'Hamed and Chemseddine, 2002). In both olive groves, a total of 23 species were identified (Chapter 3). Nine species were represented in both olive groves and years. From

these, *Chilocorus bipustulatus*, *Scymnus (Pullus) subvillosus*, *Scymnus (Mimopullus) mediterraneus*, *Scymnus (Scymnus) interruptus* and *Rhyzobius chrysomeloides* were the most abundant species completing several life cycles in this agro-ecosystem. Abundance and dominance of species was found to be different between the IPM and organic groves reflecting the impact of the management regime on this community. This may have implications on the preservation of ecological functions associated with coccinellids, namely their role as control agents of *S. oleae*. In the OF grove, the coccinellid community was dominated by *S. interruptus* and *C. bipustulatus* while in the IPM grove, *R. chrysomeloides* and *M. mediterraneus* were the dominant species.

Temporal synchrony was established for the five most abundant coccinellid species and *S. oleae* (Chapter 4). It is important to highlight the association between *S. interruptus*, *P. subvillosus*, *M. mediterraneus* and the first (principally those recently emerged) and second instars nymphs and between *C. bipustulatus* and the second and third instars nymphs of *S. oleae*. These associations were demonstrated by the Correspondence Analysis and are summarized in Fig. 8.1.

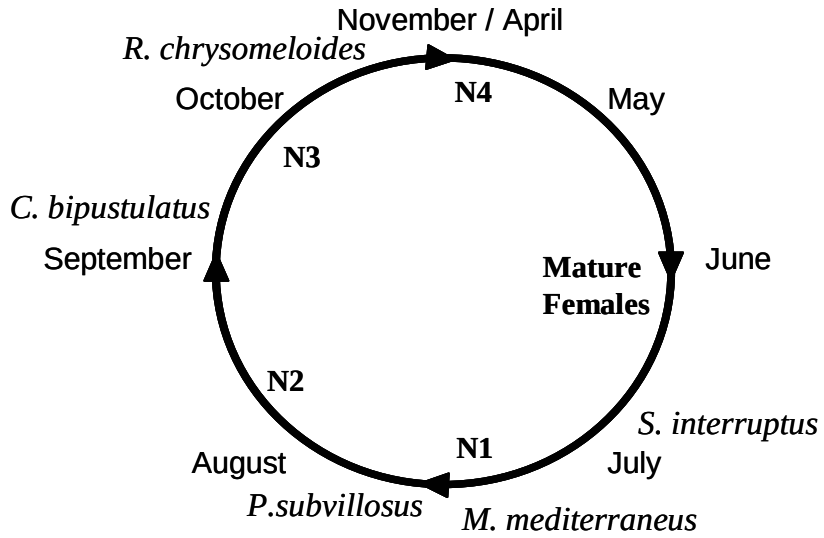

Figure 8.1. Seasonal synchrony among the most abundant coccinellid species found in olive groves and different phenological stages of *S. oleae*.

It was clear that coccinellids have frequent opportunities to prey on different phenological stages of *S. oleae* mainly due to their:

1) Abundance and diversity in the olive grove;

2) Seasonal activity patterns that showed to be well synchronized with *S. oleae* abundance;

3) High voltinism.

The indirect enzyme-linked immunosorbent assay (ELISA) showed that 21.2% of the overall field collected coccinellids (a total of 1322) reacted positively with the *S. oleae* antiserum (Chapter 5). *C. bipustulatus* and coccinellid larvae obtained the highest percentages of positives. In laboratory experiments, *C. bipustulatus* consumed different phenological stages of *S. oleae* and completed its life cycle when fed exclusively with eggs or with first instar nymphs of *S. oleae* (Chapter 6). According with the consumption of phenological stages showed by coccinellid species, it is likely that both *C. bipustulatus* larvae and adults use the black-scale as a frequent food resource and *M. mediterraneus*, *P. subvillosus* and *S. interruptus* use the black-scale as an occasional food resource. Therefore, the occurrence of different coccinellid species may result in a more effective and complementary suppression of the pest through the consumption of different nymphal stages.

In a desirable situation of biological control, a timely pest control is determinant and two aspects should be considered (Chi and Yang, 2003):

- the stage-specific predation rate of predator;
- the stage-specific vulnerability of prey.

In this context, evidences of *S. oleae* predation by *C. bipustulatus* can increase the likelihood of a successfully biological control of this pest in olive groves, bringing valuable indirect advantages to the olive tree, such as the reduction of sooty mold

proliferation, whose effects resulted in a significant decrease of both foliar light absorbance and free gas exchanges (Chapter 7).

Sooty mold attacks are commonly promoted by the presence of the pest *S. oleae*. Although insecticides can be used in integrated pest management systems (IPM) to control insect pests there effectiveness against *S. oleae* is low, at least for those that are less noxious to the environment. Instead, non-target species like coccinellids that predate on *S. oleae*, are strongly affected by their use (Fig. 8.2).

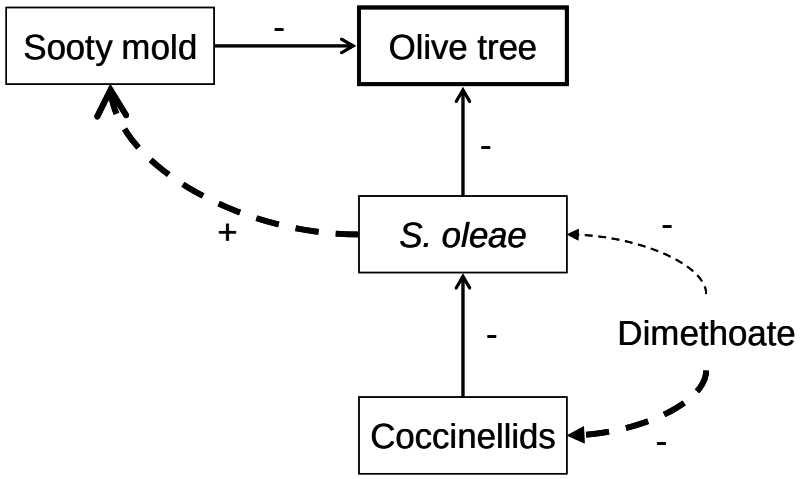

Figure 8.2. Prevailing relationships associated with sooty mold attack and *S. oleae* dynamics. Solid line represents interactions between organisms while dashed lines represent indirect effects (plus or minus signs indicate the type of effect while the thickness of the line reflects the intensity of the effect).

In conclusion, some measures can be taken to protect and also, increase natural enemies populations in the olive grove:

- To forbid the most toxic pesticides;
- To reduce the application of allowed pesticides and avoid cultural practices that reduce their populations;
- To provide supplementary food, shelters and places for hibernation in order to increase their populations;
- To promote mass production and mass release of efficient natural predators. For black scale this can be achieved by mass releasing third and fourth larval stages of

C. bipustulatus in the beginning of August, when first instar nymphs of the pest are the most abundant stage.

8.1. References

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