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THE USE OF HABITAT MANAGEMENT, ELICITORS AND AUGMENTATION TO IMPROVE BIOLOGICAL CONTROL IN VINEYARD

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Abstract

Nowadays, pesticides represent the most common tool used to control pests in agriculture. Because of the detrimental effects both on environment and human health related to chemical defence strategies, more ecological approaches need to be tested.

My dissertation aims to evaluate some sustainable biological control strategies in vineyard. Natural enemy (*Anagyrus vladimiri* Triapitsyn (Hymenoptera: Encyrtidae) and *Cryptolaemus montrouzieri* (Mulsant) (Coleoptera: Coccinellidae)) were released against mealybugs (Hemiptera: Pseudococcidae) and the role of tending ants was investigated in beneficial-mealybug-ant tritrophic relation. Habitat management approaches were adopted to mitigate *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae) infestation and enhance beneficial activity. Finally, silica gel, a silicon-derived compound, was tested to study its potential role in eliciting plant defence response, including parasitoid attraction. Field trials were carried out in Northern Italy, in important viticultural areas of Lambrusco and Pignoletto wines.

Results showed the great potentiality of *A. vladimiri* and *C. montrouzieri* in controlling both *Planococcus ficus* (Signoret) and *Pseudococcus comstocki* Kuwana infestation. The use of sugar dispensers to disrupt ant activity increased ecosystem services and reduced mealybug infestation. Ground cover management did not reduce *L. botrana* infestation, but it boosted parasitoid biodiversity. Finally, the treatments with silica gel enhanced the attraction towards three important Hymenoptera parasitoid families: Mymaridae, Encyrtidae and Braconidae.

In conclusion, these strategies showed a potential both in suppressing pest infestation and enhancing natural enemy activity and attraction. Further studies should focus on combining environmentally-friendly practices, creating a synergistic effect which has the potentiality to reduce or even avoid insecticide treatments in some vineyard context.

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Chapter 1 Introduction

In this thesis, I aim to explore some sustainable biological control strategies in vineyard ecosystem. In particular, augmentative and conservation biological control are investigated against two of the most damaging vineyard pests. Regarding augmentation, the thesis is focused on inoculative releases of the two most important natural enemies of mealybugs. Considering the critical and disruptive role that ants play in the trophic relation between predators, parasitoids and mealybugs, a faunistic study of ant population and control method are also explored.

Concerning conservation biological control, several measures can be adopted to enhance beneficial abundance and activity. Here I focus on habitat management approaches, especially the effect of ground cover management, on European grapevine moth and its parasitoids.

Finally, I focus my thesis on a new strategy based on plant defence chemical signals which allow the communication with other trophic levels. The potential of elicitors, especially silicon, on the attraction of natural enemies is investigated.

Vineyard sites that I considered for my field trials are located in two provinces of Emilia Romagna Region: Reggio Emilia, that is known for Lambrusco wine, one of the most exported worldwide, and Bologna, principally characterized by Pignoletto production.

Firstly, I will briefly introduce the necessity of biological control adoption in vineyard systems and I will make a short presentation on augmentation and conservation biological control topic, as well as on chemical ecology. Secondly, I will describe the most important Italian vineyard pests, including their economic damages and control strategies. Next, I will present the specific aims of the thesis followed by 5 chapters, each describing my field trials.

1.1 Biological control

World population is expected to reach 9.7 billion in 2050 and 11.2 billion in 2100 (McNabb, 2019). Considering these projections, the food production must be doubled to feed the entire human population (Reddy, 2017). Pesticides cannot be considered the main solution anymore. Several side effects are associated with chemical applications, such as insecticide resistance, human health issues and environmental degradation and pollution (Deguine et al., 2019; Gagic et al., 2018; Rayl et al., 2018; Rusch et al., 2017). Moreover, market access issues and decreased consumer tolerance towards pesticides represent another aspect linked to chemical use (Gagic et al., 2018; Rayl et al., 2018; Tompkins et al., 2012).

In this scenario, "sustainable intensification" and environmentally-friendly alternatives need to be adopted to guarantee production yield and quality (Deguine et al., 2019; Rayl et al., 2018).

An environmentally safe pest management method is biological control, whose aim is to reduce damaging organism population by means of the use of another organism population (van Lenteren et al., 2018). There are four different strategies of biological control: natural, classical, augmentative and conservative (van Lenteren et al., 2018). Natural biological control is achieved when there is a pest population reduction by means of their natural enemies, whereas classical biological control takes place when natural enemies, after being collected in the area of origin of the pest, are released where the pest is invasive (van Lenteren, 2012; van Lenteren et al., 2018). Augmentative biological control (ABC) consists in the mass rearing and release of natural enemies to obtain an immediate control of the pest population (inundative biological control) or for control the pest for several generations (inoculative biological control) (van Lenteren, 2012; van Lenteren et al., 2018). Finally, the goal of the conservation biological control (CBC), in particular the "top-down" effect, is to support natural enemies and enhance their fitness, increasing plant biodiversity and so providing shelter, nectar, alternative hosts/preys and pollen (SNAP) for beneficials, including habitat management (Begg et al., 2017; Gurr et al., 2017).

Another way to promote efficient ecologically-based alternatives to chemicals is boosting plant resistance (Alhousari & Greger, 2018). Plants produce Herbivore Induced Plant Volatiles (HIPVs) after being attacked by pests and these plant emissions can be exploited by natural enemies to find their hosts (Silveira et al., 2018) and so to enhance pest control. The combination of chemical ecology and conservation biological control offers new possibilities in crop defence strategies, applying for example the "attract and reward" approach (Salamanca et al., 2018; Simpson et al., 2011a). Natural enemies are attracted by volatiles emitted by plants after pest infestation or by synthetic HIPVs treatment, while nectar plants and in general plant diversity foster beneficial residency in the crop (Silveira et al., 2018).

1.1.1 Augmentative biological control

Augmentative biological control (ABC) is applied in many cropping systems, such as fruit and vegetable crops, cereals, maize, cotton, sugarcane, soybean and many greenhouse crops. A list of natural enemies used for ABC programs is available in van Lenteren et al., (2018).

It is a popular approach to manage pests on which pesticides are inefficient due to phytophagous resistance. Successful ABC strategies, such as the use of predators to control thrips and whiteflies on sweet peppers in greenhouses in Spain or hemipteran predators to manage *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) infestations, highlight the importance of augmentative biological control in agricultural areas that otherwise would have had to cease vegetable production (van Lenteren et al., 2018). Nevertheless, there are several issues that hamper biological control adoption. Regulations concerning environmental risks and poor dissemination to growers and stakeholders are only some of the aspects that impede a larger diffusion of biological control (Barratt et al., 2018).

The growth of biological control agent market is faster than pesticide one, although it represents less than 2 % of chemical product business. The commercial biological market has shown a sale increase of more than 15% per year since 2005 (van Lenteren et al., 2018).

1.1.2 Conservation biological control

Agricultural landscapes have deeply and dramatically changed due to food production intensification. This has led to a huge simplification of agroecosystem and, consequently, biodiversity loss and pest problems have intensified (Bianchi et al., 2006; Lu et al., 2014). Increasing disturbances threatens beneficial insects, generating less pest suppression and greater need for insecticides, the so-called pesticide treadmill (Jonsson et al., 2015).

It is well known that greater species diversity results in greater stability of communities and, consequently, of ecosystem functioning over time (Evans, 2016). In this context, CBC adopts two different approaches to enhance agrobiodiversity and re-establish ecosystem stability: the bottom-up effects, also called also "resource concentration hypothesis", and the top-down effect, the "enemy hypothesis". The first one acts directly on herbivores through vegetation, whereas the second one is based on natural enemies mediated techniques to control pests (Gurr et al., 2017). Beneficial complex is affected by landscape composition: different habitats can sustain and support a broad spectrum of natural enemies (Bianchi et al., 2006), promoting multi-trophic relations and, consequently, pest control. CBC encompasses several techniques (Gurr et al., 2017; Rayl et al., 2018), which can increase biodiversity both at local scale (within the crop) and at landscape level (outside the crop) (Wilson et al., 2017). Concerning the top-down effect of CBC, the main goal is to enhance natural enemy activity and fitness providing them with SNAP resources. For example, hedgerows and other non-crop areas represent important natural enemy overwintering sites and suitable places to find alternative hosts and preys. The case of *Anagrus* spp. is paradigmatic (Altieri et al., 2010; Ponti et al., 2003). This parasitoid can survive on alternative leafhopper eggs located in natural vegetation around vineyard to overwinter. In this context, hedges allow the persistence of *Anagrus* spp. in the crop and his timely activity when grape leafhoppers appear.

Hedgerows and field margins can also act as a protection site from hostile climate conditions, such as period of heat, cold, wind and rain, and pesticides (Gurr et al., 2017; Rodriguez-Saona et al., 2012). Some plants possess naturally-occurring shelter, called domatia, that offer important protected locations for predatory mites. It has been demonstrated that some grape cultivars are less susceptible to mite outbreaks thanks to domatia (Rodriguez-Saona et al., 2012).

Regarding food resources, the majority of natural enemies are omnivores, so they rely on non-prey food to survive (Rodriguez-Saona et al., 2012). It is well known that flower resources supply nectar and pollen to beneficials, increasing their longevity, fecundity and also predation and parasitization (Gurr et al., 2017; Wäckers & van Rijn, 2012; Foti et al., 2017). Sugar and pollen are required by adult natural enemies to survive and reproduce (Deguine et al., 2019). Although, parasitoids and predators can rely on extrafloral nectar (Gurr et al., 2017) and honeydew, when nectar is scarce (Gillespie et al., 2016). Nectar is a critical carbohydrate resource for parasitoids, providing them with amino acids and protective enzymes and offering them a quickly exploitable energy source, particularly for flight (Deguine et al., 2019).

Within local management strategies, several plants can be added to the main crop to manage pests. These secondary plants affect differently the trophic levels in a crop system and for this reason they are classified in several way (Parolin et al., 2012). As insectary plants, flower resources have been widely used to foster natural enemy abundance and boost ecosystem services, such parasitization and predation, in several crop systems (Balzan et al., 2016; Begum et al., 2006; Berndt et al., 2006; Burgio et al., 2016; de Pedro et al., 2020; Hoffmann et al., 2017; Irvin et al., 2016; Ramsden et al., 2015; Woltz et al., 2012).

Deep knowledge of natural enemies and flowers is required to choose the most suitable flower blend to enhance beneficial activity (Gurr et al., 2017). Flower morphology and colour, nectar composition and availability and natural enemy mouthpart structure are critical elements which define the flower suitability. Moreover, floral species needs to be carefully evaluated for the appropriate cropping system. Flowering plants may provide resources to herbivores, ants or hyperparasitoids, causing in some cases ecosystem disservices (Deguine et al., 2019; González-Chang et al., 2019). Finally, also agronomic aspects, such as competition between the crop and the flower strips for water, nutrients and light, need to be taken into account for CBC success (Gurr et al., 2017).

Among flower resources, buckwheat (*Fagopyrum esculentum* Moench.), sweet alyssum *(Lobularia marittima* L.) and phacelia (*Phacelia tanacetifolia* Benth.) are the most commonly used (Jado et al., 2018). Several studies have shown the potential of these and other flowering plants. In vineyard ecosystem, Irvin et al. (2016) highlighted that buckwheat is effective in attracting natural enemies, collecting 27 times more insects than in control plots. Burgio et al. (2016) demonstrated that alyssum, buckwheat and the "vetch and oat" mixture significantly attracted some Hymenoptera parasitoid families in Northern Italy vineyard. Not only beneficial abundance can be enhanced, but also ecosystem functions and services can be fostered through cover crops. Hoffman et al. (2017) showed that phacelia and *F. esculentum* increased the predation of *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae) eggs, whereas Berndt et al. (2006) and Begum et al. (2006) demonstrated higher parasitisation of *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae) in treatments with flowers strips compared to ground cover. Finally, Sommaggio et al. (2018) and Silva et al. (2010) confirmed a higher abundance of beneficial arthropods associated to the presence of sowed selected species in respect to monocultural plots.

A list of other useful plants for attracting natural enemies in vineyard ecosystem is provided by Altieri et al. (2010). Besides selected flower strips, also endemic plants can be used to provide essential resources for natural enemies. Endemic plants are more likely adapted to local conditions and may require less maintenance compared to exotic ones. Zanettin (2018) highlighted that non mowed spontaneous grass in vineyard inter-rows increased the presence of natural enemies in Northern Italy. Daane et al. (2018) showed that native grasses reduced leafhopper pest presence and parasitism rate was higher in grass treatment than bare soil in a Californian vineyard. Also in 2010, Daane et al. confirmed that *E. postvittana* egg predation increased in the native grass cover crop. Finally, Shields et al. (2016) demonstrated that native plant species provided multifunctional ecosystem benefits, including pest management increase.

Despite extensive literature and increasing interest in conservation biological control in the last 20 years, its application among farmers remains quite limited. Growers principally count on insecticides for pest management and the low predictability of natural enemy-based control does not foster the implementation of CBC techniques (Holland et al., 2016). Even if beneficial insects respond positively to conservation strategies, agroecological manipulations do not often achieve an effective pest regulation and suppression, probably contributing to low adoption of CBC approach by farmers. Habitat management is a complex pest suppression strategy, in which multiple factors may influence its success (Begg et al., 2017). Thus, focused field trials have to be implemented, taking the biogeography and the crop ecosystem characteristics into account.

1.1.3 Chemical ecology and multitrophic relations

The use of semiochemicals is another tool to boost natural enemy abundance and biological control, exploiting those regulating the interactions among organisms. Using plant volatiles, which represent a critical signal for multi-trophic relations, it is possible to chemically repel herbivores, but also augment, conserve and enhance natural enemy activity in cropping systems (Cook et al., 2007; Rodriguez-Saona et al., 2012).

1.1.3.1 Herbivore Induced Plant Volatiles and Elicitors

Interactions between plants and herbivores are central to all ecosystem (Mithöfer et al., 2018). In particular, several defence mechanisms have been evolved by plants to face herbivores and mites (Dicke & van Poecke, 2002). Besides direct defence responses, chemical signals can be produced by plants to attract natural enemies, employing them as "bodyguards" (Dicke & van Poecke, 2002). This recruitment phenomenon occurs by means of volatile production, called HIPVs (Herbivore Induced Plant Volatiles). The emission of these substances is triggered by herbivore oral secretions which act as elicitors. In particular, volatile compound emission is mediated by phytohormones, such as jasmonic acid (JA), salicylic acid (SA) and ethylene. Among these, JA represents the most important phytohormone mediating HIPV production (Dicke, 2009). The Octadecanoid pathway is responsible for JA production. In particular, herbivore attack induces the lipids of the membrane to release linolenic acid which is then converted into jasmonic acid (Ayoub & Afroz, 2017).

The three major classes of compounds that can be produced after an herbivore attack are green leaf volatiles (GLV), aromatic compounds and terpenoids. They can form different blends that vary with the genotype of the plant and the species and developmental stages of the herbivore (Heil, 2008). In this way, natural enemies can discriminate plants with herbivores with a different value to carnivore (Dicke, 2009).

HIPVs can be exploited not only by predators and parasitoids, but also by other community members, such as parasite plants, pollinators or herbivores themselves (Dicke, 2009). Pests, indeed, can use these volatile compounds to locate their suitable hosts or avoid plants that have been already infested (Heil, 2014). Also, phenomenon of "associational resistance" can happen. Plants can passively benefit from volatiles released by neighbours (Heil, 2014) and activate their defence response preventively (Guerrieri, 2016).

Interest in HIPVs has deeply increased since 1983, as shown in Heil (2014). Several studies on the potential of volatile compounds as tool for biological control were carried out in the last few years. The majority of researches focused on the second trophic level, repelling the herbivores, whereas only few studies concentrated on parasitoids and predators (Parrilli et al., 2019). So far works on this topic have been more commonly carried out in laboratory than in field.

James and Price (2004) demonstrated that methyl salicylate (MeSA) enhanced the attraction of some species of predators and parasitoids. Also Lucchi et al. (2017) showed that sticky traps baited with a blend of methyl salicylate, acetic acid and 2-phenylethanol were highly attractive to *Chrysoperla* adult lacewings. Orre et al. (2010) highlighted that synthetic HIPVs can have an effect not only on pest parasitoids, but also on individuals of the fourth trophic level. Indeed, in their work, synthetic methyl salicylate lured the lacewing parasitoid, *Anacharis zealandica* Ashmead (Hymenoptera: Figitidae).

Besides the use of synthetic volatiles, plant defence against pests can be enhnanced using HIPV-eliciting compounds, also called resistance inducers or elicitors. They trigger plant immune system mimicking herbivore oral secretions, leading to volatile production.

Historically, elicitors have been mainly employed to manage pathogen infections (Walters et al., 2013), but some applications have been carried out to improve plant resistance against herbivores (Turlings & Erb, 2018). Both biotic and abiotic elicitors have been used to increase plant defence against pests. For example, parasitoid species of *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae) were significantly attracted by benzo- (1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) and laminarin treated plants (Sobhy et al., 2014).

Moreover, Coppola et al. (2017) showed that tomato plants treated with *Trichoderma harzianum* T22 attracted aphid parasitoid when infested, whereas Xin et al. (2016) demonstrated that (Z)-3-hexenol increased jasmonic acid and ethylene production, attracting the parasitoid of the tea geometrid *Ectropis obliqua* Prout (Lepidoptera: Geometridae).

Among elicitors, silicon (Si) is one of the most studied. It represents the second most abundant element in the Earth crust and, even if it is not fundamental for plant development and growth, it assumes several critical roles in plant defence. The majority of researches has focused on the effect of silicon treatment on fungal disease, whereas few studies have observed the effect of this compound on vine-resistance to arthropod pests. The absorption of bioavailable Si by plants induces the strengthening, the abrasiveness and the hardness of plant tissues. High level of silicon in leaves, stems or roots hinders herbivore feeding activity, delaying or making mouthparts penetration on host tissues impossible for chewing insects. Thus, insect susceptibility to beneficials and adverse weather conditions may increase (Alhousari & Greger, 2018).

Moreover, the palatability and digestibility are strongly reduced in silicon enriched tissues, hampering pest growth and leading to herbivore midgut damages due to high Si content in plants (Alhousari & Greger, 2018; Leroy et al., 2019). High concentration of Si element act also biochemically, increasing defence gene expression and so promoting the production of defensive enzymes, such as lipoxygenase and polyphenol oxidase (Leroy et al., 2019).

Finally, silicon can have an influence on HIPV production, inducing and amplifying their release or altering their blend (Alhousari & Greger, 2018). The accumulation of this element in the plant has an impact on phytohormone level (Luyckx et al., 2017). In particular, interaction between jasmonic acid and silicon has been recently detected. Parrilli et al. (2019) showed that silica gel treated plants produced higher level of JA compared to untreated plants.

Effects on both second and third trophic levels have been demonstrated. Yang et al. (2018) revealed that the feeding of brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), was lower in Si-amended than in non-amended rice plants in the early stages post-pest infestation. *Zinnia elegans* Jacq. treated with soluble silicon increased resistance against *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), as reported by Ranger et al. (2009). Regarding effects on natural enemies, Kvedaras et al. (2010) demonstrated that cucumber plants treated with silicon and infested with *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) significantly attracted more adults of the predator *Dicranolaius bellulus* (Guèrin-Mèneville) (Coleoptera: Melyridae) compared to Si untreated and infested plants. Finally, Parrilli et al. (2019) showed that silica gel treated plants attracted more individuals of Mymaridae (Hymenoptera: Chalcidoidea), an important group of egg-parasitoids, than control in vineyard context.

1.2 Main pests and associated natural enemies in Italian vineyards

There are several arthropod vine pests in Europe. A number of leafhoppers, such as *Empoasca vitis* (Goëthe) and *Scaphoideus titanus* (Ball) (Hemiptera: Cicadellidae), and spider mites, e.g. *Panonychus ulmi* (Koch) and *Eotetranychus carpini* (Oudemnas), are of economic importance in vineyards (Pertot et al., 2017). Currently, the most detrimental pests are represented by European grapevine moth, *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae) and *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) in the Mediterranean area (Lucchi et al., 2019). Moreover, in the last few years, besides *P. ficus*, a species native to Eastern Asia, *Pseudococcus comstocki* Kuwana (Hemiptera: Pseudococcidae), is causing several damages on vine plants, resulting in important economic losses for growers.

1.2.1 Mealybugs (Hemiptera: Pseudococcidae)

Vineyard mealybugs have caused serious economic losses in the last decades. Female individuals of this family are characterized by white and powdery wax which covers their bodies.

Pseudococcidae encompasses several species which look similar but show different morphological and molecular traits, geographic origin and distribution, host plant preferences, economic injury and control strategies (Daane et al., 2012).

Italian viticulture is threatened by two main mealybug species: *P. ficus* and *P. comstocki*. The identification of the two species is based on wingless female individuals and often requires molecular analysis. Indeed, both species have female individuals characterized by oval bodies surrounded by 17 short wax filaments and just two longer posterior pair. *Planococcus ficus* has posterior wax filaments slightly longer (1/8 of body length) than others, whereas *P. comstocki* shows posterior wax filaments which are 2/3 of body length (Mani & Shivaraju, 2016).

1.2.1.1 *Planococcus ficus* (Signoret)

Planococcus ficus, whose origin is Paleartic, is one of the key vineyard pests worldwide (Cocco et al., 2018; Mansour et al., 2018; Pacheco da Silva et al., 2016). The species is also commonly known as vine mealybug and is recorded in the Mediterranean basin (Italy, France and Spain), California, South America (Argentina and Brazil) and South Africa (Lucchi et al., 2019).

Morphologically, female body is yellow when newly molted, and then pink, orange-brown when mature (Mani & Shivaraju, 2016).

Three generations occur annually in the North of Italy. The first one starts in mid-April, when the overwintering females begin to oviposit their eggs, and ends in June. Adult female of the first generation oviposit on vine trunks at the end of June, so the nymphs of the second generation can infest shoots, leaves and gradually bunches. When the juvenile mealybugs reach the maturity, the eggs of the third generation are laid and the adults of this generation start to appear at the beginning of September principally on bunches. In presence of particularly good weather conditions, a 4th generation could occur. Adult females are the main overwintering stage, but also females with ovisac, eggs or the nymphs can overwinter under vine bark (Cravedi et al., 2010).

1.2.1.2 *Pseudococcus comstocki* (Kuwana)

The Comstock mealybug (CMB) is an extremely polyphagous pest native to eastern Asia. Gradually, it was accidentally introduced in central Asia and eastern Europe (Georgia, Ukraine, Russia and Moldova), in the USA, Canada and South America (Argentina and Brazil). The first record in western Europe dates back in 2004, when this mealybug was collected on mulberry trees in north-eastern Italy. Later on, CMB infestations were discovered on several species of ornamentals and then in commercial peach, apple and pear orchards (Pellizzari et al., 2012). More recently, the Comstock mealybug has caused detrimental damages on vine plants in Italy, especially in Emilia-Romagna, Trentino Alto-Adige and Veneto region.

Pseudococcus comstocki overwinters in the egg stage and 2-4 generations occur annually, depending on the weather conditions. In the north of Italy, the pest usually develops 3 generations (Pellizzari & Mori, 2013). The egg hatching starts in March-April when plants restart vegetative growth, while first adult females appear from June until mid-July. Second generation starts between the end of June and the beginning of July, when adult females begin to lay eggs. Second generation females are observed from the beginning of August until mid-September, whereas 3rd generation adults appear at the beginning of October (Pellizzari & Mori, 2013).

Both CMB and *P. ficus* have immature instars highly mobile that spread very fast (more or less 4 cm per minute) due to their long legs (Mani & Shivaraju, 2016). In addition, CMB adults move faster and farer than *P. ficus* ones.

1.2.1.3 Damages

Mealybug spread through various means, such as air currents, ants, planting materials, animals, farm equipments and personnel, makes them extremely dangerous and increases their potential to cause severe damages to crops (Mani & Shivaraju, 2016).

The comstock and vine mealybug are responsible for similar damages in vineyard. Besides feeding activity, the main damage is caused by the excretion of large amount of honeydew which drops on leaves and bunches, fostering sooty mould formation. Moreover, severe infestation reduces plant vigour and a critical weakening can occur. All these damages compromise seriously bunch production, leading to severe economic losses for growers.

Furthermore, mealybugs are vectors of various grape pathogens, such as the grapevine leafroller-associated virus (GLRaV), the Grapevine virus A (GVA) and the corky-bark disease (Cocco et al., 2018; Lucchi et al., 2019; Mani & Shivaraju, 2016; Pacheco da Silva et al., 2016; Pellizzari et al., 2012).

1.2.1.4 Control methods

Managing mealybug infestations shows several difficulties. Besides the high number of offspring produced by each female adult, another criticism related to mealybug control is their behaviour. During their life cycle, these pests are often located in hidden places which make them difficult to be found, monitored and also to be reached by chemical products. Indeed, they overwinter under the bark of vine trunk or under the soil, next to the root zone, and colonize bunches, which are barely exposed locations, especially for compact grape varieties (Mani & Shivaraju, 2016).

Insecticides represent the main strategy to control mealybugs. A number of products can be used in Italian vineyards:

a. Mineral oils: they are usually applied in March, during bud swelling. They are not so efficient against mealybugs, also because most of them are not exposed yet, but carefully protected under the bark in the period of oil application. Oils are used mainly in organic vineyards;
b. Pyriproxyfen: this active ingredient is part of IGR insecticides. It has to be applied before blooming and acts on the most mobile mealybug stages;
c. Spirotetramat: it is a systemic pesticide which acts interrupting the lipid biosynthesis in the insects. It is particularly efficient on juvenile stages when the plants have high vegetative activity. So, it is critical the right timing of application in order to obtain satisfying results;
d. Acetamiprid: this neonicotinoid insecticide is not so effective on mealybugs. Anyway, this product is also allowed for treatments against *S. titanus*, so it can have a collateral effect also on CMB and vine mealybugs.

Pesticides do not provide satisfactory control against these pests, especially in the long period. Mealybug resistance, the wax barrier of their body and their ability to locate in hidden and protected parts of the plant reduce the effectiveness of treatments and management. Moreover, these chemicals, above all neonicotinoids, have detrimental impacts on the environment including beneficial arthropods (Mani & Shivaraju, 2016).

Agronomic control can help managing mealybug infestations. Extremely vigorous plants foster pest population, in particular adult females increase the number of eggs in each ovisac. Furthermore, excessively vigorous canopy creates the perfect environment for mealybug development, increasing the humidity, reducing lighting and providing more sheltering locations. Thus, pruning during high vegetative plant growth can be helpful in controlling these pests (Mani & Shivaraju, 2016).

Another possible tool that can be used to control mealybugs is mating disruption. Synthetic sex pheromone of *P. ficus*, S-lavandulyl senecioate, has been applied using sprayer or dispenser (Cocco et al., 2011; Lucchi et al., 2019; Walton et al., 2006). Although the knowledge about this technique is still limited and needs to be implemented, promising results have been accomplished. Lucchi et al. (2019) highlighted that a novel hand-applied reservoir dispenser, Isonet PF® reduced the percentage of vine mealybug infested bunches and also the number of this pest per bunch compared to the control. Also Cocco et al. (2018) demonstrated that consecutive applications of mating disruption over the years would increase the efficacy of vine mealybug management.

Finally, biological control has been practised for many years against mealybugs around the world by means of natural enemy augmentation. Among the several beneficials that have been reported to control mealybugs, *Anagyrus pseudococci* Girault and *Cryptolaemus montrouzieri* Mulsant represent the ones most frequently used (Daane et al., 2004; Muştu et al., 2008).

Ants can also affect the mealybug management, negatively impacting on chemical control and on natural enemy activity. Some species of ant and mealybugs create a mutualistic relationship in which trophobiont mealybugs offer their honeydew to ants, whereas attending ants transport this pest, provide better hygiene conditions due to honeydew removal and tender protection from adverse weather and natural enemies (Beltrà et al., 2017; Buckley & Gullan, 1991; Mani & Shivaraju, 2016). Ants can become very aggressive in presence of mealybug parasitoids or predators, biting them, spraying formic acid and also killing them. Consequently, managing attending ants is highly recommended for optimal natural enemy control against this pest (Beltrà et al., 2017; Mani & Shivaraju, 2016; Mgocheki & Addison, 2009).

Natural enemies: *Anagyrus vladimiri* Triapitsyn

Anagyrus pseudococci (Hymenoptera: Encyrtidae) is a solitary koinobiont endoparasitoid of mealybugs and it has been widely used against *Planococcus citri* Risso (Hemiptera: Pseudococcidae) and *P. ficus* (Bugila et al., 2014; Franco et al., 2011; Mansour et al., 2010; Suma et al., 2012a). This parasitoid also attacks distantly related species, such as *P. comstocki*, *Phenococcus herreni* Cox and Williams, *Dysmicoccus brevipes* (Cockerell) and *Maconellicoccus hirsutus* Green (Hemiptera: Pseudococcidae) (Daane et al., 2004). Also in Italy, Guerrieri and Pellizzari (2009) showed that CMB presents as antagonist *A.* sp. near *pseudococci* species.

Previously described by Triapitsyn et al. (2007) and then confirmed by Andreason et al. (2019), morphologically highly similar species are included in the *Anagyrus pseudococci* complex, the members of which share the dark or partially dark first funicle segment of the female antenna (Andreason et al., 2019). In particular, this complex encompasses two reproductively and genetically different species: *Anagyrus pseudococci* and *Anagyrus vladimiri*, so far called *A.* sp. near *pseudococci*. *Anagyrus pseudococci* can be found in Argentina and California, where it was accidentally introduced in the early 1990s, as well as in Sicily and Cyprus. On the other hand, *A. vladimiri* was identified in California, Turkmenistan, Ukraine, Spain, Israel, Italy (Suma et al., 2012b; Triapitsyn et al., 2007) and Tunisia (Mansour et al., 2018). Sicily is the only place in the Mediterranean basin where both species are present (Franco et al., 2011; Triapitsyn et al., 2007). According to Triapitsyn et al. (2007), the host preference by this two described species of *A. pseudococci* complex needs to be futher investigated and verified due to likely misidentifications of both wasps and host mealybugs. Indeed, Andreason et al. (2019) highlighted that numerous recent publications have used both names, *A. vladimiri* and *A. pseudococci*, for apparently the same parasitoid species.

Anagyrus pseudococci can oviposit on adults and both on second and third nymph instar mealybugs. Although, according to Daane et al. (2004), significant more parasitoids were reared from adults compared to juvenile stages. Adults begin to emerge after 12 days from oviposition (Daane et al., 2004). *Anagyrus pseudococci* is a strict solitary species, that lays one of multiple eggs always resulting in the emergence of a single adult (Chormanski & Cave, 2018). Moreover, parasitoid gender is influenced by host development stage; indeed, adult immature mealybugs females represent the best host, yielding significantly more adult female wasps (62%) (Daane et al., 2004). It was shown that also temperature can affect parasitoid gender (Suma et al., 2012a).

A. pseudococci is arrhenotokous species: unfertilized eggs produce males while fertilized ones produce females (Chormanski & Cave, 2018). The parasitoid oviposition and development decrease or stop below 14 °C (Daane et al., 2004). The latter occurs between 14 °C and 34°C and completely stops above 36°C (Daane et al., 2004). The female wasp takes between 14 and 18 days to complete a life cycle, whereas the male parasitoid needs 12 to 27 days (Chormanski & Cave, 2018). *Anagyrus pseudococci* can complete one generation twice as quickly as mealybug (Daane et al., 2004). Average adult lifespan is about 5-9 days (Chormanski & Cave, 2018).

The parasitoid needs to be released when there are the best temperatures for achieving an optimal control on mealybugs. *A. vladimiri* acts efficiently at low host densities thanks to its excellent searching capacity, also in concealed locations. Generally, 1000-1500 adult individuals per hectare are released when the pest infestation starts or even when mealybug individuals are not visible yet. The releases can be defined as preventive in vineyards where mealybug problems are commonly recorded along the years. Despite the well-known role of *A. vladimiri* as mealybug biocontrol agent, only a few field evaluation of its efficacy have been reported in literature.

Natural enemies: *Cryptolaemus montrouzieri*

The predator *C. montrouzieri* is one of the most released biological control agent worldwide (Booth & Pope, 1986; Kairo, 2013). This ladybird beetle is native to Australia and firstly it was introduced into California in 1882 against *P. citri* (Rahmouni & Chermiti, 2013). Since that year, the predator has been imported in at least 64 countries to control more than 16 phytophagous species. In particular, *C. montrouzieri* can feed on more than 60 mealybug species (Mani, 2018).

Both juvenile stages and adults can feed on mealybugs, although, forth instar larvae and adult have higher predation rates compared to other larval instars. Moreover, among adults, females are more voracious than male individuals (Kairo, 2013). Each predatory grub can consume either 900-1500 eggs or 300 nymphs or 30 adults of *M. hirsutus* during its development (Kairo, 2013), whereas an adult female can devour 4355 eggs and 240 nymphs of the mealybug (Mani, 2018).

Adult predator females lay 1 to 6 eggs close to mealybug colony (Mani, 2018). The ladybird beetle locates its prey by means of chemical signals. Indeed, the mealybug wax and the honeydew attract *C. montrouzieri* and act as a stimulant for oviposition (Kairo, 2013). The predator takes 30 days to complete its development from egg to adult in optimal conditions (at about 30°C) (Kairo, 2013; Mani, 2018). Indeed, 21°C is the minimum temperature at which the ladybird can feed and lay eggs. Nevertheless, there are some tolerant biotypes of the predator which can survive in colder climates (Kairo, 2013).

The average lifespan for adult males is about 55 days, whereas females lives for 80 days (Mani, 2018). The average fecundity of *C. montrouzieri* is 211 eggs per female (Kairo, 2013).

Cryptolaemus montrouzieri use in biological control is highly influenced by the size of the host population. Indeed, the predator needs to be released only if mealybug density is relatively high in the field; otherwise, at low pest density, cannibalism can occur coupled with reduced egg production, oviposition and development problems (Kairo, 2013). It is recommended to release 10-15 individuals on each mealybug colony.

As described above for *A. vladimiri*, few field validation of *C. montrouzieri* efficacy in suppressing mealybug infestation are present in literature (Mani & Thontadarya, 1988; Pérez-Rodríguez et al., 2019).

1.2.2 *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae)

Lobesia botrana is one of the most destructive vineyard pests around the world (Xuéreb & Thiéry, 2006). This pest, commonly called European grapevine moth (EGVM), is native to the Paleartic Region and is widespread in Italy, southern France, central and southern Spain, Portugal, Greece and in the Mediterranean basin island. More recently, it was introduced in Chile in 2008, California in 2009 and Argentina in 2010 (Ioriatti et al., 2011; Ioriatti & Lucchi, 2016). The moth was declared eradicated in the western part of United States in 2016; indeed, no more male adults have been captured in this region since 2014 (Schartel et al., 2019).

Lobesia botrana is a very polyphagous insect; apart from grapevine, EGVM can feed on 40 other plants. In wine-growing regions, the European grapevine moth can develop two to five annual generations, depending on latitude, climate and microclimate. In Italy, it usually completes 3 generations in the north and 4 in the south. The pest overwinters in the pupal stage under the bark and in the crack of vine trunk and cordons (Ioriatti et al., 2011). *Lobesia botrana* is characterized by one antophagous generation, which is the first one, and two consecutive carpophagous generations, which correspond with the second and third generation.

First generation adults start to fly at the end of April and continue in May, laying eggs on inflorescences (around 50 eggs per female). The larvae of this generation create individual silky netsts, called glomerulae, where they develop until pupal stage emerge. The second flight is visible from the end of June to mid-July, whereas third generation adults can be observed from the beginning of August until mid-September. Both last two generations oviposit and develop on bunches, creating important yield losses (Pollini, 2013). On the other hand, the first generation does not usually require control, especially in abundant inflorescence varieties in which the pest can even act and favour a natural fruit drop.

1.2.2.1 Damages

European grapevine moth is responsible of both direct and indirect damages, especially its second and third generations.

The antophagous generation destroys the inflorescences, creating glomerulae, but the reduction of flowers and little berries is compensated by an increased size and weight of healthy bunches (Ioriatti et al., 2011). On the other hand, carpophagous generations penetrate the berries, producing their shrivelling and fall. Besides these direct damages, EGMV infestations also promote fungal and bacterial diseases, such as botrytis and sour rot (Lucchi et al., 2018; Pavan et al., 2018).

1.2.2.2 Control methods

The management of *L. botrana* carpophagous generations is usually carried out by means of pesticides, *Bacillus thuringiensis* Berliner (Bt) and mating disruption (Pavan et al., 2018). In Emilia-Romagna, integrated pest management regulations do not allow insecticide treatments against the first generation of the pest and authorise pesticide control against the second and third generations only if more than 5% of vine bunches show penetration holes or are infested with *L. botrana* eggs or/and larvae. In IPM, chemical management exploits several insecticides categories, such as insect grow regulators (Tebufenozide), spinosyns, oxadiazines (Indoxacarb), avermectines and anthranilic diamides (Chlorantraniliprole). All these chemicals must be applied against the most susceptible stages of the moth. Consequently, appropriate monitoring programs, such as pheromone traps and forecasting techniques to predict the presence of developmental stage of the insect, must be used to plan the best timing for pesticide application (Ioriatti et al., 2011).

Spinosyns and Bt are the only products among the insecticide listed above that can be used in organic vineyards. Although, mating disruption is another valid and very effective tool against the European grapevine moth, which is usually applied before EGVM first flight. Over 249,000 ha of European vineyards exploited mating disruption to manage *L. botrana* infestation in 2017, with about 36,000 ha just in Italian wine regions (Lucchi et al., 2018). Dispensers that release continuously defined amounts of (7E,9Z)-7,9-dodecadien-1-yl acetate, the main component of female sex pheromone, are currently used. Recent studies tested new aerosol devices, which release the pheromone at programmed time intervals and require less dispensers in the field. These alternatives showed promising results in controlling *L. botrana* and would contribute in saving labor costs and reducing plastic devices in agricultural settings (Lucchi et al., 2018).

Bacillus thuringiensis represents another sustainable alternative for EGVM control (Ifoulis & Savopoulou-Soultani, 2004). This microbial agent requires accuracy and the right timing of application, which is the black head stage. In several cases, it is necessary to carry out two treatments (Disciplinari di Produzione Integrata Vite Emilia Romagna 2020).

Regarding biological control through natural enemy release, several attempts have been carried out in order to find an effective solution against *L. botrana*. *Trichogramma* sp. egg parasitoids have been mass-released but without satisfying results (Ioriatti et al., 2012; Scaramozzino et al., 2017; Thiéry et al., 2018).

Natural enemies of *Lobesia botrana*

Campoplex capitator Aubert (Hymenoptera: Ichneumonidae) is one of the most frequent species in some regions of Italy and, in general, in Europe (Scaramozzino et al., 2017). This larval endoparasitoid has shown a level of parasitism up to 90% on EGVM in some occasions (Moreau et al., 2019; Scaramozzino et al., 2018), but the limited knowledge about its behavior, development and ecology, together with the difficult mass-rearing, represent an obstacle to its larger exploitation (Ioriatti et al., 2012; Moreau et al., 2019; Scaramozzino et al., 2017, 2018). Recent cooperation between Italian and Chilean entomologists seems very promising in overcoming these difficulties (Scaramozzino et al., 2018).

Besides *Trichogramma* spp. and *C. capitator*, *L. botrana* has more than 70 parasitoid species, mainly belonging to ten families of Hymenoptera (Braconidae, Icheumonidae, Chalcididae, Eulophidae, Eupelmidae, Eurytomidae, Pteromalidae, Torymidae, Trichogrammatidae and Bethylidae) and one family of Diptera (Tachinidae) (Scaramozzino et al., 2017). Among Tachinidae species, *Phytomyptera nigrina* (Meigen) is one of the most common EGVM parasitoid, which is often found by researchers in Italian studies. This Diptera species is a larval endophagous parasitoid which is usually associated with the first generation of *L. botrana*.

Within Icheumononoidea superfamily, *Tranosemella prerogator* (Linneaus) (Hymenoptera: Ichneumonidae) and *Ascogaster quadridentata* Wesmael (Hymenoptera: Braconidae) can parasitize the European grapevine moth. The first one has been reported in Veneto and Piedmont region, emerging from larvae of first and second generation (Scaramozzino et al., 2018), whereas the second is commonly found at low densities especially on carpophagous generations (Scaramozzino et al., 2017).

The gregarious *Dibrachys affinis* Masi (Hymenoptera: Pteromalidae) is recorded as a gregarious parasitoid of *L. botrana*. It parasitizes both larvae and pupal stages, developing up to 14 individuals/host. It can reproduce on overwintering pupal stages and continue its activity on *L. botrana* first generation, without using alternative hosts (Marchesini & Della Montà, 1998).

It is also possible to find parasitoid individuals belonging to the fourth and fifth trophic level. Larvae of *T. prerogator* and C. *capitator* can be parasitized by *Elasmus steffani* (Viggiani) (Hymenoptera: Elasmidae), which in turn can be parasitized by *Eutetrastichus amethystinus* (Ratzeburg) (Hymenoptera: Eulophidae).

To enhance the fitness and activity of these several parasitoid species, habitat management strategies could be exploited to reduce *L. botrana* infestations. Few studies have been carried out on conservation biological control against vineyard pests in Italy (Girolami et al., 2000; Duso et al., 2010), especially *L. botrana* (Serra et al., 2006).

Agroecological practices may be employed especially for the control of EGVM first generation, which does not usually require insecticide treatments, and consequently may help in reducing the population of *L. botrana* carpophagous generations.

1.3 Aim of this research study

Considering the necessity to adopt and increase the sustainable methods for pest management, this thesis aims to explore and deeply investigate and field test biocontrol and innovative strategies for the management of the most important vineyard pests in Italy. In literature, very few field works have assessed the effect of companion plant species as a habitat management tool to boost *L. botrana* first generation parasitization in Italy, or of inoculative releases of Pseudococcidae natural enemies against vine and comstock mealybugs. The same considerations apply to the study of ant control method on mealybug infestation, whereas no work has investigated the role of ant attendance on ecosystem services in vineyard. Finally, silicon field application to enhance beneficial attraction towards vine plants represents an innovative method which appears partially unexplored.

Therefore, the thesis investigates the following hypothesis:

1. The combined release of the parasitoid *A. vladimiri* and the predator *C. montrouzieri* would control effectively *P. ficus* and *P. comstocki* infestations in vineyards of Reggio Emilia Province;
2. The use of sugar dispersers would disrupt ant-attendance and improve biological control against mealybugs;
3. Conservative ground cover management would boost the parasitization of *L. botrana* first generation and increase trophic level complexity in vineyards of Reggio Emilia Province;
4. Vine plants treated with silica gel would enhance the attraction of Hymenoptera parasitoids, strengthening plant resistance.

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Chapter 2 Evaluation of the efficacy of combined release of *Anagyrus vladimiri* and *Cryptolaemus montrouzieri* against mealybugs

2.1 Abstract

Mealybugs currently represent a key pest in Italian vineyards. Together with *Planococcus ficus* (Signoret), a species native to Eastern Asia, *Pseudococcus comstocki* Kuwana (Hemiptera: Pseudococcidae), is causing several damages on vine plants, resulting in important economic losses for growers. Chemical applications using non-selective products have increased to cope with mealybug infestations, leading to detrimental effects in the environment. However, the increased insecticide inefficacy and the limits imposed to pesticide residues by import legislation have prompted the research for the adoption of environmentally friendly alternatives. The aim of this work is to evaluate the combined release of *Anagyrus vladimiri* Triapitsyn (Hymenoptera: Encyrtidae) and *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae), the two most important natural enemies of mealybugs. Beneficial releases were carried out both in pesticides-free trial plots, using exclusion cage method, and in commercial vineyards following an integrated approach including the use of insecticides. Both *P. ficus* and *P. comstocki* were parasitized by *A. vladimiri*, but parasitization showed to be higher on the former. Also the predator demonstrated to be effective in controlling Pseudococcidae infestation, showing a clear density dependent activity. Overall, natural enemy releases showed a huge potential in controlling mealybugs and represent a critical tool in integrated pest management. A high parazitization and predation variability among vineyards occurred, and a multi-year evaluation of the strategy is recommended.

2.2 Introduction

Mealybugs are the major pests in several agricultural crops and ornamentals around the world. Pseudococcidae encompasses more than 2000 described species in 290 genera (Mani & Shivaraju, 2016). Among them, *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae), commonly called the vine mealybug, represents one of the most damaging pest of grapevine worldwide (Cocco et al., 2018). Besides *P. ficus*, Italian viticulture has to cope with infestations of a new invasive mealybug species, *Pseudococcus comstocki* Kuwana (Hemiptera: Pseudococcidae), which is native to eastern Asia. Also called the Comstock mealybug, *P. comstocki* was collected for the first time in Italy in 2004 on mulberry and then it started spreading and attacking ornamentals and fruit trees such as peaches, apples and pears (Guerrieri & Pellizzari, 2009). Since 2018, *P. comstocki* has been found in some viticultural areas of Italy (Veneto, Trentino-Alto Adige and Emilia-Romagna regions), causing huge yield losses. Mealybugs cause direct damages, feeding on plants and excreting honeydew, which encourages the growth of sooty mould (le Vieux & Malan, 2016). This fungus infection fouls leaves and bunches (Tacoli et al., 2018), rendering fruit unmarketable (le Vieux & Malan, 2016). Moreover, *P. ficus* transmits several viruses, including Grapevine virus A (GVA), grapevine leafroll-associated viruses (GLRaVs) 1,3,4,5 and 9 and corky-bark disease (Cocco et al., 2018), whereas Grapevine virus E (GVE) is transmissible by *P. comstocki* (Coetzee et al., 2010).

Chemical control represents the most common strategy carried out against mealybug pests (Mansour et al., 2018). However, repeated applications of pesticides create resistance to active substances of several insecticides and negatively impact on natural enemies, such as parasitoids and predators (Mansour et al., 2018). Consequently, alternative strategies are being examined by scientists to find alternative and more sustainable practices, such as biological control, which could provide effective control against this deleterious pest (Mansour et al., 2018) also in organic agriculture. In addition, a growing awareness of the environmental issues is leading to a rising demand for verifiably sustainable products (Tompkins et al., 2012).

Mating disruption is considered one of these environmentally friendly techniques which can be applied against vine mealybug. This strategy has been tested in several control programs in USA, Argentina and the Mediterranean Basin (Cocco et al., 2018; Mansour et al., 2017; Sharon et al., 2016). Recent studies conducted in Northern and Southern Italy demonstrated that the use of dispenser IsonetPF® reduced the percentage of *P. ficus* infested bunches and the number of specimens per bunch compared to untreated controls (Lucchi et al., 2019). Innovative control methods have been also attempted to reduce mealybug infestation, exploiting the endophytic fungus *Beauveria bassiana* (Rondot & Reineke, 2018).

Another alternative to chemical products is augmentative biological control (Daane et al., 2006). Natural enemy releases can be adopted together with mating disruption, also because mealybug pheromone can act as attractant for beneficials (Franco et al., 2008).

Anagyrus sp. near *pseudococci*, now called *Anagyrus vladimiri* Triapitsyn (Andreason et al., 2019), has been often used for the biological control of mealybugs (Daane et al., 2004; Romano et al., 2018). Previous studies highlighted that *A. pseudococci* parasitized more or less 90% of *P. ficus* collected near harvest time in California vineyard (Daane et al., 2008). Probably most of these records likely refer to *A. vladimiri* (Andreason et al., 2019).

Besides *A. vladimiri*, another effective mealybug biocontrol agent is the ladybird *Cryptolaemus montrouzieri* Mulsant (Mani, 2018; Muştu et al., 2008). Rahmouni and Chermiti (2013) showed that this predator is able to control *Planococcus citri* Risso (Hemiptera: Pseudococcidae) populations and maintain them at an economically tolerable threshold one month after its release in citrus orchards. Also in Yemen, *P. ficus* population was reduced after the release of *C. montrouzieri* (Mani, 2018).

The aim of this research is to evaluate the efficacy of the combined release of *A. vladimiri* and *C. montrouzieri* in controlling *P. ficus* and *P. comstocki* infestations in Reggio Emilia (Emilia Romagna, Italy), a province famous for Lambrusco wine production. Mealybug infestations have increased in Reggio Emilia in the last 5 years and chemical products have not been reliable and effective in managing this pest. Combined releases of *A. pseudococci* and *C. montrouzieri* have been already carried out against *P. ficus*, showing promising results (Varner et al., 2015). In our trial, inoculative releases were carried out especially to verify the success of *P. comstocki* parasitization by *A. vladimiri* and to try providing new solutions for growers to control mealybugs.

Augmentative biological control was tested both in pesticide-free trial plots, using exclusion cage method, and in commercial vineyards following an integrated pest management approach including the sustainable use of insecticides. In this way, natural enemy performance has been tested and checked both in vineyard with optimal conditions for beneficials, that is without the use of mealybug chemical control, and in vineyard where growers continue using insecticides against these hemipteran sucking pests.

2.3 Material and methods

2.3.1 Exclusion cage trial

2.3.1.1 Field sites

The trials were carried out in 4 vineyards of Reggio Emilia Province in 2018, whereas 5 vineyards enjoyed the experiment in 2019 (Table 1). Vineyards were selected based on the pest pressure recorded by extension services in recent years.

Table 1. Details of vineyard sites. Location (Province, Longitude, Latitude), year of the trial, vine variety and pest management are showed.

Site	Province	Longitude	Latitude	Year	Variety	Pest management
A	Reggio nell'Emilia	10°49'17.13" E	44°46'52.39" N	2018	Lambrusco Salamino	IPM
B	Reggio nell'Emilia	10°41'07.57" E	44°37'21.72" N	2018 2019	Ancellotta	organic
C	Reggio nell'Emilia	10°43'54.41" E	44°51'36.36" N	2018 2019	Lambrusco Marani	IPM
D	Reggio nell'Emilia	10°36'15.41" E	44°41'25.27" N	2018 2019	Lambrusco Salamino	IPM
E	Reggio nell'Emilia	10°44'50.15" E	44°47'29.55" N	2019	Ancellotta	IPM
F	Reggio nell'Emilia	10°48'28.38" E	44°47'22.71" N	2019	Lambrusco Salamino	IPM with prophylactic use of insecticides

The trial was carried out in an area of 1 ha in vineyard A, B, C and D in 2018, whereas vineyard B, C, D, E and F joined the experiment in 2019.

2.3.1.2 *Scaphoideus titanus* treatment

Firstly, *Scaphoideus titanus* Ball. (Hemiptera: Cicadellidae) treatment was carefully planned in order to minimize the negative impact on the released natural enemies. In Emilia-Romagna region, the Flavescence dorée leafhopper vector must be treated as indicated in the regional regulations of Integrated Pest Management. Moreover, we anticipated the treatment in order to release natural enemies and allow their action as soon as possible. In 2018, thiamethoxam (ACTARA ® 25 WG, Syngenta, Italy) was used for *S. titanus* treatment in IPM vineyards (only 1 application) (Vineyard A: 21/06/18; Vineyard C: 27/06/18; Vineyard D: 28/06/18), whereas tau-fluvalinate (MAVRIK 20 EW, Adama, Italy) was used in 2019 (Vineyard C: 21/06/19; Vineyard D: 28/06/19; Vineyard E: 22/06/19; Vineyard F: 18/06/19). On the other hand, pyrethrum was used twice/year in organic vineyard both in 2018 (Vineyard B: 21/06/18, 03/07/18) and 2019 (Vineyard B: 22/06/19, 02/07/19).

2.3.1.3 Exclusion cages and natural enemy release

The exclusion cage method was used as control to evaluate the mealybug population growth in absence of released natural enemies. Three cages per vineyard were built using tulle mesh (3m x 3m), cable ties and stapler before natural enemy release (Figure 1). These cages were placed at a minimum distance of 20 metres from each other, selecting plants with mealybug infestation (when possible).

Figure 1. Exclusion cage on vine plant.

Parasitoid release was carried out after more or less one week from mandatory treatment against *S. titanus* (Figure 2). A total of 1500 individuals of *A. vladimiri* per hectare were released randomly in two consecutive dates to guarantee their permanence in the field. In particular, the first release of 1000 individuals was followed by other 500 individuals after one week.

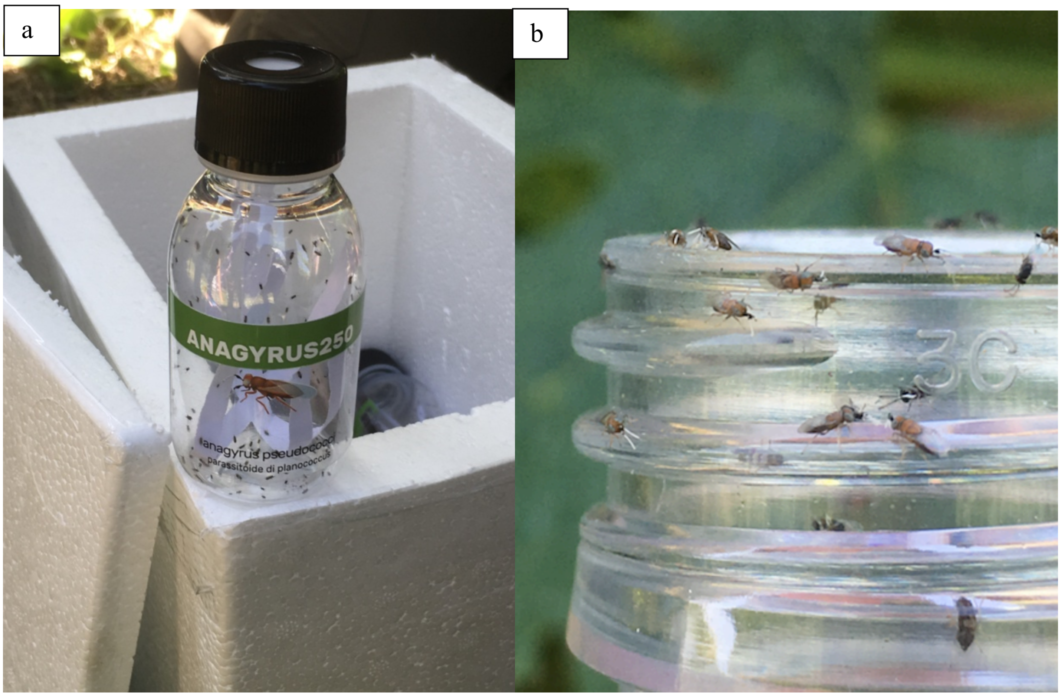

Figure 2. Anagyrus vladimiri *pack which contains 250 individuals (a), both male and female parasitoids (b).*

The predator *C. montrouzieri* was released one to two weeks after *A. vladimiri*, using minimum 300 individuals per hectare (Figure 3). Both parasitoid and predator individuals were supplied by Bioplanet (Cesena, Italy).

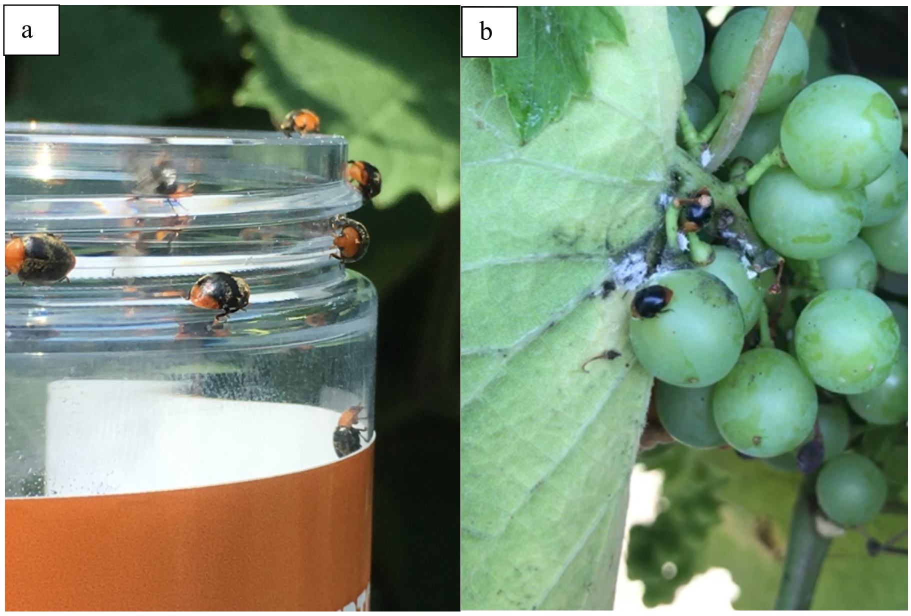

Figure 3. Cryptolaemus montrouzieri *individuals during the release (a) and on vine canopy (b).*

2.3.1.4 Infestation, parasitization and predation sampling

Bunches were collected between late August and early September, slightly before harvest time. 25 bunches were harvested per each cage, for a total of 75 bunches. To assess the infestation outside the cages, 70 bunches were randomly collected in the trial area of each vineyard, whereas 8-12 infested bunches were harvested to estimate parasitization and predation (Figure 4). Infested bunches, hereafter also referred as colony uncaged bunches, were selected based on a high presence of mealybugs on them. Besides assessing parasitization on colony uncaged bunches, parasitized mealybugs were also estimated on randomly selected bunches (used to determine infestation and hereafter also referred as random uncaged bunches) in order to evaluate the parasitoid activity at different densities of mealybug infestation. Also predation was assessed both on colony uncaged bunches and on random uncaged bunches.

Figure 4. Example of damages on bunches caused by mealybugs.

Infestation was assessed by two different way, estimating bunch infestation rate and the number of mealybugs per bunch. In particular, the percentage of infested bunches was evaluated in the field, counting the bunches with sign of damage/infestation such as honeydew, sooty mould and mealybug presence. The number of mealybugs per bunch was evaluated in laboratory

(Department of Agricultural and Food Sciences, University of Bologna), counting individuals of each development stage (nymphs, adults (females), females with ovisac) (Figure 5).

Figure 5. Bunch analysis counting mealybug individuals, parasitized and predated mealybugs and C. montrouzieri *larvae.*

Parasitization was estimated as the ratio between parasitized mealybugs and the total number of mealybugs and assessed only on adults and females with ovisac, as they represent the most suitable stages for *A. vladimiri*. Moreover, as young stages do not show visible sign of parasitisation, they can lead to a dramatic underestimation of parasitism.

Presence of a single hole in the back of mealybug body (Figure 6a) or swollen pest body (Figure 6b) were considered sign of parasitization.

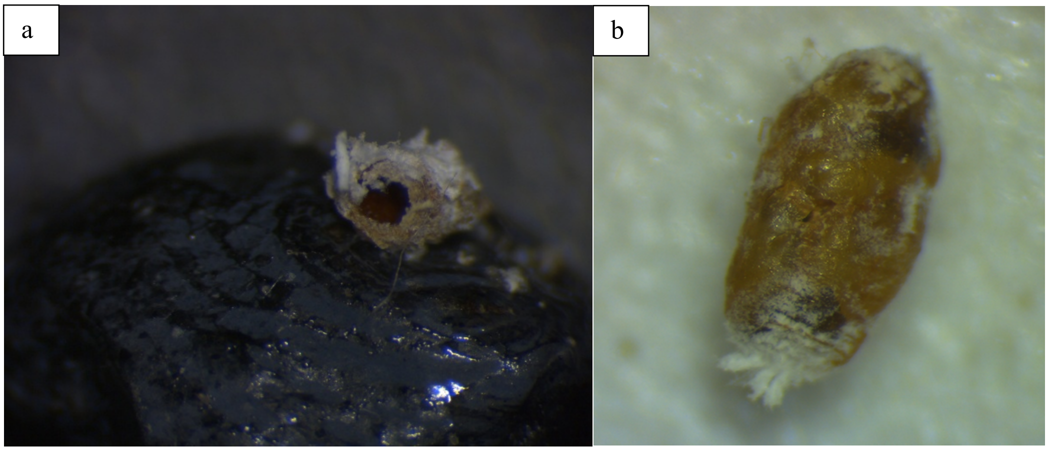

Figure 6. Example of two signs to recognize parasitized mealybugs: single hole in the back of mealybug (a) and swollen body (b).

Lacerated bodies (Figure 7a) and eggs without adults were considered sign of predation, so the ratio between predated mealybugs and the total number of mealybugs was used to calculate the percentage of predation. Also predation rate was estimated only on adult stage (adult females and females with ovisac) because it gives a better evaluation of *C. montrouzieri* activity compared to predation on all mealybug stages. Indeed, nymphs are normally devoured entirely, thereby making predation on nymphs impossible to assess. Underestimations are associated with this predation assessment, but they are replicated in the same way in each vineyard of the trial.

Finally, larvae of *C. montrouzieri* found on bunches were counted to assess the mean number of predator larvae per bunch (Figure 7b).

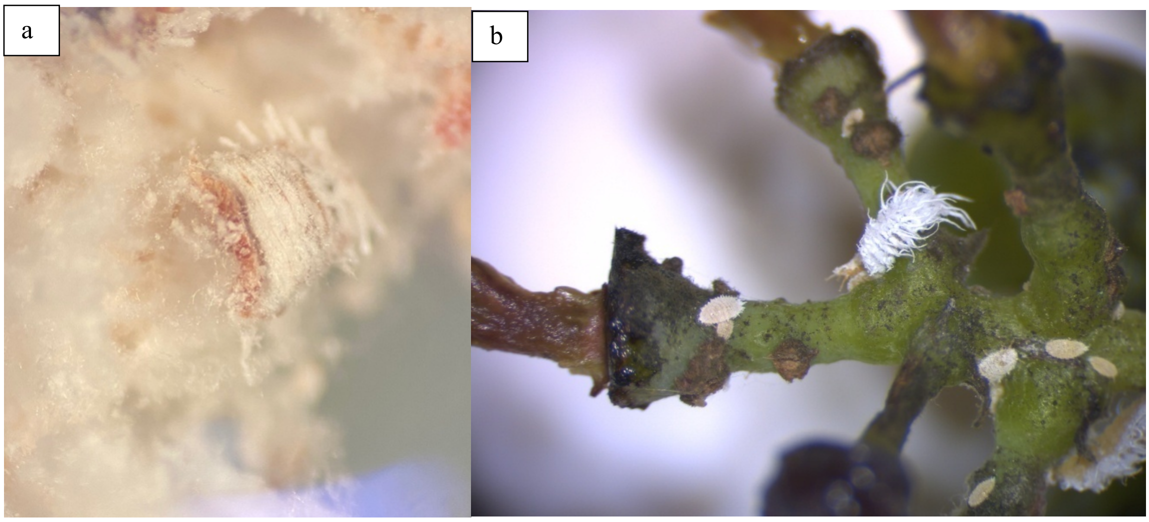

Figure 7. Mealybug body lacerated by C. montrouzieri *(a) and larvae of* C. montrouzieri *(b).*

2.3.1.5 Statistical analysis

Standard errors of infestation, parasitism and predation rates were calculated according to a binomial distribution (binomial SE).

Log linear analysis was used to analyse the cumulative ratio of infested bunches, parasitization and predation rates in the overall experiment, including years and vineyards. Log-linear analysis resembles a factorial analysis of variance and allows for simultaneous evaluation of multiple interactions among categorical variables (Burgio et al., 2020). Here, the response variables were the percentage of infested bunches, parasitization and predation rates. In infestation data analysis, the independent variables were:

- treatments (caged bunches, random uncaged bunches);
- year (2018, 2019) and
- vineyards (N= 4-5).

In parasitisation and predation analysis, the independent (or design) variables were:

- treatments (caged bunches, random uncaged bunches, colony uncaged bunches);
- year (2018, 2019) and
- vineyards (N= 4-5).

Log linear analysis was performed using Statistica version 10 software (StatSoft^{TM}, Tulsa, OK, USA).

A chi square analysis was carried out to detect significant effect of treatment on categorical (binomially distributed) variables (i.e. infestation, parasitisation, predation rate) in each vineyard site and year, when Log linear analysis showed significant interactions between the tested variables. This analysis was performed using GraphPad software (GraphPad Software Inc., CA, USA).

Finally, a z-test was performed to rank the efficacy of the treatments when the levels were > 2 (i.e. caged bunches, random uncaged bunches and colony uncaged bunches). Parasitization and predation rates were considered as efficacy variables. Bonferroni correction was implemented to adjust the p-level of the z-test. This procedure was performed, when necessary, separately for each vineyard and year, using the IBM SPSS 23 statistics package (IBM corporation, Armonk, NY, USA) (Burgio et al., 2020).

2.3.2 Natural enemy release in commercial vineyards

2.3.2.1 Field sites

A biological control program was conducted by Cantine Riunite & Civ, an important cooperative society of Lambrusco wine producers, and Bioplanet. Inoculative releases of *A. vladimiri* and *C. mountrouzieri* were carried out in about 10000 ha of Lambrusco vineyard area, accomplishing one of the widest territorial releases of natural enemies in Emilia-Romagna region.

Within the area interested to the biological control program, 6 pilot vineyards were selected and monitored to evaluate the efficacy of the inoculative releases; moreover, 3 field sites of the same area, where no beneficials were released, were considered as control. Vineyards were selected based on the pest pressure recorded by Cantine Riunite consultants in recent years. Table 2 presents the location and variety of each vineyard.

Table 2. Details of vineyard sites. Location (Province, Latitude and Longitude) and vine variety are showed.

Site	Province	Longitude	Latitude	Variety
1	Reggio nell’Emilia	10°45’02.59” E	44°45’00.91” N	Ancellotta; Lambrusco Salamino
2	Modena	10°50’50.78” E	44°48’18.62” N	Lambrusco Salamino
3	Reggio nell’Emilia	10°79’42.80”	44°77’75.95”	Ancellotta; Lambrusco Sorbara
4	Modena	10.58’10.87” E	44°30’39.46” N	Lambrusco Grasparossa
5	Modena	11°01’58.33” E	44°49’15.52” N	Lambrusco Sorbara; Lambrusco Salamino
6	Modena	11°00’31.31” E	44°45’30.41” N	Lambrusco Sorbara; Lambrusco Salamino
7	Reggio nell’Emilia	10°40’55.65” E	44°44’36.29” N	Ancellotta
8	Reggio nell’Emilia	10°50’44.8” E	44°47’26.2” N	Lambrusco Salamino
9	Reggio nell’Emilia	10°47’45.90” E	44°52’46.64” N	Ancellotta

Figure 8 represents the spatial distribution of the field sites in Reggio Emilia and Modena Province.

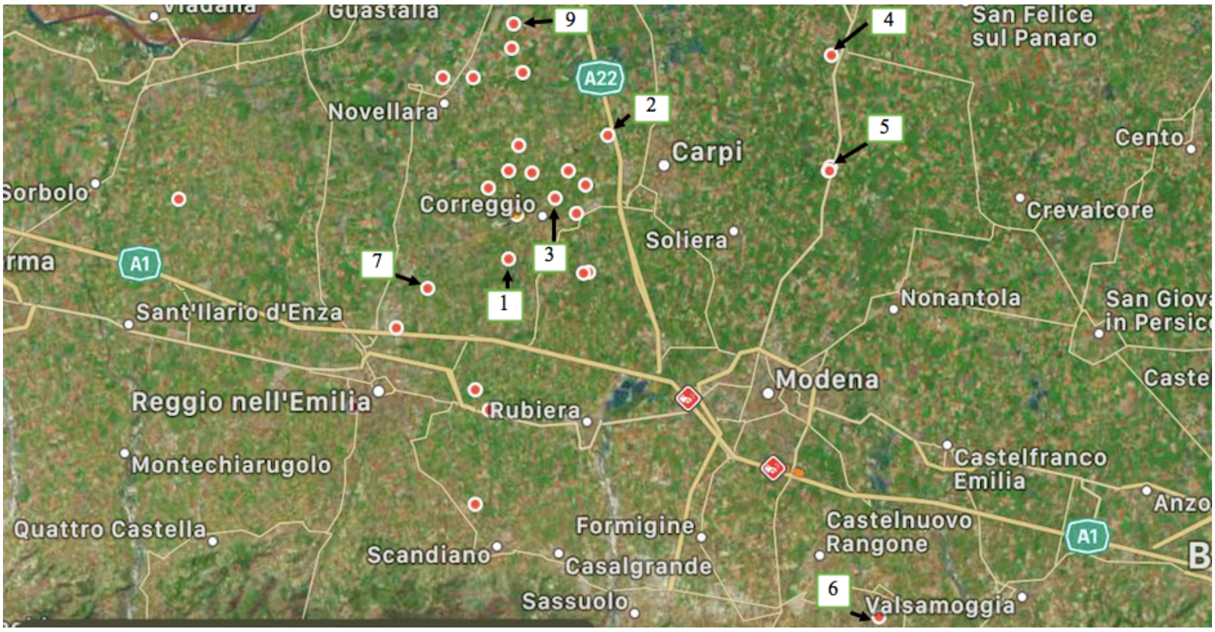

Figure 8. Spatial distribution of field sites in Reggio Emilia and Modena Province.

2.3.2.2 Natural enemy release and insecticide treatments

Natural enemy releases were carried out by Cantine Riunite consultants during the season, according to infestation level, and integrated with insecticide treatments (Table 3).

Table 3. Insecticide treatments and natural enemy releases in each vineyard site.

Site	N° of insecticide treatments	Active ingredients	*A. vladimiri* releases	*C. montrouzieri* releases
1	6	Pyriproxyfen, Spirotetramat, Flupyradifurone, Acetamiprid	29th of May; 16th of July	16th of July
2	7	Pyriproxyfen, Spirotetramat, Flupyradifurone, mineral oils	22nd of May	16th of July
3	5	Pyriproxyfen, Spirotetramat, Flupyradifurone, Etofenprox	Post harvest; 26th of April; 16th of July	16th of July
4	1	Flupyradifurone	6th of May	15th of July
5	5	Pyriproxyfen, Spirotetramat, Acetamiprid, mineral oils	6th of May	No releases
6	3	Oli minerali, pyrethrins	15th of May	16th of July 29th of August
7	5	Pyriproxyfen, Spirotetramat, Flupyradifurone, Acetamiprid	No releases	No releases
8	2	Pyriproxyfen, Flupyradifurone	3rd of August	No releases
9	4	Flupyradifurone, Spirotetramat, Acetamiprid, mineral oils	No releases	No releases

Vineyard 8 was considered as a control vineyard in July, but it was excluded in September due to the release of *A. vladimiri* on 3rd of August.

2.3.2.3 Infestation, parasitization and predation sampling

Bunches were collected in two different moment: in July and before harvesting in September to evaluate the activity of natural enemies and their efficacy along the season. In each sample, 50 bunches were randomly collected from each vineyard to evaluate the infestation, parasitization and predation, whereas 10 infested bunches (hereafter also referred as colony bunches) were harvested to estimate the percentage of parasitization and predation on mealybug colonies.

Infestation, parasitism and predation were assessed in the same way of exclusion cage trial (see chapter 2, subchapter 2.3.1).

Indeed, the percentage of infested bunches, the number of mealybugs per bunch, parasitism and predation rates were calculated.

2.3.2.4 Statistical analysis

Standard errors of infestation, parasitism and predation rates were calculated according to a binomial distribution (binomial SE).

The relationship between infestation rate and the mean number of mealybugs per bunch was analysed by curvilinear regression ($y=a*\exp(bx)$). The relationships between parasitism rate and the number of insecticide treatments, as well as between the mean number of mealybugs per bunch and the number of insecticide treatments, were analysed by linear regression ($y=a+bx$).

2.4 Results

2.4.1 Exclusion cage trial

Log linear analysis showed a significant effect of treatment, vineyard and year on the percentage of infested bunches (Table 4).

Table 4. Log linear results showing the effect of each factor (T=treatment; V=vineyard; Y=year) and their interactions on infestation rate (I=infestation). T= treatments (caged bunches-random uncaged bunches); I = Infestation; Y=years (2018-2019); V= Vineyard (N=4-5)

Effect	df	Chi square (Partial association test).	P (Partial Association test)	Chi square (Marginal association test)	P (Marginal association test)
T x I	1	419,48	< 0,001	342,76	< 0,001
V x I	4	179,88	< 0,001	22,32	<0,001
Y x I	1	13,29	< 0,001	15,94	< 0,001
T x V x I	4	7,30	> 0,05	5,13	> 0,05
T x Y x I	1	38,26	< 0,001	130,71	< 0,001
V x Y x I	4	171,37	< 0,001	186,96	< 0,001

Treatment and vineyard significantly affected also the parasitism rate (Table 5).

Table 5. Log linear results showing the effect of each factor (T=treatment; V=vineyard; Y=year) and their interactions on parasitism rate (Pa=parasitism). T= treatments (caged bunches-random uncaged bunches-colony uncaged bunches); Pa=Parasitization; Y=years (2018-2019); V= Vineyard (N=4-5)

Effect	df	Chi square (Partial association test).	P (Partial association test)	Chi square (Marginal association test)	P (Marginal association test)
T x Pa	2	130,62	< 0,001	294,39	< 0,001
V x Pa	4	9218,37	< 0,001	9153,52	< 0,001
Y x Pa	1	142,54	< 0,001	0,87	>0,05
T x V x Pa	8	66,52	< 0,001	203,56	< 0,001
T x Y x Pa	2	5,56	>0,05 (0,06)	22,66	< 0,001
V x Y x Pa	4	53,74	< 0,001	554,00	< 0,001

Marginal association test of log linear analysis showed a significant effect of treatment, vineyard, year and their interactions on *P. comstocki* parasitization (Table 6).

Table 6. Log linear results showing the effect of each factor (T=treatment; V=vineyard; Y=year) and their interactions on parasitization rate on P. comstocki *(PaPc=parasitization* P. comstocki*). T= treatments (caged bunches-random uncaged bunches-colony uncaged bunches); Pr = Predation; Y=years (2018-2019); V= Vineyard (N=3)*

Effect	df	Chi square (Partial association test).	P (Partial association test)	Chi square (Marginal association test)	P (Marginal association test)
T x PaPc	2	3,31	> 0,05	55,66	< 0,001
V x PaPc	3	973,32	< 0,001	1051,17	< 0,001
Y x PaPc	1	0,70	> 0,05	20,09	< 0,001
T x V x PaPc	6	288,86	< 0,001	271,61	< 0,001
T x Y x PaPc	2	5,12	> 0,05	12,09	< 0,01
V x Y x PaPc	3	31,62	< 0,001	53,33	< 0,001

Treatment, vineyard and year and their interactions all significantly affected the number of predated mealybugs (Table 7).

Table 7. Log linear results showing the effect of each factor (T=treatment; V=vineyard; Y=year) and their interactions on predation rate (Pr=predation). T= treatments (caged bunches-random uncaged bunches-colony uncaged bunches); Pr = Predation; Y=years (2018-2019); V= Vineyard (N=4-5)

Effect	df	Chi square (Partial association test).	P (Partial association test)	Chi square (Marginal association test)	P (Marginal association test)
T x Pr	2	366,07	< 0,001	559,91	< 0,001
V x Pr	4	9650,18	< 0,001	9739,66	< 0,001
Y x Pr	1	311,78	< 0,001	28,62	< 0,001
T x V x Pr	8	43,58	< 0,001	78,48	< 0,001
T x Y x Pr	2	7,76	0,02	20,18	< 0,001
V x Y x Pr	4	52,40	< 0,001	126,78	< 0,001

2.4.1.1 Year 2018

Vineyards were not infested by the same mealybug species. *Pseudococcus comstocki* was responsible of bunch damages in vineyard A, C and D, whereas *P. ficus* was found only in vineyard B.

In general, caged bunches presented higher infestation compared to uncaged bunches, both in terms of the percentage of infested bunches and the mean number of mealybugs per bunch (Figure 9a and 9b). Infestation was principally characterized by third generation nymphs.

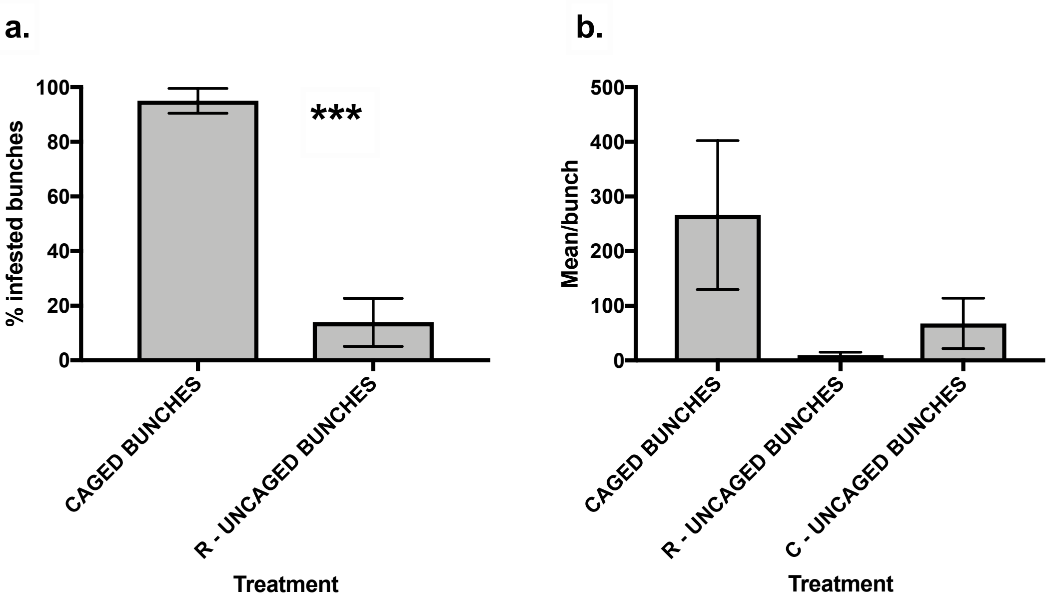

*Figure 9. Average infested bunch rate (± SE) (a) and mean mealybugs per bunch (± SE) (b) in 2018 (n=4). R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches. Chi square test showed a significant difference of infested bunch rate between the two treatments (***p<0,001) (a).*

Vineyard A presented significantly more infested bunches (100%) in cage treatment compared to random uncaged one (40%) (df=1; χ^2= 63,35; p<0,001) (Figure 10a). Conversely, the mealybug mean number per bunch on colony uncaged bunches was twice that of caged ones, showing that exclusion cage system did not overestimate infestation (Figure 10b).

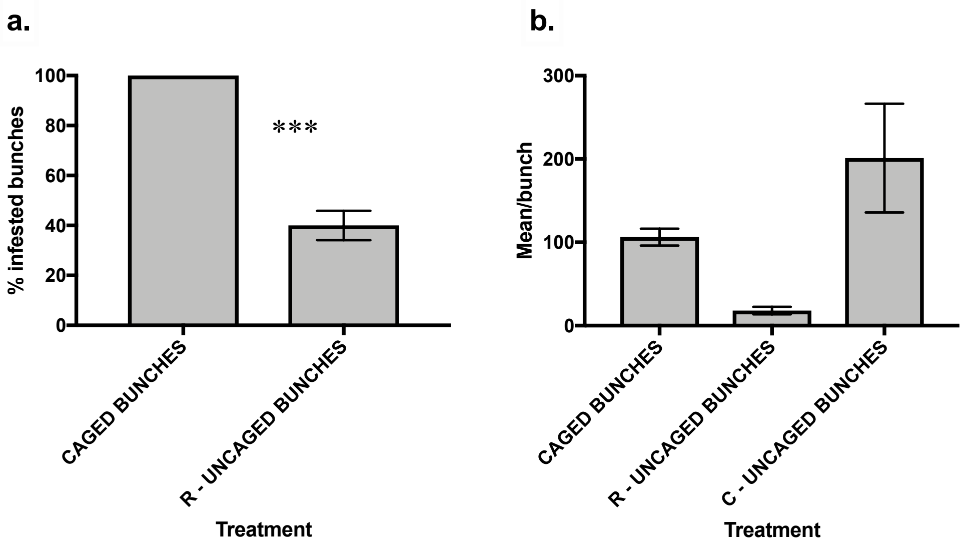

*Figure 10. Vineyard A. Infested bunch rate (± binomial SE) (a) and mean mealybugs per bunch (± SE) (b) in 2018. R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches. Chi square test showed a significant difference of infested bunch rate between the two treatments (***p<0,001) (a).*

In vineyard B, 100% of caged bunches was infested, whereas just 7% of random uncaged bunches was damaged (Figure 11a), highlighting a significant difference between the two treatments (df=1; χ^2=126,2; p<0,001). Also the mean number of mealybugs per bunch of cage treatment was six times higher compared to uncaged one (Figure 11b).

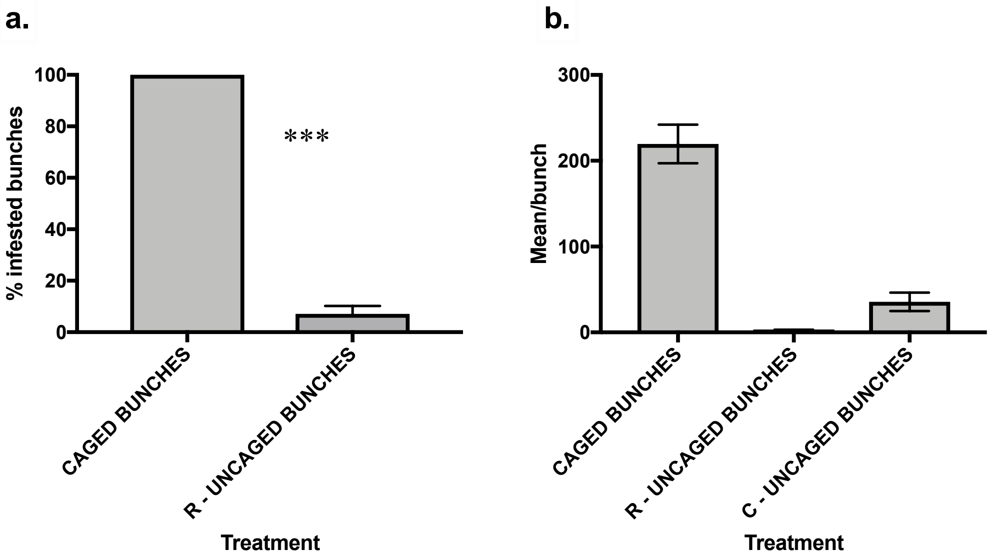

Figure 11. Vineyard B. Infested bunch rate (± binomial SE) (a) and mean mealybugs per bunch (± SE) (b) in 2018. R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged

*bunches. Chi square test showed a significant difference of infested bunch rate between the two treatments (***p<0,001) (a).*

Also vineyard C was characterized by a significant difference of infestation between caged and random uncaged bunches (df=1; χ^2=122,3; p<0,001) (Figure 12a). Caged treatment presented the highest damage, both in terms of percentage of infested bunches and the mealybug mean per bunches (Figure 12a and 12b).

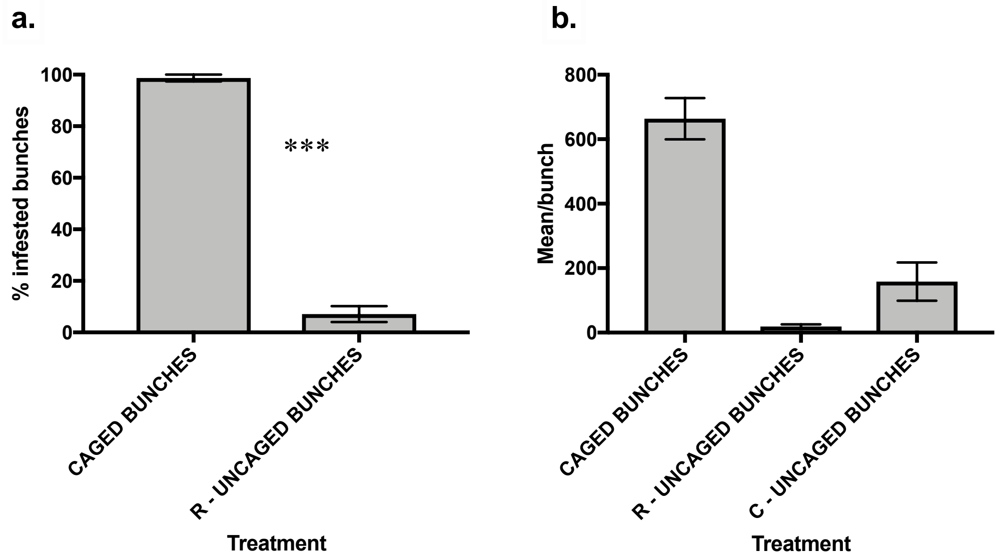

*Figure 12. Vineyard C. Infested bunch rate (± binomial SE) (a) and mean mealybugs per bunch (± SE) (b) in 2018. R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches. Chi square test showed a significant difference of infested bunch rate between the two treatments (***p<0,001) (a).*

Finally, vineyard D showed significantly less infested random uncaged bunches compared to caged ones (df=1; χ^2= 94,45; p<0,001) (Figure 13a). Also the mean number of mealybugs per bunch was lower in uncaged bunches than in caged ones (Figure 13b). A very low infestation was confirmed outside the cages by the absence of mealybug colonies (Figure 13b).

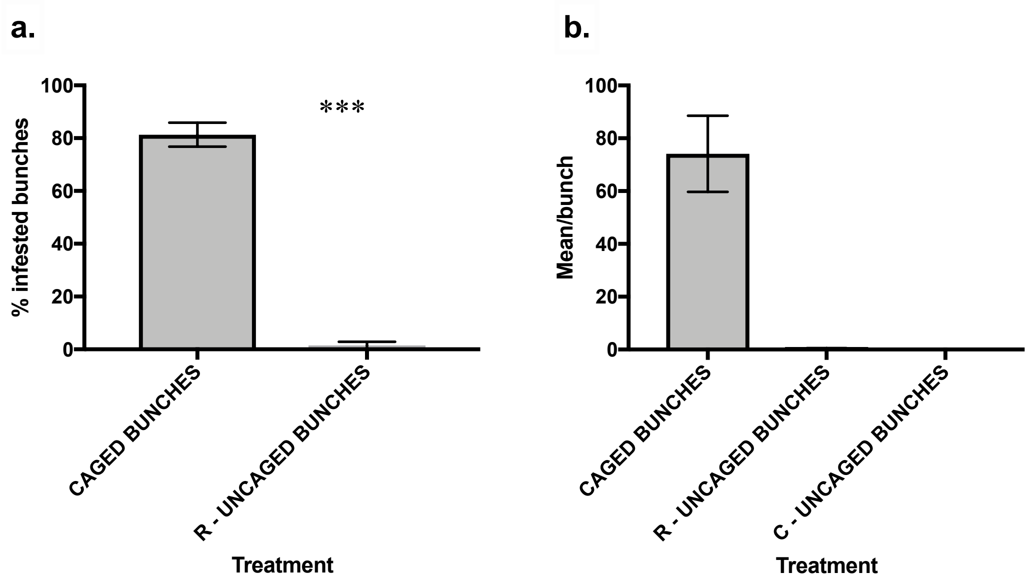

*Figure 13. Vineyard D. Infested bunch rate (± binomial SE) (a) and mean mealybugs per bunch (± SE) (b) in 2018. R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches. Chi square test showed a significant difference of infested bunch rate between the two treatments (***p<0,001) (a).*

Parasitization was higher on *P. ficus* than on *P. comstocki*. Indeed, vineyard B, which was mainly infested by *P. ficus*, presented the highest level of parasitization rate with over 80% of mealybugs parasitized both outside and inside the cages. A significant difference of parasitism rate was detected between caged bunches and random uncaged bunches by z-test (χ^2=17,16; df=2; p=0,02) (Figure 14b).

The percentage of parasitization was significantly higher in caged bunches than in colony uncaged ones in vineyard C by z-test (χ^2=53,90; df=2; p<0,001). Less than 2% of mealybugs were parasitized in colony uncaged bunches (Figure 14c).

The parasitization was less than 1% in vineyard A and no statistical differences were observed among treatments (Figure 14a).

Finally, parasitism rate was significantly higher on random uncaged bunches compared to caged bunches in vineyard D (χ^2=130; df=1; p<0,001) (Figure 14d). Although, few adult individuals were collected in this vineyard, probably overestimating the parasitization percentage on random uncaged bunches. No mealybug colonies were observed, thereby making impossible the collection of colony uncaged bunches.

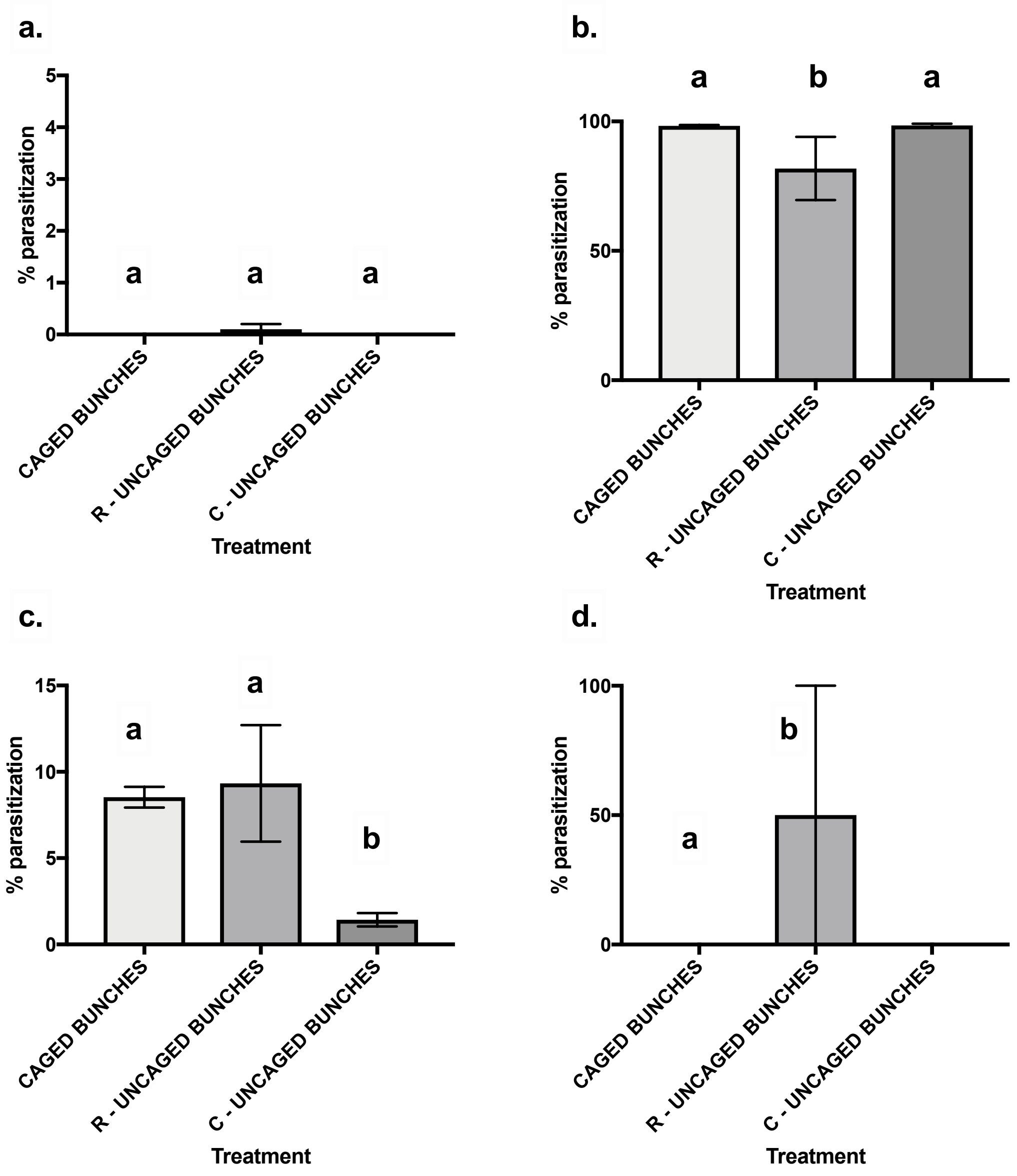

Figure 14. Parasitization rate of adult and female with ovisac mealybug stages (± binomial SE) of vineyard A (a), vineyard B (b), vineyard C (c), vineyard D (d) in 2018. R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches. Different letters indicate significant statistical differences (z-test: p<0,05). Chi square test was performed for vineyard D (d) due to the absence of colony uncaged bunches.

Despite the higher efficacy of *A. vladimiri* towards *P. ficus* compared to *P. comstocki*, figure 15 shows a good level of parasitization rate also on the latter; indeed, the mean percentage of parasitization in random uncaged bunches was 17% higher than in caged bunches where this mealybug was present (n=3).

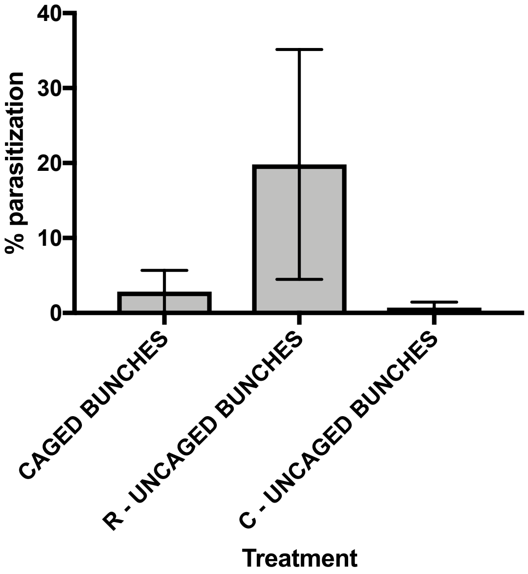

Figure 15. Average adult and female with ovisac parasitism rate (±SE) in vineyards infested by P. comstocki *(n=3) in 2018. R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches.*

Significantly more predated mealybugs were collected in colony uncaged bunches (98%) compared to caged ones (84%) in vineyard C (χ^2=119,56; df=2; p<0,001) (Figure 16b).

Even though bunch sampling occurred earlier compared to other vineyards, predation rate was significantly higher in colony uncaged bunches compared to caged ones in vineyard A (χ^2=225,42; df=2; p<0,001) (Figure 16a). Vineyard D did not have any data on colony uncaged bunches because of the absence of mealybug colonies (Figure 16c). This impeded the correct assessment of the Australian ladybird performance which is highly density-dependent. Finally, vineyard B did not present any sign of predation.

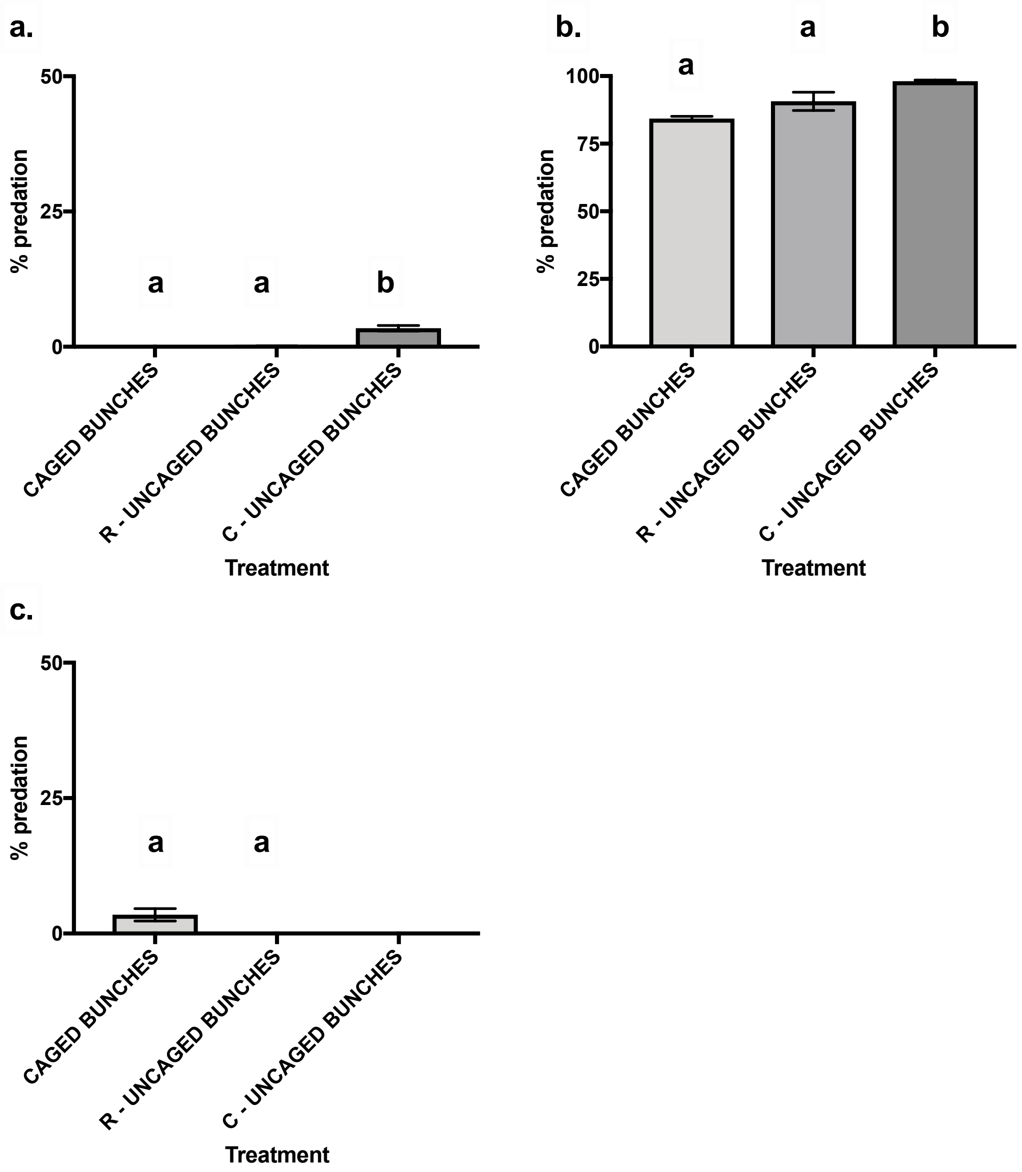

Figure 16. Predation rate of adult and female with ovisac mealybug stages (± binomial SE) of vineyard A (a), vineyard C (b), vineyard D (c). R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches. Different letters indicate significant statistical differences (z-test: p<0,05). Chi square test was performed for vineyard D (c) due to the absence of colony uncaged bunches.

The density-dependent nature of *C. montrouzieri* was also demonstrated by the graphs of the mean number of larvae per bunch (Figure 17 and figure 18a, 18b and 18c).

Figure 17 shows that, on average, the number of coccinellids per bunch was two times higher in colony uncaged bunches compared to caged ones.

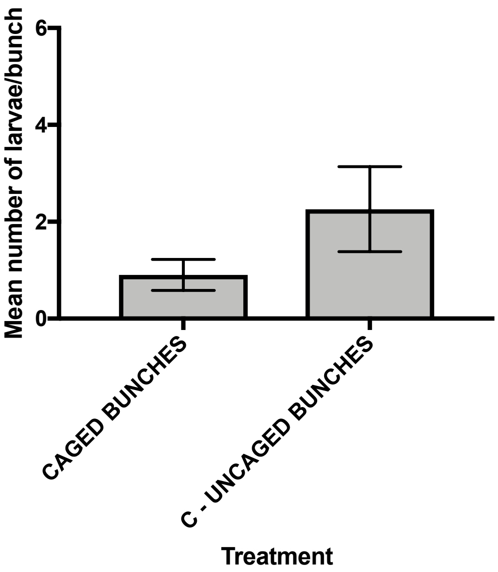

Figure 17. Average C. montrouzieri *larvae per bunch (± SE) (n=4) in 2018. C – uncaged bunches refer to colony uncaged bunches.*

Also analyzing each vineyard, the density dependence of the predator was confirmed. Indeed, both vineyard A and B showed the presence of higher number of larvae in colony uncaged bunches compared to caged ones (Figure 18a and 18b). On the other hand, less *C. montrouzieri* larvae per bunch were collected in colony uncaged bunches than in caged ones in vineyard C (Figure 18c).

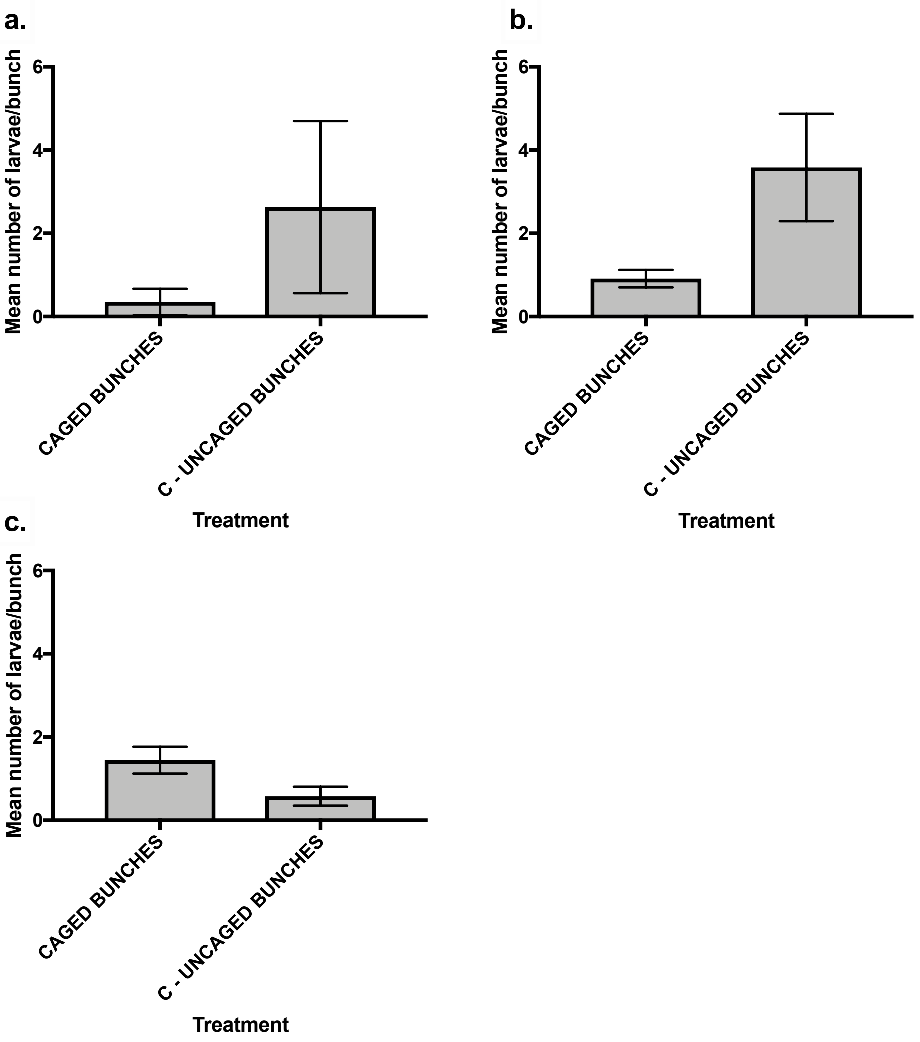

Figure 18. Mean C. montrouzieri *larvae per bunch (± SE) of vineyard A (a), vineyard B (b) and vineyard C (c) in 2018. C – uncaged plants refer to colony uncaged bunches.*

2.4.1.2 Year 2019

Mealybug infestation was lower in 2019 compared to 2018 inside the cages, after the second year of natural enemy release. The percentage of infested bunches dropped by 26%, whereas the mealybug mean decreased from 266 to 85 individuals per bunch (Figure 19a and 19b). As recorded in 2018, the percentage of infested bunches and also the number of individuals per bunch were higher on caged bunches than on random uncaged ones in 2019 (Figure 19a and 19b).

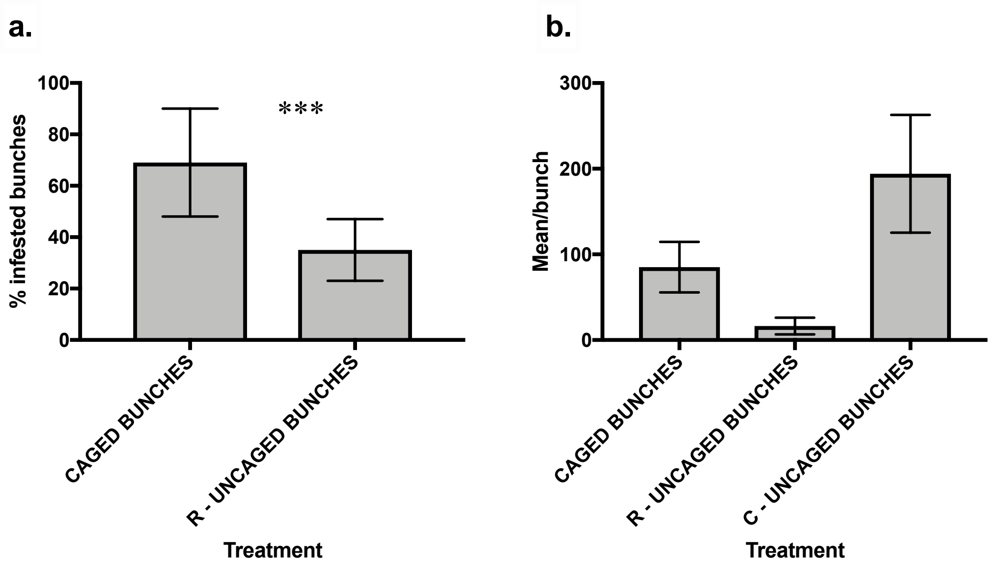

*Figure 19. Average infested bunch rate (± SE) (a) and mean mealybugs per bunch (± SE) (b) in 2019 (n=5). R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches. Chi square test showed a significant difference of infested bunch rate between the two treatments (***p<0,001) (a).*

Vineyard B presented a sharp decrease of damaged bunches in 2019. Indeed, no infested bunches were observed in caged and random uncaged treatment, whereas less than 100 mealybugs per bunch were found in colony uncaged bunches (Figure 20).

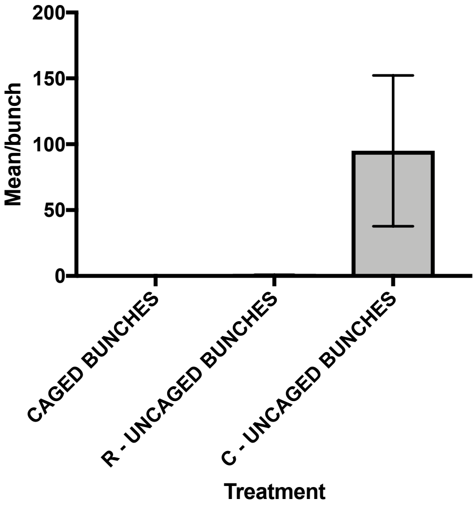

Figure 20. Vineyard B. Mean mealybugs per bunch (± SE) in 2019. R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches.

Significantly higher infestation rate was observed in caged bunches (56%) compared to random uncaged ones (21,43%) in vineyard C (df=1; χ^2=18,14; p<0,001) (Figure 21a). In particular, less than 100 individuals per bunch were found in caged bunches, whereas more or less 7 mealybugs per bunch were counted in the random uncaged bunches (Figure 21b). More than 350 of this hemipteran sucking pests were observed in colony uncaged bunches (Figure 21b), confirming once again that exclusion cage system did not overestimate infestation.

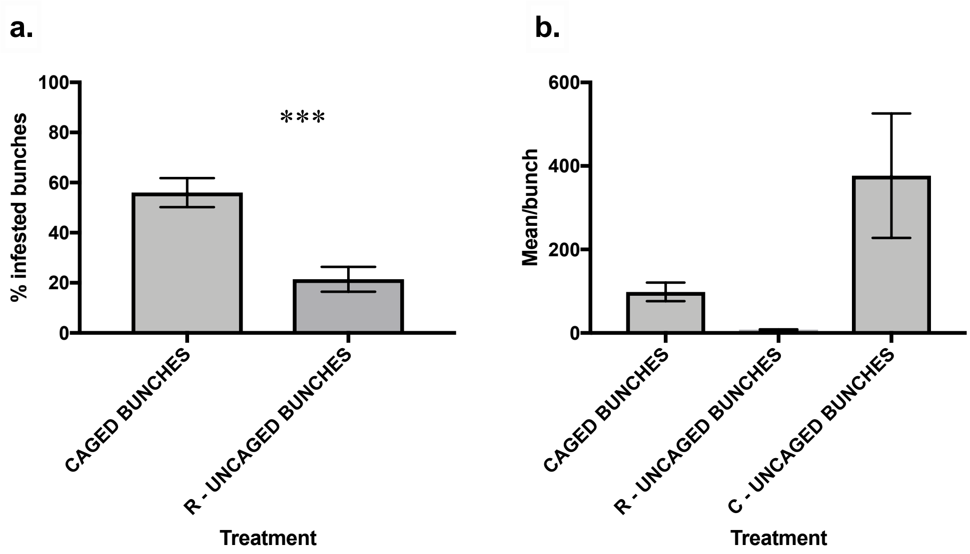

*Figure 21. Vineyard C. Infested bunch rate (±binomial SE) (a) and mean mealybugs per bunch (±SE) (b) in 2019. R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches. Chi square test showed a significant difference of infested bunch rate between the two treatments (***p<0,001) (a).*

Random uncaged treatment of vineyard D presented significantly less infested bunches than in caged one (df=1; χ^2=29,29; p<0,001) (Figure 22a). Regarding the number of mealybugs, 177 individuals per bunch were found in caged bunches, whereas 54 mealybug per bunch were observed in random uncaged bunches. On colony uncaged bunches, 347 mealybugs per bunch were counted (Figure 22b).

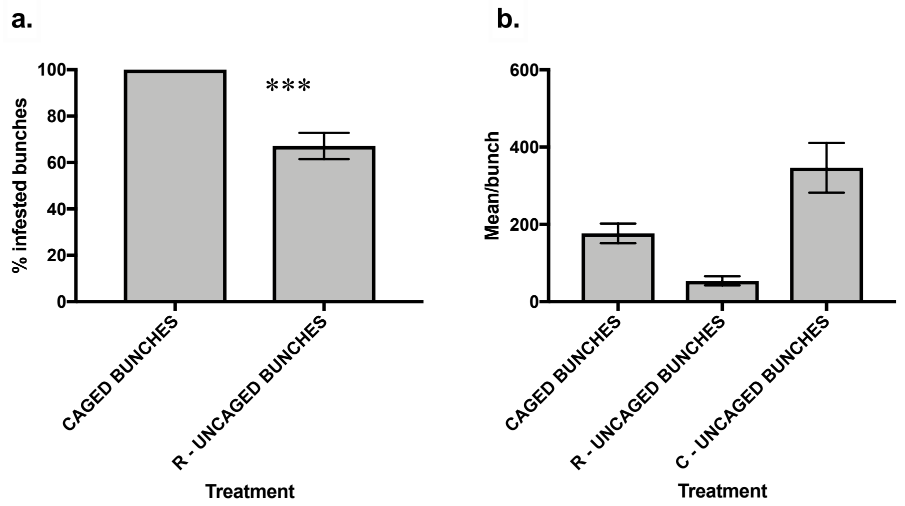

*Figure 22. Vineyard D. Infested bunch rate (± binomial SE) (a) and mean mealybugs per bunch (± SE) (b) in 2019. R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches. Chi square test showed a significant difference of infested bunch rate between the two treatments (***p<0,001) (a).*

In the cage treatment, 100 % of infested bunches was found compared to only 40 % in random uncaged bunches in vineyard E (Figure 23a), showing a significant difference between the two treatments (df=1; χ^2=26,89; p<0,001). Concerning the mean number of mealybugs per bunch, more individuals were observed in caged bunches than in colony and random uncaged bunches, which presented only 16 mealybugs per bunch (Figure 23b).

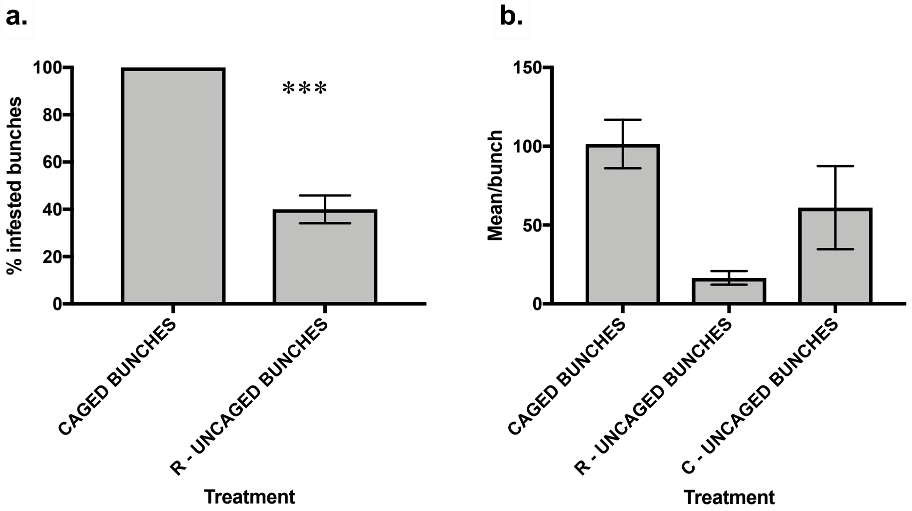

*Figure 23. Vineyard E. Infested bunch rate (± binomial SE) (a) and mean mealybugs per bunch (± SE) (b) in 2019. R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches. Chi square test showed a significant difference of infested bunch rate between the two treatments (***p<0,001) (a).*

Lastly, cage treatment presented significantly 39 % more infested bunches than random uncaged one in vineyard F (Figure 24a) (df=1; χ^2=26,3; p<0,001). Colony uncaged bunches showed the highest number of individuals per bunch (91,25), whereas more or less 49 mealybug per bunch were observed in caged bunches and only 4 in random uncaged bunches (Figure 24b).

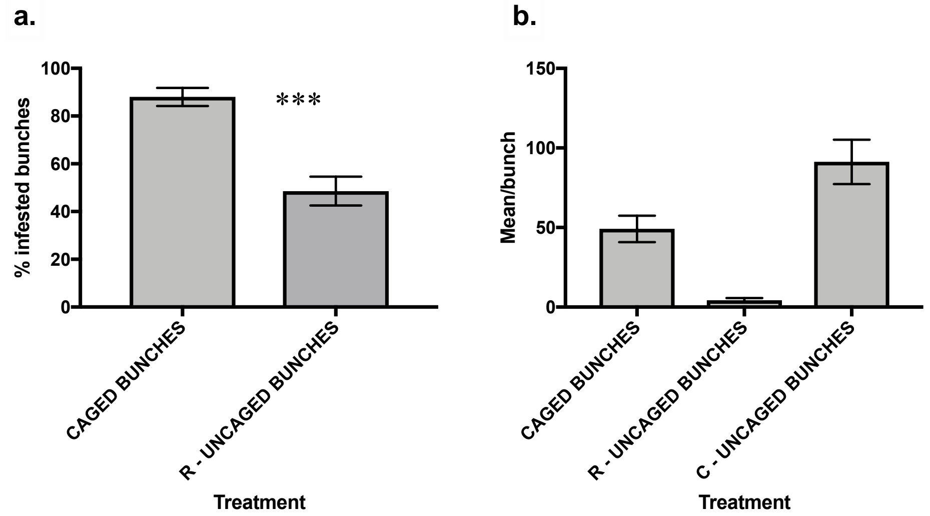

*Figure 24. Vineyard F. Infested bunch rate (± binomial SE) (a) and mean mealybugs per bunch (± SE) (b) in 2019. R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches. Chi square test showed a significant difference of infested bunch rate between the two treatments (***p<0,001) (a).*

Concerning mealybug parasitization, there was a high variability among vineyards in 2019. Vineyard B had a very low infestation during that year. Consequently, the percentage of parasitized mealybug was more or less 0% both inside and outside the cages (Figure 25a), impeding z-test. Parasitization was higher in the caged bunches compared to random and colony uncaged bunches in vineyards C and E (Figure 25b and 25d). In particular, less than 2% of mealybugs were parasitized outside the cages, whereas 19% of *P. comstocki* presented sign of parasitization in the cage treatment of vineyard C (Figure 25b). Chi square test followed by z-test confirmed the significant difference between parasitism rate of caged bunches and colony uncaged bunches in vineyard C (χ^2=10,69; df=2; p=0,009). In vineyard E, where infestation was principally caused by *P. ficus*, less than 50% of individuals were parasitized in random and colony uncaged bunches, while parasitism rate was 61,54 % on caged ones (Figure 25d). However, these differences were not statistically relevant.

On the other hand, *P. comstocki* parasitization was significantly higher in the colony uncaged bunches (20,9%) compared to caged ones (2,82%) in vineyard D (χ^2=15,42; df=2; p<0,001) (Figure 25c).

Finally, less than 0,5% of mealybugs were parasitized in vineyard F (Figure 25e). This datum can be explained by the grower's choice of treating mealybug infestation, causing a premature disappearance of the parasitoid and incomplete assessment of its action.

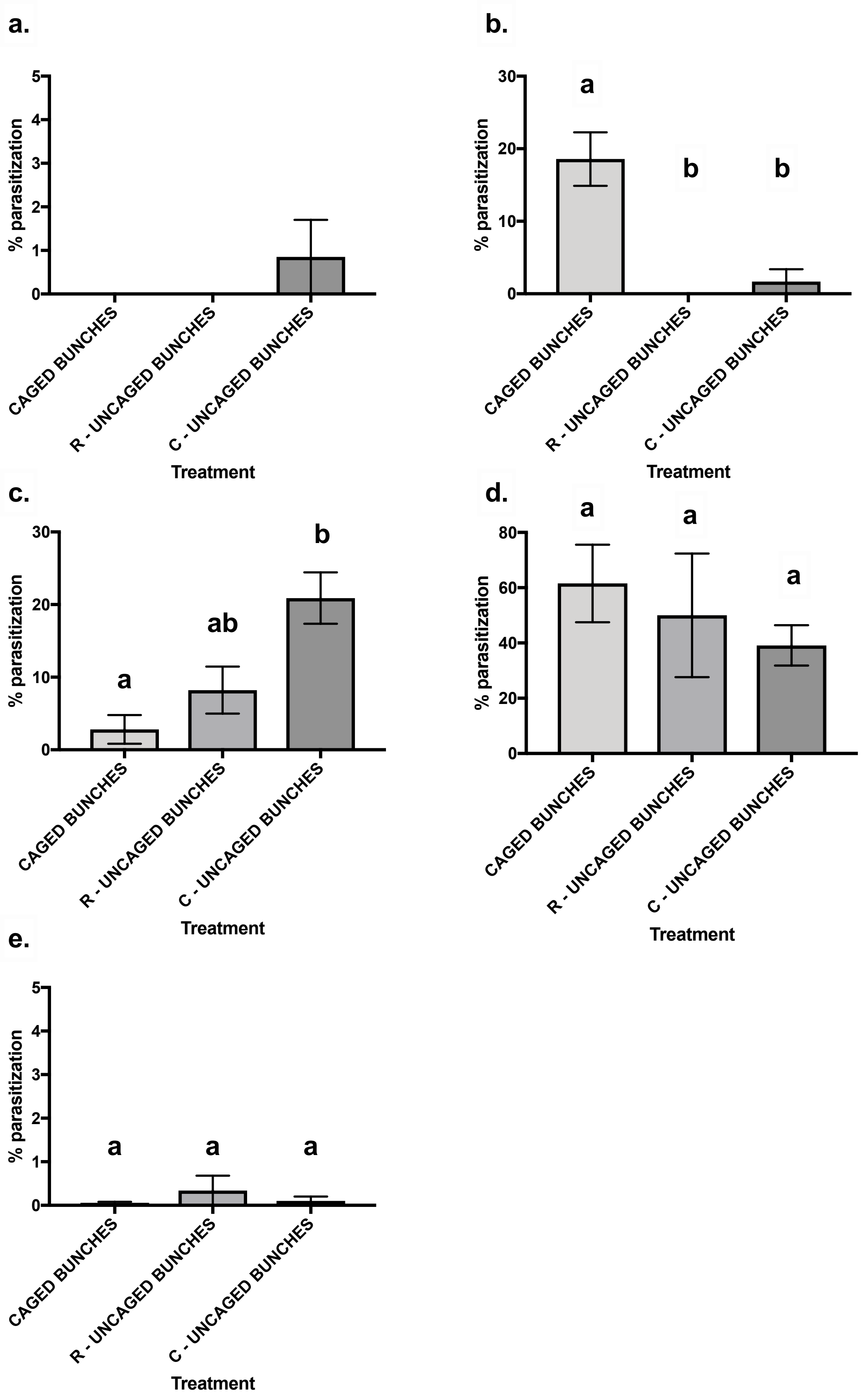

Figure 25. Parasitization rate of adult and female with ovisac mealybug stages (± binomial SE) of vineyard B (a), vineyard C (b), vineyard D (c), vineyard E (d), vineyard F (e) in 2019. R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches. Different letters indicate significant statistical differences (z-test: p<0,05).

Parasitism rate of *P. comstocki* was higher in caged bunches in 2019 compared to 2018. This result is line with marginal association test of log linear analysis which showed a significant interaction between treatment and year on parasitization rate (Table 6). Nevertheless, the number of the parasitized Comstock mealybugs was the same between caged and colony uncaged bunches, whereas a lower parasitism percentage was observed in random uncaged bunches compared to caged ones (Figure 26).

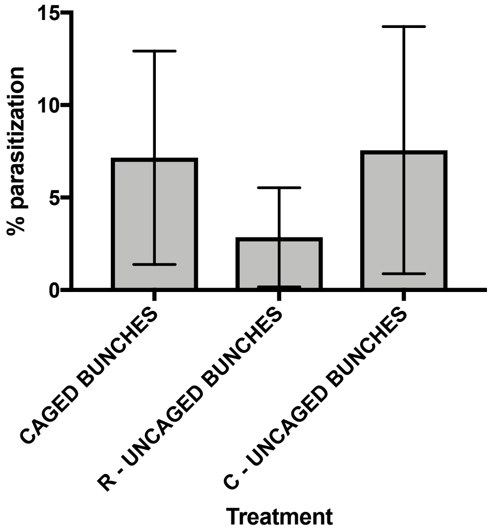

Figure 26. Average parasitism rate of adult and female with ovisac (± SE) considering all vineyards infested by P. comstocki *mealybugs (n=3) in 2019. R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches.*

Regarding predation, *C. montrouzieri* acted effectively on mealybug infestations in all vineyards, apart from vineyard B and F where predation rates were less than 2% (Figure 27a and 27e). As already mentioned above for the parasitization, vineyard B had a very low infestation in 2019, whereas in vineyard F chemical treatments against mealybugs were carried out one month earlier bunch harvesting. For this reason, it was not possible to run a z-test in vineyard B.

On the other hand, predation significantly increased by more or less 30% on colony uncaged bunches compared to caged ones in vineyard C (χ^2=15,42; df=2; p<0,001) (Figure 27b). Also in vineyard D, the number of predated mealybugs was more abundant on colony uncaged bunches (17,91%) compared to caged ones (8,45%) (Figure 27c), even though the difference was not significant.

Finally, vineyard E showed a higher predation on random uncaged bunches than caged ones. Unlike other vineyards, the percentage of predation was higher on random uncaged bunches (33,33%) compared to colony uncaged ones (6,52%) (Figure 27d). Nevertheless, these differences were not significant.

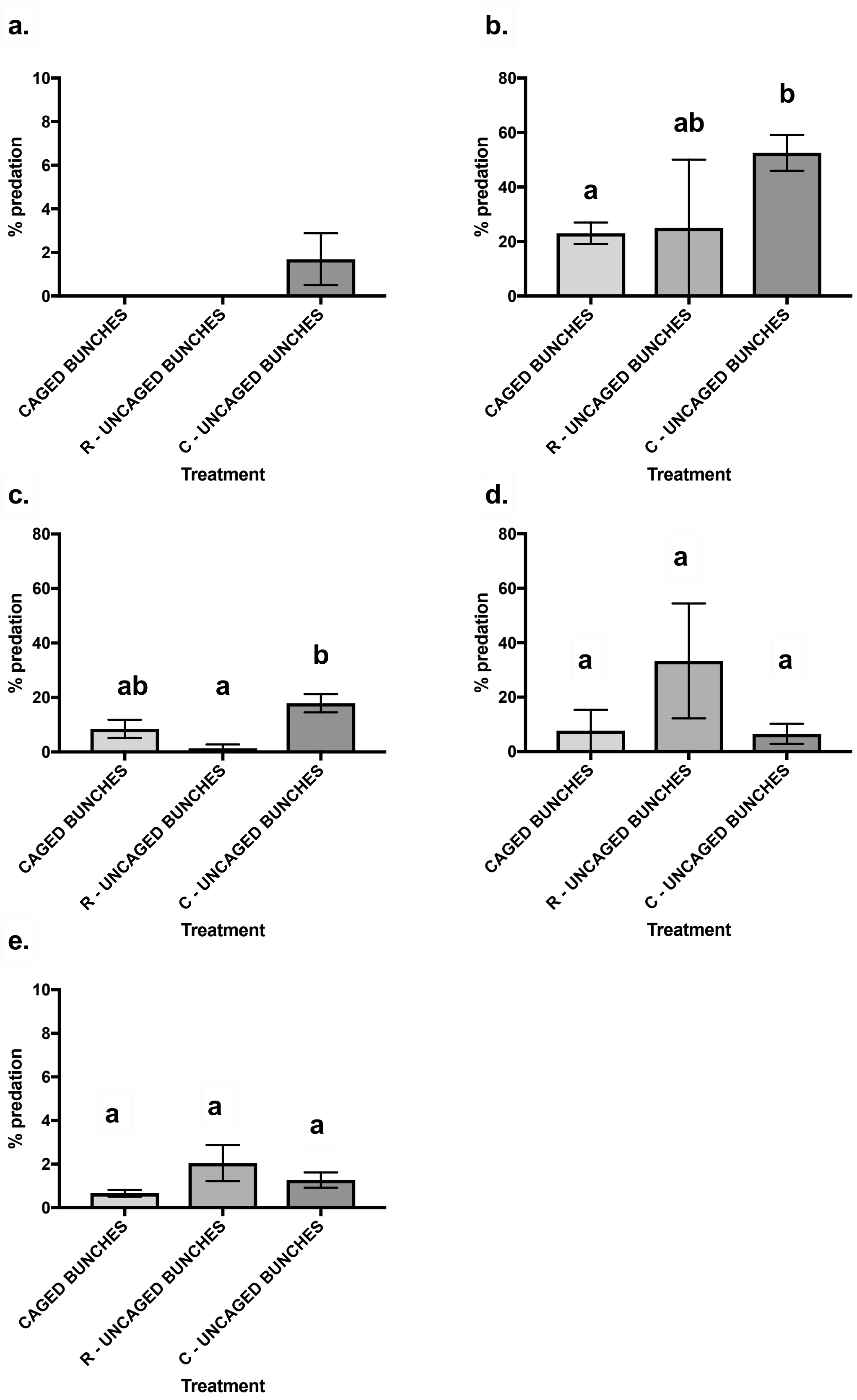

Figure 27. Predation rate of adult and female with ovisac mealybug stages (± binomial SE) of vineyard B (a), vineyard C (b), vineyard D (c), vineyard E (d), vineyard F (e). R - uncaged bunches refer to random uncaged bunches, whereas C – uncaged bunches refer to colony uncaged bunches. Different letters indicate significant statistical differences (z-test: p<0,05).

On average, the mean number of *C. montrouzieri* larvae per bunch was higher in colony uncaged bunches compared to caged ones (Figure 28).

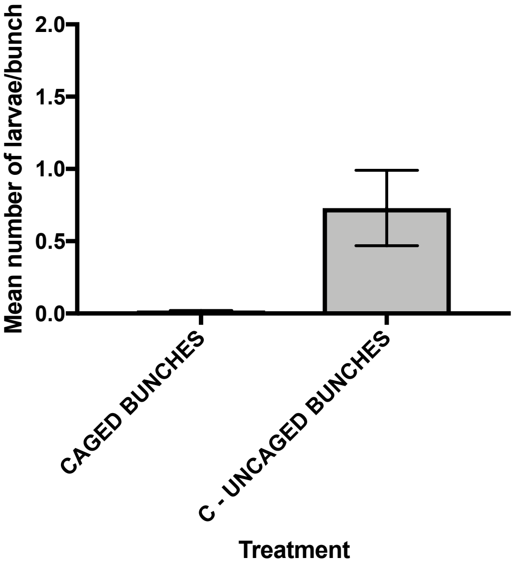

Figure 28. Average C. montrouzieri *larvae per bunch (± SE) (n=4). C – uncaged bunches refer to colony uncaged bunches.*

This density dependent trend of the predator was also observed in the majority of vineyards of the trial in 2019 (Figure 29b, 29c and 29d). Only vineyard C presented a similar mean number of the coccinellid per bunch (Figure 29a).

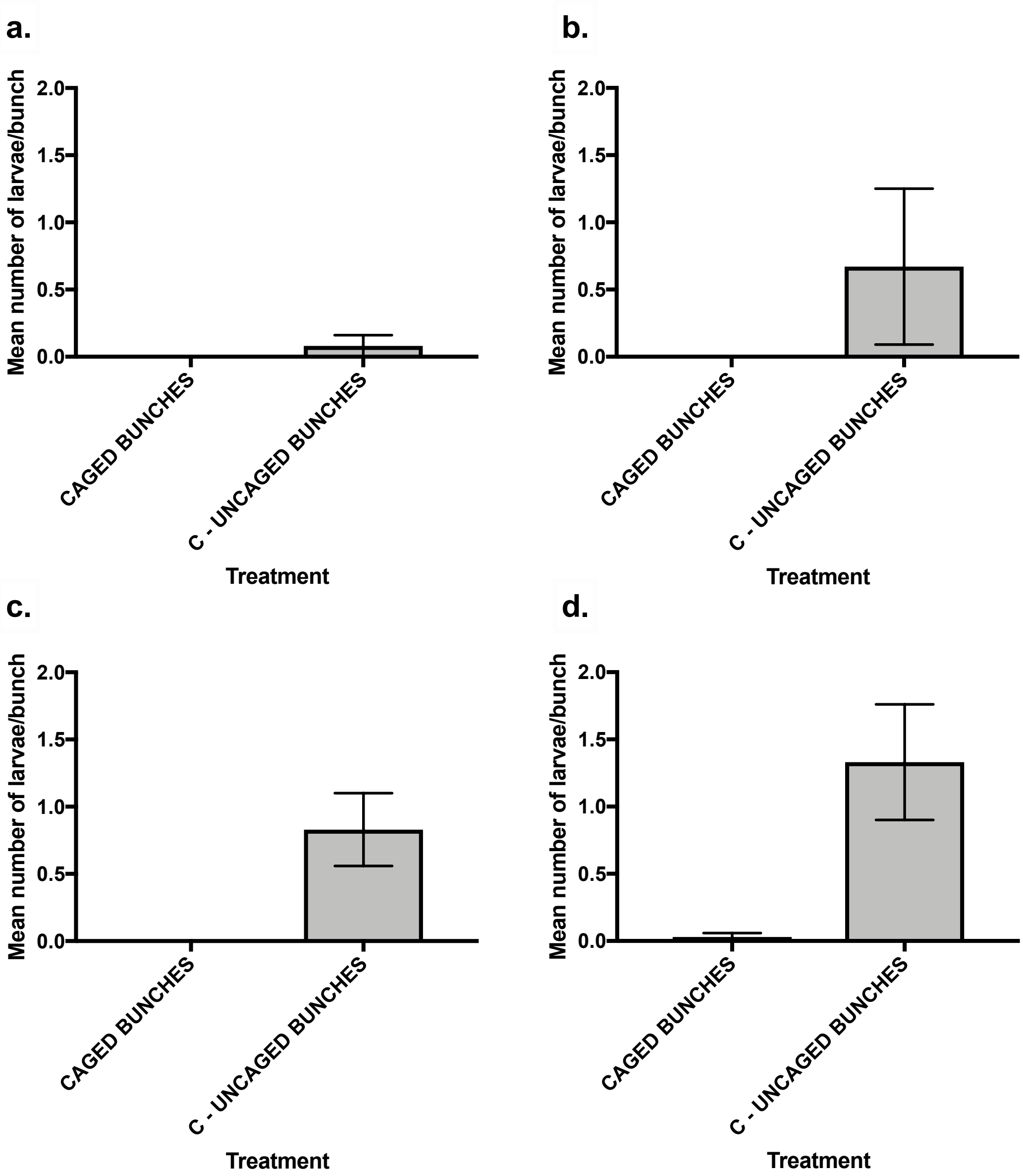

Figure 29. Mean C. montrouzieri *larvae per bunch (± SE) of vineyard C (a), vineyard D (b), vineyard E (c) and vineyard F (d) in 2019. C – uncaged bunches refer to colony uncaged bunches.*

2.4.2 Natural enemy release in commercial vineyards

In July sampling, low infestation on bunches was detected in most of the vineyards. Vineyard 1 presented the highest level of infestation rate (28%), whereas vineyard 5 showed the lowest number of infested bunches (2%).

Figure 30 shows the different level of bunch infestation in each vineyard in September sampling. The percentage of infestation rose in September and the number of infested vineyards was higher compared to July. The minimum level of infestation was in vineyard 7 (4%), while vineyard 1 presented the highest percentage of infested bunches (58%) (Figure 30a). Also the mean number of mealybugs per bunch increased in September compared to July, following the trend of infested bunch rate. Vineyards with higher infestation rates had also higher number of mealybugs per bunch (Figure 30b).

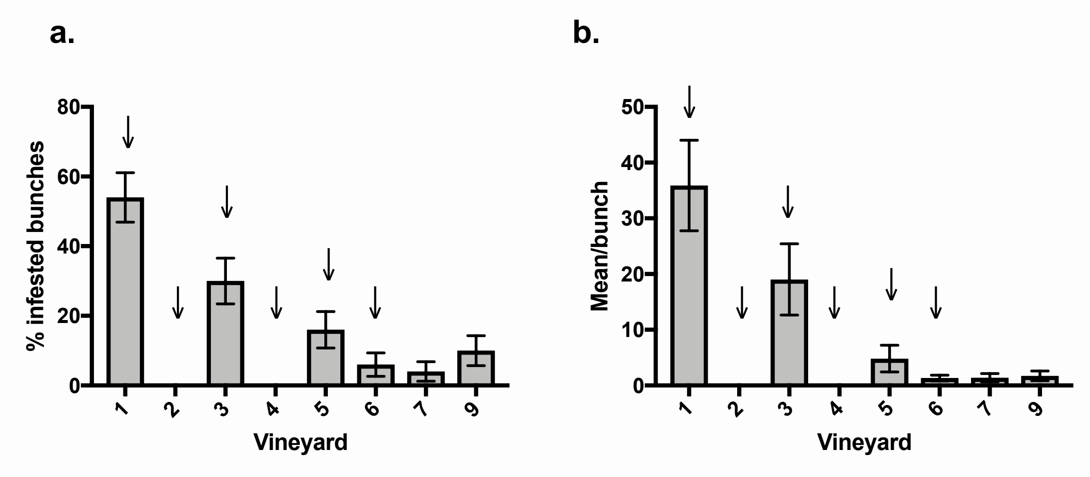

Figure 30. Infested bunch rate (±binomial SE) (a) and total mealybug mean per bunch (±SE) (b) in each vineyard in September sampling. Arrows show in which vineyards natural enemy releases were carried out. Vineyard 7 and 9 were considered as control vineyards (no natural enemies were released).

Figure 31 shows the positive correlation between infested bunch rate and the mean number of mealybugs per bunch.

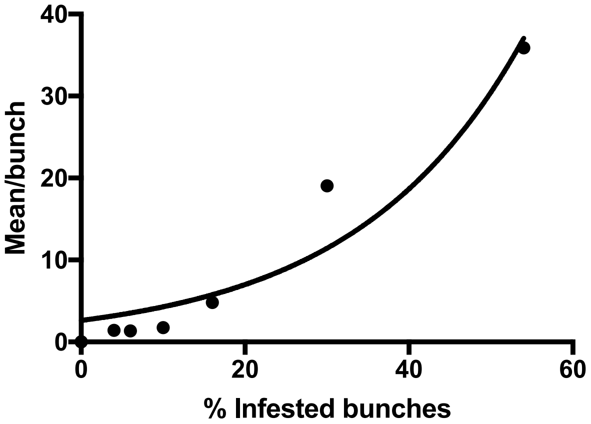

*Figure 31. Curvilinear correlation between the percentage of infested bunches and mealybug mean per bunch (Equation: y=2,627*exp(0,04x); R= 0,92; p<0,05)*

Vineyard 2 and 4 did not have any infested bunch both in July and in September.

As shown in figure 32, no correlation was observed between infestation and insecticide treatments. Only mealybug and *Scaphoideus titanus* treatments were considered for this correlation.

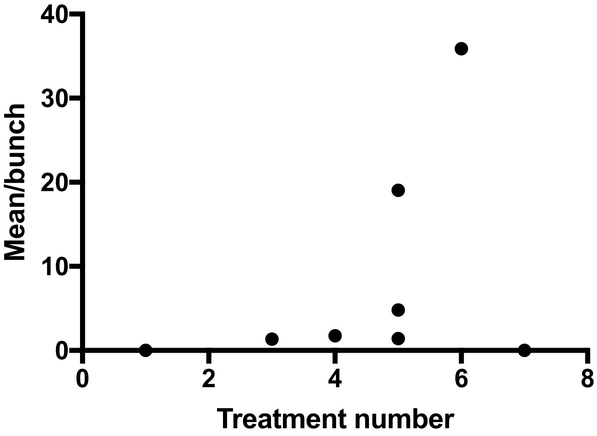

Figure 32. Relationship between the number of insecticide treatment and the mean number of mealybugs per bunch. p>0.1 (linear correlation analysis).

Only September data on parasitization are reported because of the lack of parasitised mealybugs on bunches in July. There was high variability among vineyards. Apart from vineyard 6, the parasitization showed a higher percentage in random bunches compared to colony ones in vineyards where the inoculative releases were carried out (Figure 33). Indeed, the percentage of parasitization in random bunches was between 10% and 27 % (Figure 33a), whereas less than 10% of mealybug individuals were parasitized on colony bunches (Figure 33b). Only in vineyard 6, the parasitism rate was 0% on random bunches, whereas 36,59% of parasitized mealybugs were observed on colony bunches (Figure 33).

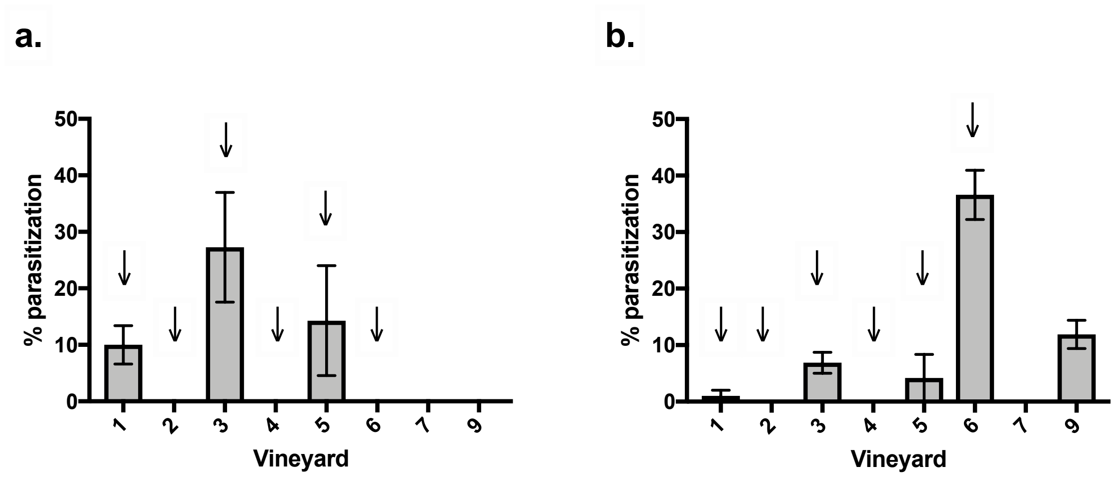

Figure 33. Parasitism rate of female adults and females with ovisac (± binomial SE) on random bunches (a) and on colony bunches (b). Arrows show in which vineyards A. vladimiri *releases were carried out. Vineyard 7 and 9 were considered as control vineyards (no* A. vladimiri *individuals were released).*

Furthermore, figure 34 shows that the parasitism rate decreased as the number of insecticide treatments increased.

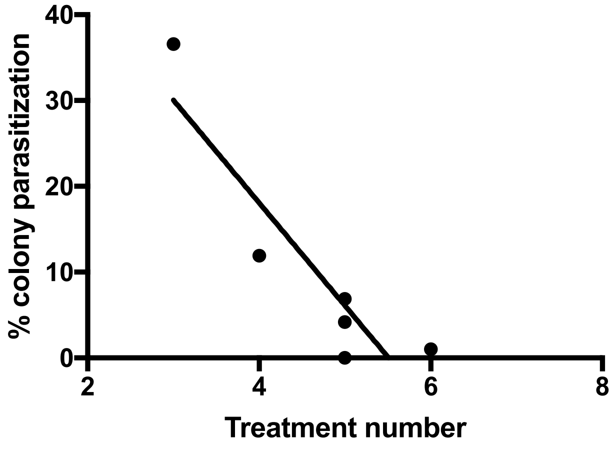

Figure 34. Correlation between the number of insecticide treatment and the parasitization rate of adult and females with ovisac on colony bunches (Equation: y=49,5013-8,7573x; R=0.78; p<0.05)

On average, 12,89% of female adult and female with ovisac mealybugs were parasitized on random bunches of vineyards where *A. vladimiri* was released (n=4). On the other hand, in those vineyards, the percentage of parasitization on colony bunches was only 12,16% (n=4). Anyway, the parasitization was higher both on colony and on random bunches in vineyards where inoculative releases were carried out compared to vineyards without them. Less than 6% of parasitized mealybugs were observed where the parasitoid was not released (n=2).

Parasitization data showed that *P. ficus* was more parasitized by *A. vladimiri* than *P. comstocki*. Indeed, parasitism rate peaks was 36,59% and 27,27 % on *P. ficus* and *P. comstocki* respectively.

The activity of *C. montrouzieri* in terms of predation on adult mealybug stages was higher in colony bunches compared to random bunches in vineyards where it was released. *C. montrouzieri* predated 80,61% and 52,85% of mealybugs in vineyard 1 and 3 respectively, whereas predation rates were less than 30 % in control vineyards (Figure 35).

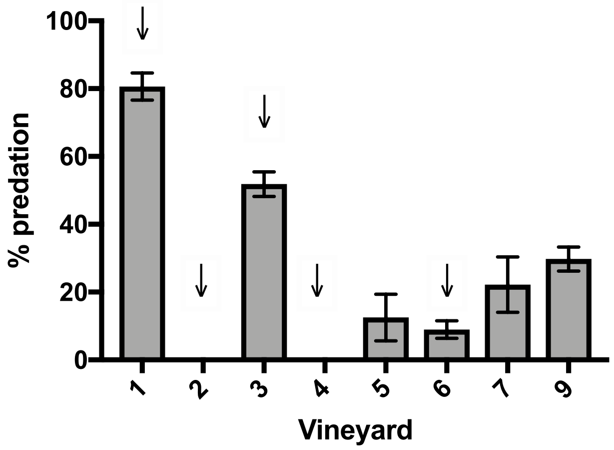

Figure 35. Predation rate of adult and female with ovisac mealybug stages (± binomial SE) on colony bunches. Arrows show in which vineyards C. montrouzieri *releases were carried out. Vineyard 7 and 9 were considered as control vineyards (no* C. montrouzieri *individuals were released).*

On average, 47,14% of adult mealybugs were predated on colony bunches of vineyards where *C. montrouzieri* was released (n=3). On the other hand, only 21,49 % of mealybugs were devoured where no inoculative releases were performed (n=3).

Finally, larvae of *C. montrouzieri* were recorded in all vineyards where the predator was released (Figure 36), whereas none of them was found in control vineyards. Even if predator releases were carried out in vineyard 2 and 4, no *C. montrouzieri* individuals were observed, probably because no mealybug infestation was detected.

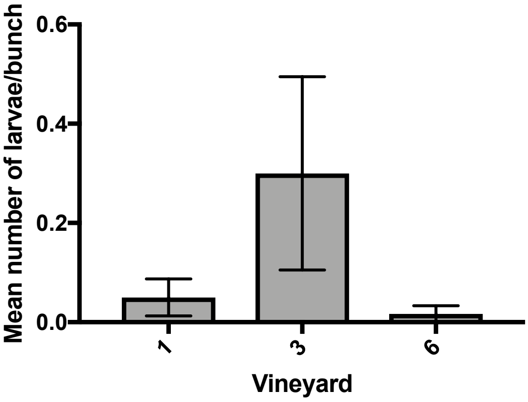

Figure 36. Mean C. montrouzieri *larvae per bunch (± SE) in vineyards where the predator was released.*

2.5 Discussion and conclusion

Both *A. vladimiri* and *C. montrouzieri* provided a successful control against mealybugs in both field trials, even if some variability was observed depending on mealybug species and their level of infestation.

Our results of exclusion cage trial confirmed that this technique is reliable and effective to evaluate the efficiency of natural enemies released for the biological control pest infestation. This technique allows to assess the contribution of natural occurring antagonists and that of released ones to the total impact of biological control. One of the major risks of exclusion technique is the large growth rate of pests inside the cages which is created by an extremely favourable microenvironment in terms of temperature and humidity. This excessive growth of pest population may overestimate the infestation parameters of cage treatment. However, our data showed that this is not the case. Indeed, mealybug infestation was higher outside than inside the cages in several vineyards of the trial. Several researchers have carried out exclusion studies to assess natural enemy potential in suppressing pests in different crop systems (Ameixa & Kindlmann, 2011; Meihls et al., 2010; Grasswitz & Burts, 1995).

Average higher infestation in cage treatment compared to random and colony uncaged bunches confirmed that natural enemy performance was effective in controlling mealybugs, even if there was variability among vineyards.

In both mealybug trials, variability was particularly noticeable in parasitism rate among treatments and among vineyards. Vineyards presented infestations which were arranged differently on spatial level and this characteristic may have influenced the presence of higher parasitism rate on colony bunches rather than on random bunches or viceversa. In some cases, mealybug populations were extremely localised, as in vineyard B in 2019 and vineyard 6, whereas it spread more widely in other field sites, such as vineyard F.

Mealybug species-specificity may have contributed to infestations spatially different. The Comstock mealybug is more mobile, has a higher reproductive rate and spreads faster compared to *P. ficus*, thereby making *P. comstocki* more dangerous.

Also parasitism rate was highly influenced by mealybug species. The efficacy of *A. vladimiri* on *P. ficus* was widely demonstrated by previous studies. Conversely, *A. vladimiri* and the Comstock mealybug represents a new association and no published data are present about the parasitoid releases against this pest if we exclude what reported by Ricciardi et al. (2021) who calculated a parasitization rate in laboratory of 50%. *Acerophagus malinus* (Hymenoptera: Encyrtidae) releases have been carried out against the Comstock mealybug in the former USRR and USA, whereas *Allotropa burrelli* (Hymenoptera: Platygastridae) have been used to control this Asian pest in the USA (Malausa et al., 2016; Meyerdirk & Newell, 1979). Malausa et al. (2016) investigated possible biological control agents of *P. comstocki* in France and identified *A. vladimiri* as one of the parasitoid species that emerged from the Comstock mealybug.

In the exclusion cage trial, the parasitism rate of *P. comstocki* was 18% higher in colony uncaged bunches than caged ones only in vineyard D in 2019. Considering the mean among vineyards in 2019, the number of *P. comstocki* individuals parasitized by *A. vladimiri* outside the cages (colony uncaged bunches) was similar to parasitism rate of cage treatment (caged bunches). In 2018, parasitism rate was higher in random uncaged bunches compared to caged ones. Vineyard D may have determined this result, but probably the parasitism rate of this field site may have overestimated the parasitization due to the low number of mealybug individuals collected on random uncaged bunches.

P. comstocki parasitim was more than double in vineyards where the parasitoid was released (11%) than in field sites where it was not released (3%). Moreover, up to 27% of *P. comstocki* mealybugs were parasitized in vineyard 3.

Not only *A. vladimiri* was responsible of parasitism rate of *P. comstocki*. Indeed, also gregarious and solitary parasitoids which did not belong to Encyrtidae family were found, probably Pteromalidae individuals.

Sign of parasitism by *A. vladimiri* on the Comstock mealybug was detected in both field trials, although this encyrtid parasitoid was surely more effective on *P. ficus*. In vineyard B, almost 100% of vine mealybug were parasitized in all treatments in 2018. A high level of parasitism that was observed in cage treatment can be justified by a previous release of *A. vladimiri,* carried out for a semi-field trial in 2017 (Gambirasio, 2017). This first release of the parasitoid may have fostered its permanence in the following year. Also in commercial vineyard trial, in field site 6, 36% of *P. ficus* were parasitized.

The predator *C. montrouzieri* clearly showed to be very effective in controlling both mealybug species. A high predation rate was observed in the majority of vineyards in both field trials. Ladybird larvae were principally collected on colony bunches, whereas no *C. montrouzieri* adults were found within most of the cages or vineyards where no inoculative releases were not carried out. This result demonstrated that this Australian coccinellid is highly density dependent and particularly voracious so its capacity of suppressing mealybugs, especially on mealybug colonies, is very high. Its characteristics can be also exploited in vineyards where the infestation is extremely localised and affects just a few vine plants.

While *C. montrouzieri* activity has to be estimated on colony bunches due to its density dependence (Kairo, 2013), both colony and random bunch sampling were necessary for *A. vladimiri* parasitism evaluation, for its variable response to mealybugs density. Assessing parasitized mealybugs on colony bunches was fundamental when vineyards were characterized by low infestation. Nevertheless, intraguild predation (IGP) may occur in vineyards with medium-high infestation level and random bunch sampling becomes crucial to evaluate the impact of *A. vladimiri* on mealybugs. Previous studies reported IGP phenomenon on several predator-parasitoid-pest systems (Fu et al., 2017; Pirzadfard et al., 2020; Prieto et al., 2018). On colony bunches, *C. montrouzieri* may have provided such a great control, leaving very few hosts suitable for the parasitoids; also, the predators could have eaten parasitized mealybugs, leading to local underestimation of parasitisation. Muştu et al. (2008) showed that *C. montrouzieri* consumption of *A. pseudococci* parasitized mealybugs tended to exceed that of non-parasitized mealybugs. Although, the consumption of parasitized *P. citri* and *P. ficus* became impossible as mummification advanced.

Natural enemy releases in commercial vineyards demonstrated that insecticides against vine and the Comstock mealybugs did not provide a complete control, as no correlation was observed between infestation and the number of pesticide treatments. Moreover, it was observed that the intensity of chemical control decreased significantly the parasitism rates. Acetamiprid, which is used against mealybug infestation, represents one of the most harmful active ingredient for *A. pseudococci* (Satar et al., 2018).

The positive correlation between infested bunch rate and the mean number of mealybugs per bunch (or binomial sampling) was an important result which may simplify and accelerate the estimation of mealybug infestation. Counting the number of mealybug individuals, especially before harvesting, is extremely complex, especially in some vine varieties and it is not practical in field survey. On the other hand, defining the percentage of infested bunches is easier and could be assessed directly in the field by means of visual sampling, in order to have an easier evaluation of the severity of pest infestation.

To summarise, augmentative biological control by means of combined release of *A. vladimiri* and *C. montrouzieri* provided a successful control against mealybugs. In particular, both the predator and the parasitoid demonstrated to be effective against *P. ficus* (Daane et al., 2008; Mansour et al., 2012). Previous field trial recorded between 27% and 70 % of *P. ficus* parasitism rate carried out by *A. vladimiri* in Tuscany region (Lucchi & Benelli, 2018; Lucchi et al., 2018; Varner et al., 2015). In a preliminary small scale field experiment in Emilia (Gambirasio, 2017), a parasitisation rate of P. *ficus* mealybugs ranged from 50 to 70%.

The Australian ladybird confirmed its efficient performance against the Asian mealybug, whereas further studies need to be carried out on the encyrtid parasitoid, in order to better understand the trend of parasitisation during the years. Parasitism rate improved after *A. vladimiri* release, but still a high variability was recorded among vineyards and other parasitoids of *P. comstocki* were observed (Figure 37). Moreover, also ant attendance could contribute to the variability of parasitisation rate on *P. comstocki.*

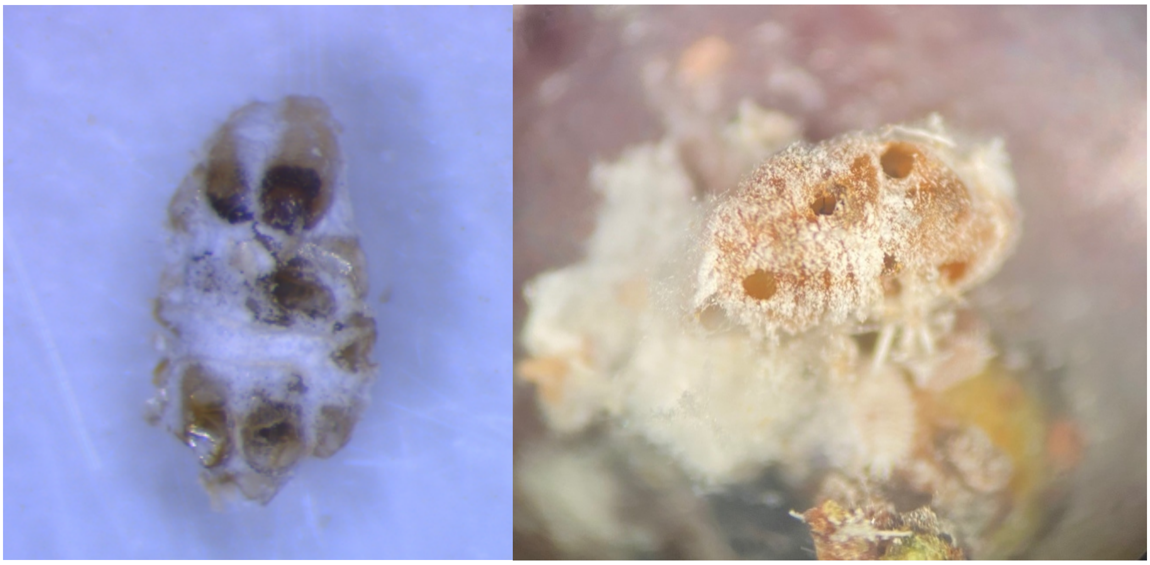

Figure 37. Examples of mealybugs parasitized by gregarious parasitoids.

Individuals of *P. comstocki* should be collected from vineyards and reared in laboratory, in order to carry out an integrative characterization of its parasitoids. This approach would provide a more precise estimation on real possibility of using *A. vladimiri* biological control programmes against *P. comstocki*.

Finally, the role of tending ants should be investigated. Several studies demonstrated that beneficials' activity was often disturbed by highly aggressive ants (Fanani et al., 2020; Mansour et al., 2012; Mgocheki & Addison, 2009). Mitigating ant attendance, from which mealybugs benefit, may help to boost natural enemy action against this pest.

Beneficial releases carried out in these trials represented an important innovation in Emilia-Romagna region and increased growers' awareness on the great potentiality of *A. vladimiri* and *C. montrouzieri* in controlling mealybug infestation. For the first time, a wide territorial approach of augmentative biological control was accomplished, releasing the mealybug parasitoid and predator on 10000 ha vineyard area (DISTAL, 2020). Considering the low efficacy of insecticides and their negative impact on environment as well as on human health, combined releases of these natural enemies should be normally applied as a sustainable mealybug integrated control strategy in vineyards and as a routine technique in organic farming.

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Chapter 3 Use of sugar dispensers to disrupt ant attendance and improve biological control of mealybugs in vineyard[1]

3.1 Abstract

Mealybugs (Hemiptera: Pseudococcidae), nowadays considered severe agricultural pests, are small, soft-bodied plant sap-sucking insects. *Planococcus ficus* (Signoret) and *Pseudococcus comstocki* (Kuwana) are economically important pests occurring in vineyards, causing severe economic losses for growers and compromising bunch production.

The partial effectiveness of insecticides used in controlling mealybug infestations, their high impact on environment and on human health have prompted the research of alternative and sustainable control methods, including biological control. Several natural enemies are reported to be effective against mealybugs, but their activity may be hindered by tending ants, which are known to exhibit a mutualistic relationship with mealybugs, resulting extremely aggressive against beneficial insects. Consequently, this study explored a method to mitigate ant attendance by means of sugar dispensers in order to improve ecosystem services, thus decreasing mealybug infestation in vineyards. Field trials were run in four commercial vineyards of Northern Italy infested by mealybugs, in which *Anagyrus vladimiri* Triapitsyn (Hymenoptera: Encyrtidae) and *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) were released as biological control agents. Ant activity was estimated by visually counting the number of ants on vine trunks. The rate of infested bunches was assessed by a visual sampling at harvest, whereas bunches were collected and analysed in laboratory to estimate parasitization and predation rates.

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[1] This chapter has been published in a slightly different form as: Parrilli, M., Profeta, M., Casoli, L., Gambirasio, F., Masetti, A., Burgio, G. Use of sugar dispensers to disrupt ant attendance and improve biological control of mealybugs in vineyard. Insects 2021, 12, 330.

The use of sugar dispensers reduced ant activity and in turn mealybug infestation, leading to a significant enhancement of ecosystem services. The technique showed a great potential in boosting biological control against mealybugs in field conditions, though the field application seemed to be labour intensive and needs to be replicated for a multi-year evaluation.

3.2 Introduction

Mealybugs (Hemiptera: Pseudococcidae) are key pests of vineyard. Their feeding activity, as well as the excretion of large amount of honeydew, are responsible for severe damages on plants, especially on fruit production (Cocco et al., 2018; Lucchi et al., 2019; Ricciardi et al., 2021). Pesticides still represent the most common strategy to control mealybugs. Nevertheless, mealybug habit to stay in concealed plant parts and the waxy cover of these insects make chemical control somewhat poorly effective (Mani & Shivaraju, 2016). New tactics, alternative to chemicals, show a potential to reduce and suppress mealybug infestations, including biological control (Cocco et al., 2021). Previous studies showed the potential of natural enemies, such as *Anagyrus vladimiri* Triapitsyn (Ricciardi et al., 2021) and *Cryptolaemus montrouzieri* Mulsant, in controlling these detrimental pests (Daane et al., 2008; Rahmouni & Chermiti, 2013). However, few studies reporting evaluation of the field effectiveness of these biological agents are available. In Northern and Central Italy, several releases of *A. vladimiri* and *C. montrouzieri* have been carried out in the last few years and a field efficacy evaluation of these biological agents is in progress (Parrilli, unpublished data). Preliminary assessments of biological control programs reported a high effectiveness of inoculative releases (Varner et al., 2015), characterized by some degree of variability depending on geographic area, phytosanitary treatments of vineyard, and occurrence of attending ant (Hymenoptera: Formicidae) species (Parrilli, unpublished data).

It is well known that the activity of biological control agents on mealybugs may be hindered by mealybug-tending ants which create a strong association with these sap sucking pests. Some ant species develop a mutualistic relationship with mealybugs due to their honeydew-consuming habit. These species feed on honeydew, offering in return many benefits to mealybugs. Tending ants build earthen shelters to protect mealybugs from adverse weather conditions and prevent nymphs getting stuck in their honeydew (Mani & Shivaraju, 2016). Moreover, ants facilitate mealybug dispersal and provide them protection against natural enemies. Parasitoids and predators are often attacked by tending ants, which are particularly hostile to any possible harmful movement around honeydew source (Mani & Shivaraju, 2016). Ant aggressiveness often disturbs natural enemy activity, thereby causing biological control disruption (Daane et al., 2007) or reduced effectiveness of the released beneficials. Mani and Shivaraju (2016) showed several examples of natural enemy failures in controlling mealybugs due to the presence of ant attendants.

Ant aggressiveness depends on species and Buckley and Gullan (1991) demonstrated that coccoids attended by relatively inoffensive ants were more parasitized than those attended by more aggressive species. Mgocheki and Addison (2009) proved that the ant species *Anoplolepis steingroeveri* (Forel), *Crematogaster peringueyi* Emery and *Linepithema humile* (Mayr) (Hymenoptera: Formicidae) significantly interfered with biological control of *Planococcus ficus* (Signoret). Also *Tapinoma nigerrimum* (Nylander) was found to hinder and disrupt the activity of two main natural enemies of the vine and citrus mealybugs (Mansour et al., 2012). Fanani et al. (2020) demonstrated that the parasitoid of cassava mealybug, *Anagyrus lopezi* (De Santis) (Hymenoptera: Encyrtidae), exhibited higher parasitism and emergence rates on ant-excluded plants compared to ant-attended plants in laboratory conditions.

Granular insecticides, exclusion methods, liquid baits as well as sugar source provisioning have been already tested to control ants or mitigate their activity (Beltrà et al., 2017; Daane et al., 2006; Klotz et al., 1998; Nondillo et al., 2016), showing promising results in reducing mealybug abundance and infestations (Beltrà et al., 2017; Daane et al., 2008; Daane et al., 2006; Itioka & Inoue, 1996). Stanley (2004) reviewed the efficacy of baits deployed for ant control and eradication. Liquid sucrose-based baits were particularly effective against tending ants, whose diet consists mainly of sugar (Daane et al., 2008; Nondillo et al., 2016). Artificial sugar dispensers, with or without insecticides, have been tested (Beltrà et al., 2017; Daane et al., 2008). The inclusion of insecticides can provide a control of ant population, whose members feed other colony individuals by trophallaxis. Insecticide addition may have detrimental effects on non-target insects, such as pollinators and natural enemies. Nevertheless, the small amount of pesticides and bait delivery system reduce the undesirable effects compared to broad-spectrum insecticide sprays (Daane et al., 2008; Tay et al., 2017).

In field conditions, the impact of sugar dispensers on ecosystem services, such as parasitization and predation, has not been deeply evaluated yet. Developing alternative sugar sources to reduce the population of mealybug tending ants should also help increasing natural enemy performance in vineyards. The goal of this work was to investigate if the use of sugar dispensers can reduce ant activity and attendance, thus enhancing the biological control against mealybugs, including the efficacy of the inoculative releases of *A. vladimiri* and *C. montrouzieri* in infested vineyards. Moreover, this study was aimed at describing ant assemblages in Northern Italy, an important area of grape cultivation, and evaluating the different level of protection to mealybugs that ant species foraging in vineyards can provide.

3.3 Material and methods

3.3.1 Field sites

The experiment was carried out in 4 vineyards infested by mealybugs, in Reggio Emilia Province, in 2020 (Table 1). Vineyards were selected based on the pest pressure recorded by extension services in recent years.

Table 1. Details of the vineyard sites used for the field trial.

Site	Province	Longitude	Latitude	Variety	Training system	Mealybug species	Pest management
1	Reggio nell'Emilia	10°48'28.38" E	44°47'22.71" N	Lambrusco Salamino	Espalier	*P. comstocki*	IPM with prophylactic use of insecticides
2	Reggio nell'Emilia	10°44'50.15" E	44°47'29.55" N	Ancellotta	GDC; Espalier	*P. ficus* and *P. comstocki*	IPM
3	Reggio nell'Emilia	10°43'54.41" E	44°51'36.36" N	Lambrusco Marani	Espalier	*P. comstocki*	IPM
4	Reggio nell'Emilia	10°43'29.18" E	44°50'01.45" N	Lambrusco Marani	Espalier	*P. ficus* and *P. comstocki*	IPM with prophylactic use of insecticides

Inter row ground cover vegetation was mowed close to the ground and no mealybug insecticides were applied in the trial areas.

The trial was carried out in a 0.6-1.5 ha area inside each vineyard. Two plots were selected within each area: sugar dispenser and control plots. Their sizes varied between 1200 and 2800 m^2 and the minimum distance between plots was 20 m.

3.3.2 Sugar dispensers

Sugar dispensers (Figure 1) were set up using a similar method to that of Daane et al. (2008). Centrifuge tubes (175 mL Falcon, Corning) were used as sugar dispensers. One cm hole was drilled in each cap and 10 cm x 10 cm square of permeable plastic mesh was placed between the cap and tube. A plastic net (4 mm mesh) was placed on dispenser caps to avoid honeybee access to sucrose liquid.

Sugar dispensers were positioned on vine trunks randomly chosen (height 40-50 cm from the soil) at the beginning of June and were removed at the beginning of September. Sugar dispensers were deployed at a density of about 120 dispensers/ha, evenly spaced throughout the sugar dispenser plot of each vineyard (Vineyard 1: 16 sugar dispensers; Vineyard 2: 15 sugar dispensers; Vineyard 3: 34 sugar dispensers; Vineyard 4: 30 sugar dispensers). Each sugar dispenser was filled with 25% sucrose aqueous solution and refilled fortnightly.

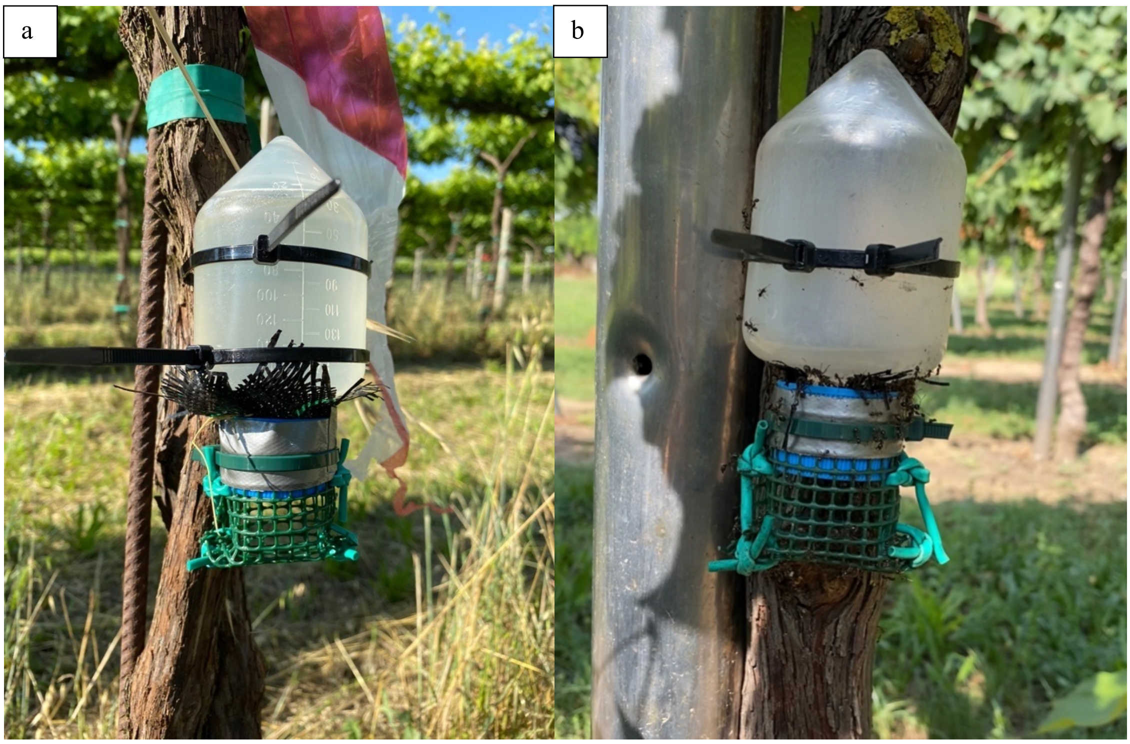

Figure 1. Sugar dispenser which was just refilled with sucrose liquid (a) and ants feeding on dispenser sucrose liquid (b).

3.3.3 Natural enemy release

Natural enemies were released in order to ensure a comparable level of ecosystem services in each vineyard, mitigating as much as possible the variability of natural parasitization and predation among sites.

At the beginning of July (2nd and 8th of July), *A. vladimiri* was randomly released at a rate of 1.500 individuals/ha in two different moments to guarantee its permanence in the field. The first release included 1.000 wasps and, one week after, other 500 individuals were released. Parasitoid releases were carried out after two weeks from mandatory treatment (applied on 18th-22nd of June) with tau-fluvalinate (MAVRIK 20 EW, Adama, Italy) against *Scaphoideus titanus* Ball. (Hemiptera: Cicadellidae). *Anagyrus vladimiri* releases were carried out near vine plants while walking along vineyard rows. The host-seeking activity of this parasitoid allows the release even when mealybug infestation is not clearly visible in field.

The predator *C. montrouzieri* was released at a minimum density of 300 individuals/ha in all the sites starting at the end of July (29th of July), except in vineyard 1. In this vineyard, three consecutive releases (8th, 29th of July and 5th of August) of 300 *C. montrouzieri* individuals (for a total of 900 individuals) were carried out, due to the presence of early and severe mealybug infestation. In vineyard 4, two consecutive releases (29th of July and 5th of August) of 300 individuals/ha were performed to improve control of mealybug infestation, considering unexpected colony appearance in the previous years. *Cryptolaemus montrouzieri* releases were targeted on plants with high mealybug infestation. Honeydew and wax secretions are fundamental to stimulate the predator oviposition (Kairo, 2013), so a high prey density is needed to ensure *C. montrouzieri* permanence in field.

Both *A. vladimiri* and *C. montrouzieri* were supplied by Bioplanet (Cesena, Italy), with which releases of natural enemies were set.

3.3.4 Ant species and activity

Samples of ants were collected fortnightly during the experiment from canopy, branches or from sugar dispensers, to identify the species. Ant individuals were killed in a refrigerator and stored in test tubes (70% ethanol) until identification, which was accomplished by means of identification keys (Seifert, 2018). Relative abundance of each ant species was calculated as the ratio between the number of each ant species and the total number of ants collected.

Ant activity was estimated fortnightly by counting the number of ants crossing an imaginary line placed on vine trunk (at about 15-20 cm below the vine canopy) during 1-min period (Beltrà et al., 2017). The imaginary line was a transect whose length was equal to vine trunk diameter. In sugar dispenser plot, ant activity assessment was carried out on each vine on which sugar dispensers were hung, with the imaginary lines placed approximately 30 cm above sugar dispensers. It was expected to detect less ant individuals crossing the imaginary line because of the presence of sugar dispensers. Vines for ant counting in the controls were randomly selected to evenly represent the whole plot.

3.3.5 Infestation, parasitization and predation sampling

Bunches were collected between the end of August and early September, just before harvest.

To evaluate mealybug infestation in sugar dispenser plot, one randomly selected bunch was collected on each plant where dispensers were placed, for a total of 15-34 bunches/plot. Exactly the same number of bunches was collected randomly in control plots picking one bunch per vine on the same plants where ant activity was estimated.

To more precisely estimate parasitization and predation, 6-10 infested bunches (hereafter also referred as colonies) per plot were actively searched and collected. Vines where ant activity was estimated were excluded by the picking of infested bunches. Besides assessing parasitization on infested bunches, parasitized mealybugs were also estimated on randomly selected bunches (used to determine infestation) in order to evaluate the parasitoid activity at different density of mealybug infestation.

Infestation was assessed in two different ways, estimating bunch infestation rate and counting the number of mealybugs per bunch. In particular, the percentage of infested bunches was evaluated by a visual sampling in the field, counting the bunches with sign of infestation such as honeydew, sooty mould and mealybug presence. Once infested bunch rate was assessed, the same bunches were taken to the laboratory (Department of Agricultural and Food Sciences, University of Bologna), and the number of mealybugs was assessed counting individuals of each development stage (nymphs, adults (females), females with ovisac) and distinguishing mealybug species, when possible. Parasitization was estimated as the ratio between parasitized mealybugs and the total number of mealybugs considering only adult and females with ovisac as they are the most suitable stages for *A. vladimiri*. Also, parasitization of nymph stage can lead to strong underestimation due the lack of certain symptoms of parasitoid attack. Presence of a single hole in the back of mealybug or swollen pest body were considered sign of parasitization.

Lacerated bodies and eggs without adult females were considered as sign of predation, so the ratio between predated mealybugs and the total number of mealybugs was used to calculate the percentage of predation. Underestimations are associated with this predation assessment, but they are replicated in the same way in each vineyard of the trial.

Finally, mean number of *C. montrouzieri* larvae per bunch was recorded as well.

3.3.6 Statistical analysis

The mean number of ants counted on vine trunks was analyzed using a generalized linear mixed model (GLMM) with normal probability distribution and identity log-link function. Treatment (sugar dispenser and control) was included as fixed factor and sampling dates as repeated measures. Vineyards were considered as random block factor. Restricted maximum likelihood with Kenward-Roger's approximation of the degrees of freedom (df) was selected.

A correspondence analysis was performed in order to correlate ant species with vineyards and to better describe potential variations in ant communities in the investigated sites.

Log linear analysis was used to analyse the average ratio of damaged bunches, parasitization on randomly-collected bunches and colonies, and predation on colonies. Log-linear analysis allows for simultaneous evaluation of multiple interactions among categorical variables, using a method that resembles a factorial analysis of variance (Steel et al., 1997). Here, the response variables were the percentage of infested bunches, parasitization and predation, whereas the independent variables were treatment (sugar dispenser and control) and vineyards (n=4). In the results, both the partial association and the marginal association tests were shown.

In each single vineyard, the effect of treatment on damaged bunches, parasitization and predation was evaluated by chi square test (χ^2).

The software IBM SPSS Statistics (ver. 26) (IBM corporation, Armonk, NY, USA) and Statistica version 10 (StatsoftTM, Tulsa, OK, USA) were used for the analyses.

3.4 Results

3.4.1 Ant species and activity

Overall 11 ant species were recorded in field sites (Figure 2), for a total of 232 ant individuals collected. The highest number of species (9) was found in vineyard 1, whereas the lowest number of ant species (4) was observed in vineyard 3, that was characterized by the dominance of the aggressive *Lasius niger* (L.). Most of the ant species collected display a sugar feeding behavior and only the genus *Messor* consists of seed harvesting ants (Plowes et al., 2013). As integration of figure 2, figure 3 provides a statistic support of dominant ant species in each site, explaining near the 80% of inertia.

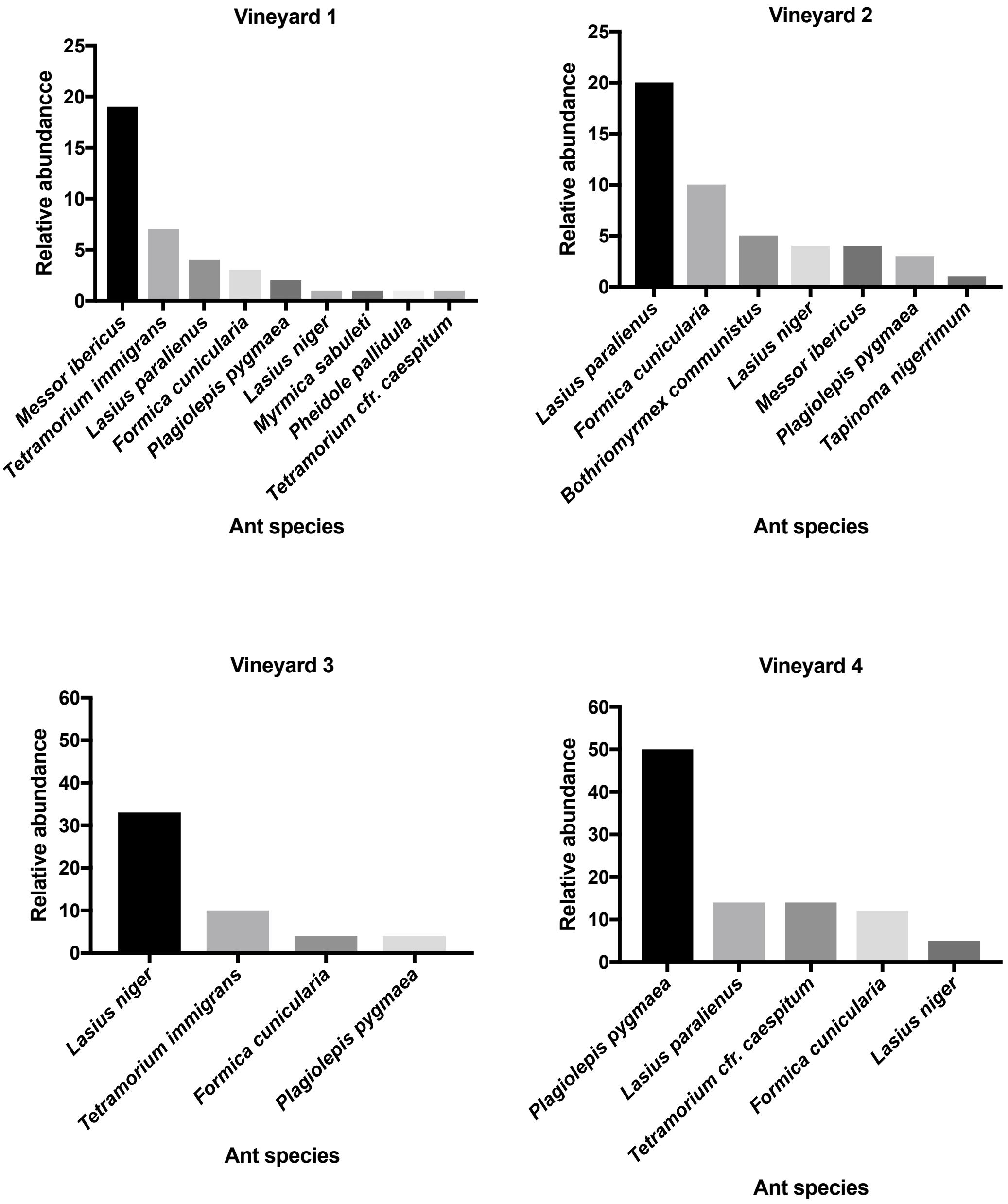

Figure 2. Relative abundance (%) of ant species collected in each vineyard. Relative abundance was calculated as the ratio between the number of each ant species and the total number of ants collected.

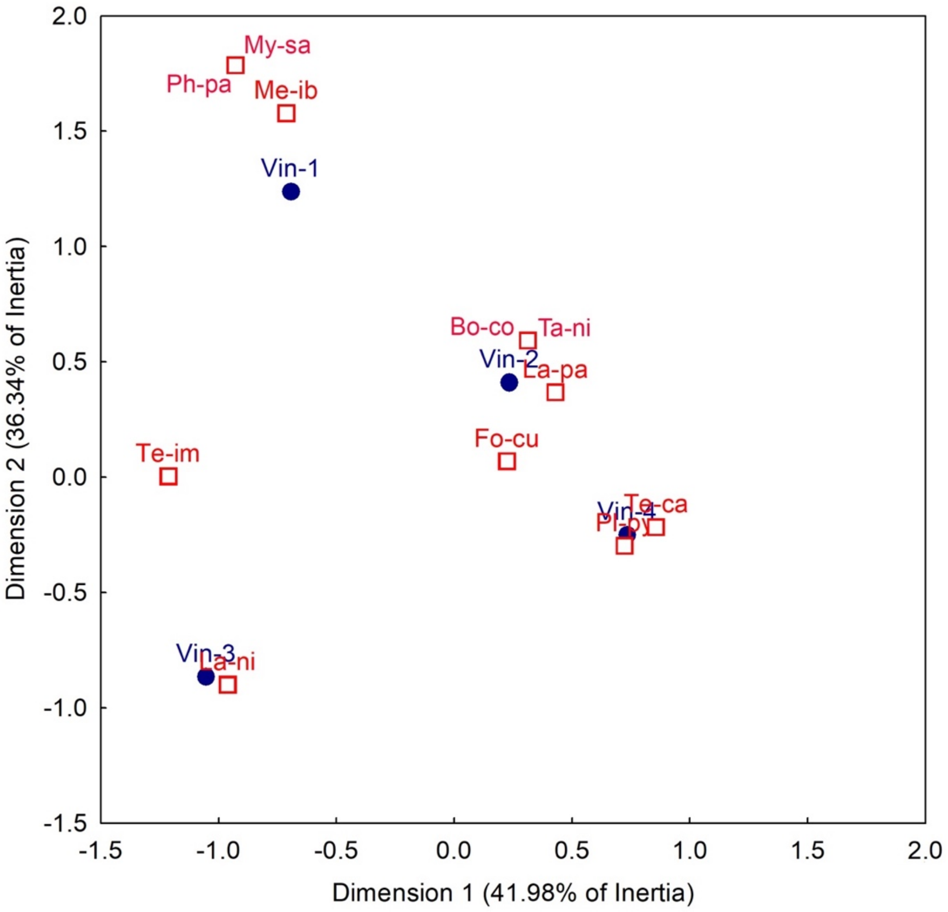

Figure 3. Biplot of the two first axes of the Correspondence Analysis relating ant species (represented by open red squares) and vineyards (represented by blue circles). Ant species: My-sa= Myrmica sabuleti*; Ph-pa=*Pheidole pallidula*; Me-ib=*Messor ibericus*; Bo-co=*Bothriomyrmex communistus*; Ta-ni=*Tapinoma nigerrimum*; La-pa=*Lasius paralienus*; Fo-cu=*Formica cunicularia*; Te-im=*Tetramorium immigrans*; Te-ca=*Tetramorium cfr. caespitum*; Pl-py=*Plagiolepis pygmaea*; La-ni=*Lasius niger*. Vineyards: Vin-1=vineyard 1; Vin-2=vineyard 2; Vin-3=vineyard 3; Vin-4=vineyard 4.*

Figure 4 shows the average ant activity during the summer 2020. In all monitoring dates, the mean ant activity was significantly lower in sugar dispenser plot compared to that of control. A significant effect of sampling dates on ant activity was also detected (Table 2). GLMM did not detect any significant effect of the vineyards, which were included in the statistical analysis as a random block factor (Z = 0.74; p = 0.46).

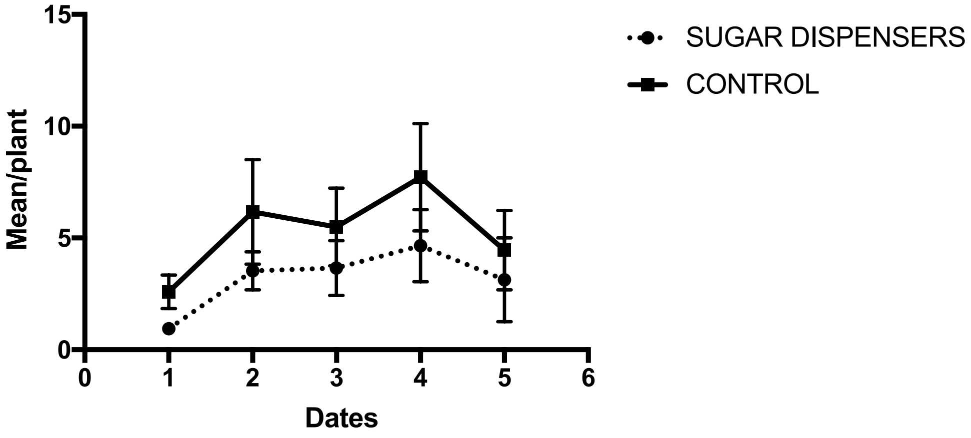

Figure 4. Average ant activity (± SE) in sugar dispenser and control treatments during summer 2020 (n=4). The sampling period started on 17th -19th of June (Date 1) and ended on 26th -28th of August (Date 5). Ant activity was estimated fortnightly. GLMM showed a significant effect of sugar dispensers on ant activity (p=0.034), as well as a significant effect of sampling dates (p=0.017).

Table 2. Factor effects in the generalized linear mixed model carried out on mean number of ants counted on the vines.

Factor	F	df1	df2	p
Treatment	5,15	1	21,33	0,034
Date	4,94	4	10,44	0,017
Treatment x date	0,12	4	10,44	0,973

A trend of ant counting on vines is also reported separately for each vineyard to better evaluate the behaviour of ants (Figure 5). Vineyard 1 was characterized by a higher ant activity in control than sugar dispenser treatment in 3 dates out of 5. A similar mean number of tending ants was counted in the two treatments in the last monitoring dates (Figure 5). Lower ant activity in sugar dispenser treatment compared to control plot was detected in vineyard 2 during the entire season (Figure 5), with strong differences between the two treatments. On the other hand, more tending ants were counted in sugar dispenser treatment than control in four out of five sampling dates in vineyard 3 (Fig 5). Finally, in vineyard 4 ant activity was lower in sugar dispenser plot than control, apart from the third date (Figure 5).

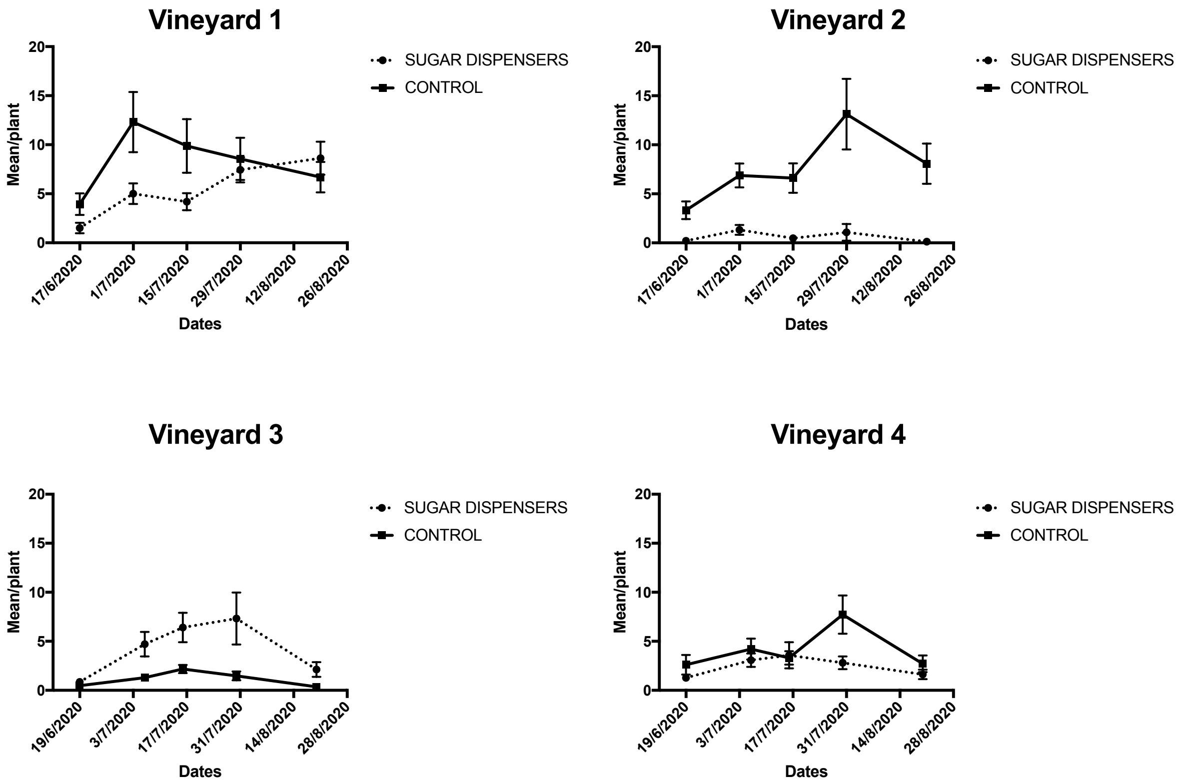

Figure 5. Ant activity (± SE) in sugar dispenser and control plots in each vineyard. Ant activity was estimated by counting the number of ants crossing an imaginary line on vine trunk in 1-minute period.

3.4.2 Meaybug infestation

The use of sugar dispensers significantly reduced the percentage of infested bunches in dispenser treatment compared to control (p<0.01); partial association test showed the same result of marginal association one (Table 3, Figure 6a). A vineyard effect was also observed on the percentage of infested bunches (Table 3). On the other hand, the mean number of mealybugs per bunch registered by random sampling was similar in control and sugar dispenser plots (Figure 6b).

Table 3. Log linear results showing the effect of each factor (T=treatment; V=vineyard) on the percentage of infested bunches (I=infestation).

Effect	df	Chi square (Partial association test).	P (Partial association test)	Chi square (Marginal association test)	P (Marginal association test)
T x I	1	10,70	0,001	8,33	0,004
V x I	3	47,96	<0,001	45,60	< 0,001

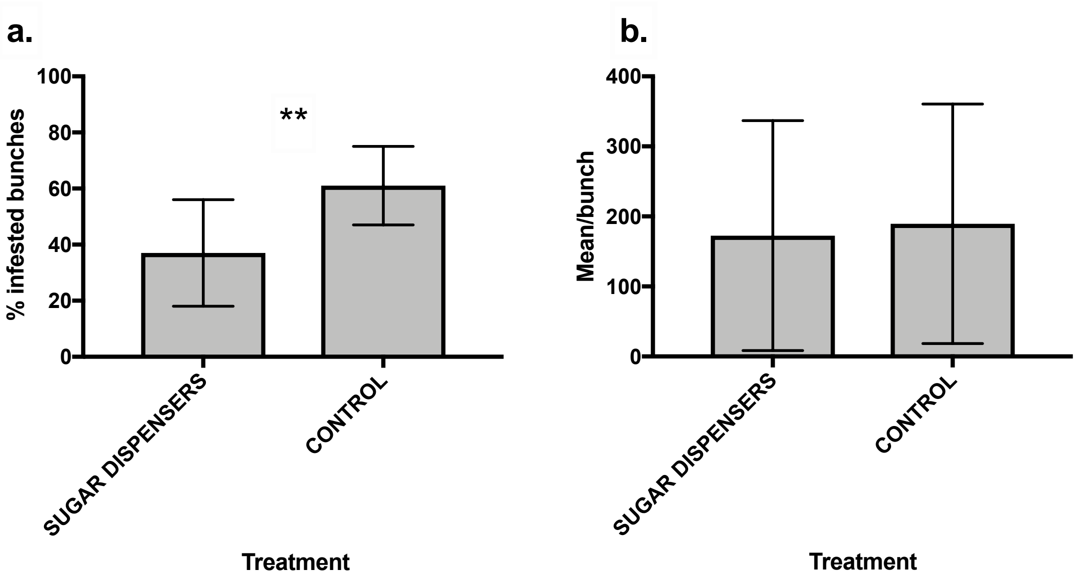

*Figure 6. Average bunch infestation (n=4): percentage of damaged bunches (± SE) (a) and the mean number of mealybugs per bunch (± SE). Log linear analysis showed a significant difference of infested bunch rate between the two treatments (**=p <0.01).*

A further analysis of bunch infestation was carried out in each vineyard, in order to better explain the infestation dynamics in each site. Two vineyards out of 4 had a significantly higher percentage of infested bunches in control plot compared to sugar dispenser (Figure 7). In vineyard 2, 13% of bunches were infested by mealybugs in sugar dispenser treatment, whereas in control the infestation level was 73% (df=1; χ^2=11; p<0.001) (Figure 7). Also vineyard 4 showed a significantly lower infestation in sugar dispenser treatment plot (17%) than in control one (43%) (df=1; χ^2=5.08; p=0.02) (Figure 7). On the other hand, approximately the same level of infestation was found in vineyard 1 and 3 in control and sugar dispenser plots (Figure 7).

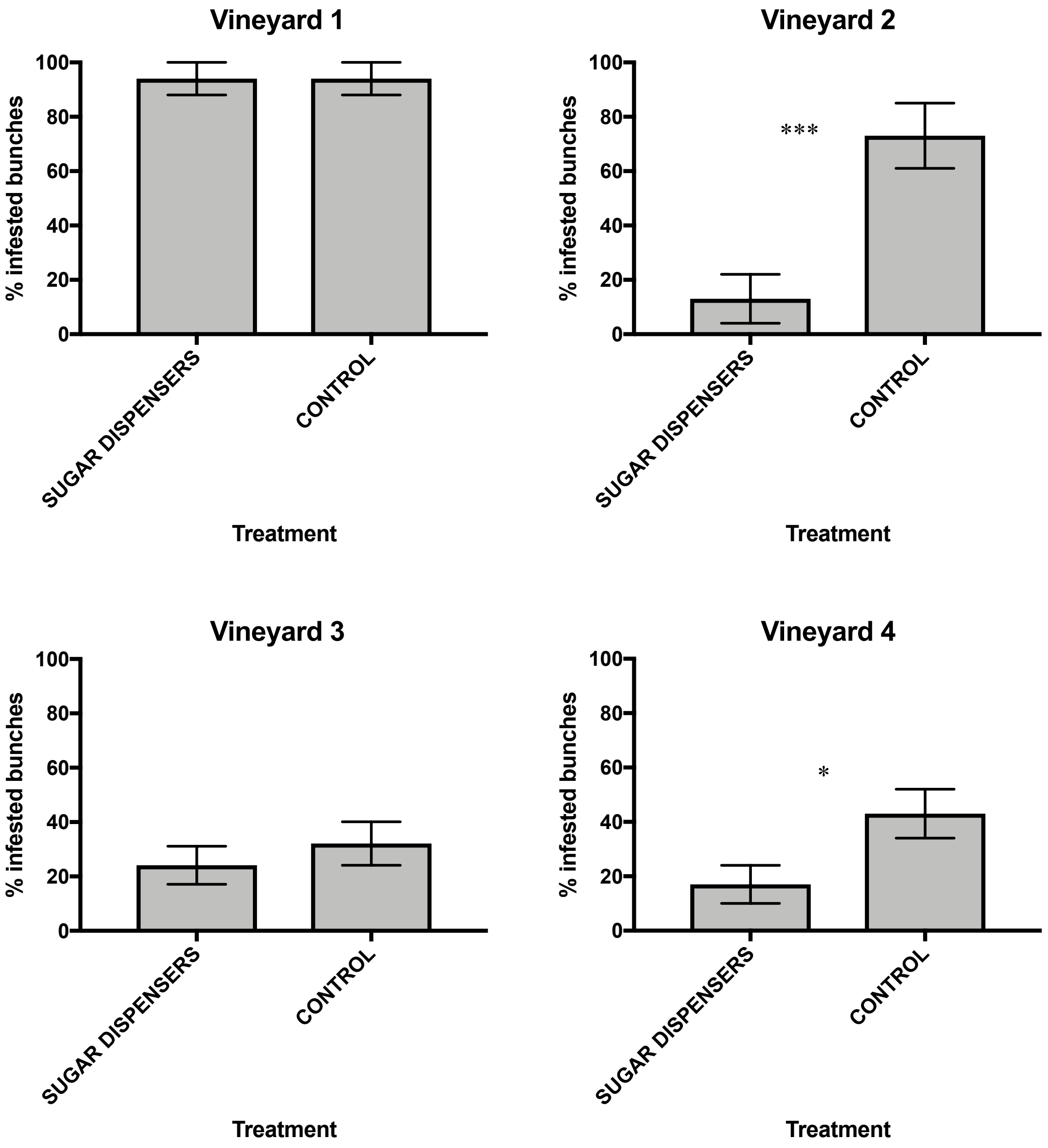

*Figure 7. Percentage of infested bunches (± binomial SE) in sugar dispenser and control plots in each vineyard. Chi square test showed a significant difference of infested bunch rate between the two treatments in vineyard 2 (***p< 0,001) (b) and in vineyard 4 (*p <0,05) (d).*

These infestation patterns are also confirmed by the mean number of mealybugs per bunch (Figure 8). Indeed, 3.73 ± 2.66 mealybugs per bunch were found in sugar dispenser treatment, while 18.80 ± 5.35 mealybugs per bunch were counted in control plot of vineyard 2 (Figure 8). Also vineyard 4 presented a higher number of mealybugs per bunch in control treatment (17.73 ± 5.70) compared to dispenser one (3.43 ± 2.51) (Figure 8). A similar number of mealybugs per bunch was observed in the control and sugar dispenser treatment of vineyard 1 and 3 (Figure 8).

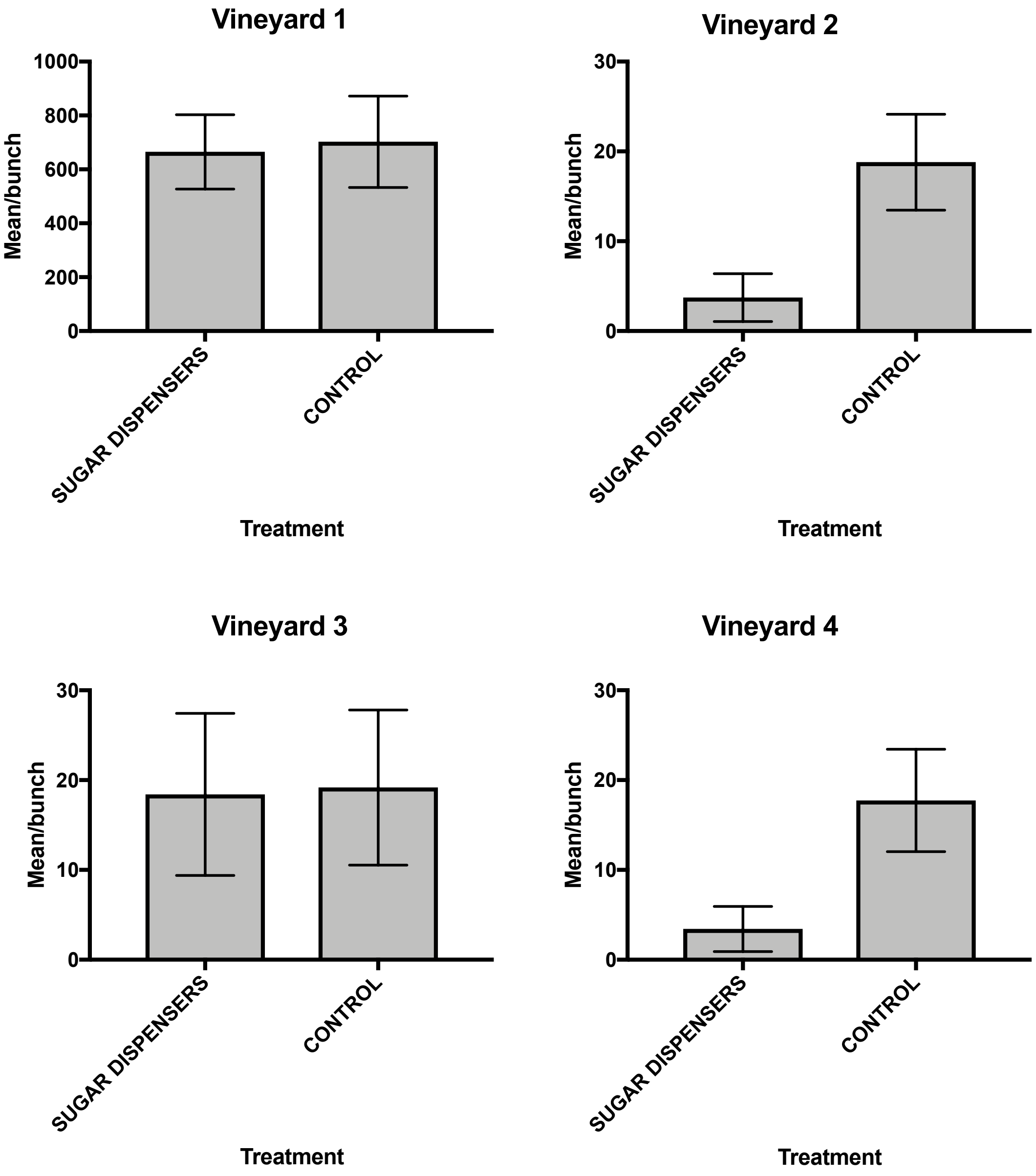

Figure 8. Mean number of mealybugs per bunch (± SE) in sugar dispenser and control plots in each vineyard.

3.4.3 Parasitization and predation

Log linear analysis showed a significantly higher parasitization rate in colonies from sugar dispenser treatment (Table 4, Figure 9a), whereas no significant difference was observed on parasitisation detected in randomly-collected bunches (Table 5, Figure 9b). Also, a significant effect of vineyard was detected on randomly-collected bunch parasitization (Table 5).

Table 4. Log linear results showing the effect of each factor (T=treatment; V=vineyard) on colony parasitization rate (PaC=parasitization rate on colonies).

Effect	df	Chi square (Partial association test).	P (Partial association test)	Chi square (Marginal association test)	P (Marginal association test)
T x PaC	1	11,46	<0,001	10,68	0,001
V x PaC	3	7,46	0,06	6,67	0,08

Table 5. Log linear results showing the effect of each factor (T=treatment; V=vineyard) on parasitization rate on random bunches (PaR=parasitization rate on random bunches).

Effect	df	Chi square (Partial association test).	P (Partial association test)	Chi square (Marginal association test)	P (Marginal association test)
T x PaR	1	1,82	>0,05	1,39	>0,05
V x PaR	3	17,57	<0,001	17,14	<0,001

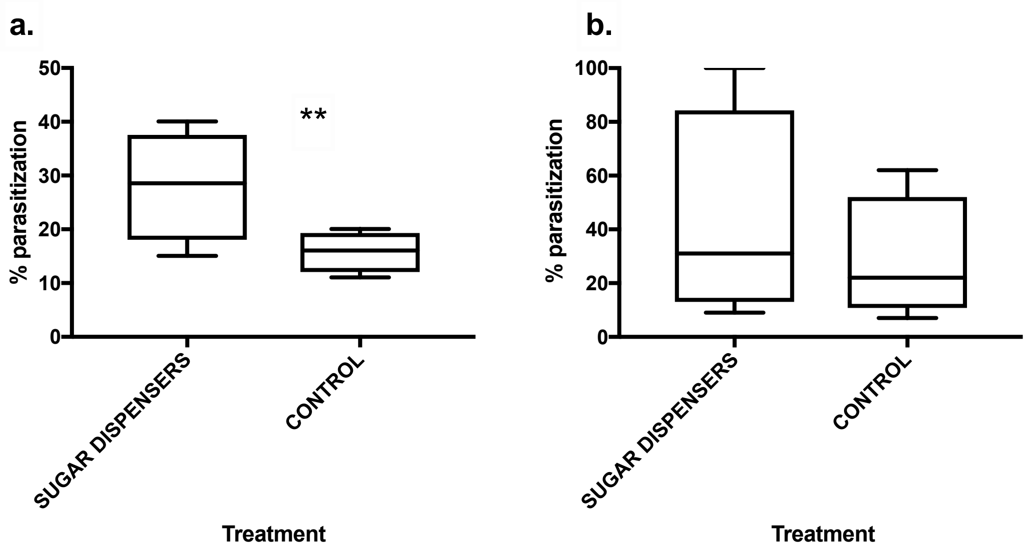

*Figure 9. Mean percentages of parasitization (n=4) on colonies (a) and randomly-collected bunches (b). Box plots indicate the median (solid line) and the range of dispersion (the lower and upper quartiles); the whiskers (vertical lines) represent the minimum and maximum parasitization rates observed. Log linear analysis detected a significant difference of parasitization rate between the two treatments on colonies (**=p<0.01) (a), whereas no significant difference of parasitization rate was observed between sugar dispenser and control plots on randomly-collected bunches (p>0.05) (b).*

Regarding colony parasitization, only vineyard 4 presented significantly more parasitized mealybugs in dispenser treatment compared to control one (df=1; χ^2=14.32; p<0.001) (Figure 10). The parasitized mealybugs were twofold higher in sugar dispenser plot in comparison with that of control in vineyard 2 (Figure 10), but this difference was not supported by chi-square test. A level of parasitization of 27% was observed in sugar dispenser treatment, while just 20% of parasitized mealybugs were found in control plot in vineyard 3 (Figure 10). Vineyard 1 presented about the same percentage of parasitized mealybugs in the control and sugar dispenser treatment (Figure 10).

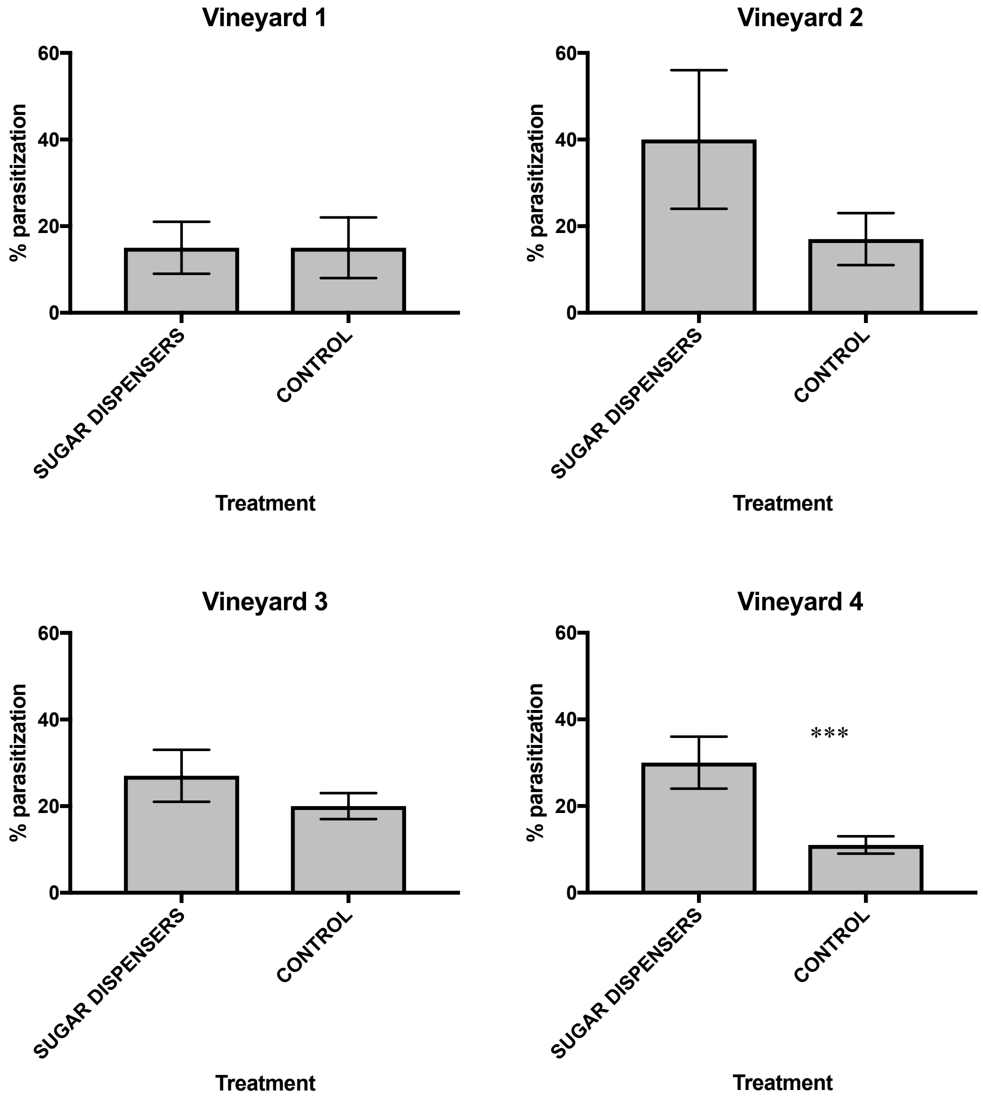

*Figure 10. Parasitization rate on colonies (± binomial SE) in sugar dispenser and control treatments in each vineyard. Chi square test showed a significant difference of parasitization rate between the two treatments in vineyard 4 (***=p<0,001).*

Parasitization rate in randomly-collected bunches was higher in sugar dispenser than in control plot in vineyard 1 (Figure 11), even if this difference was not supported by chi-square test. The percentage of parasitized mealybugs was slightly higher in sugar dispenser treatment compared to control on randomly-collected bunches of vineyards 3 and 4 (Figure 11). On the other hand, in vineyard 2, a 100% of parasitization was found in sugar dispenser plot, whereas 62% of parasitized mealybugs were observed in control plot (Figure 11).

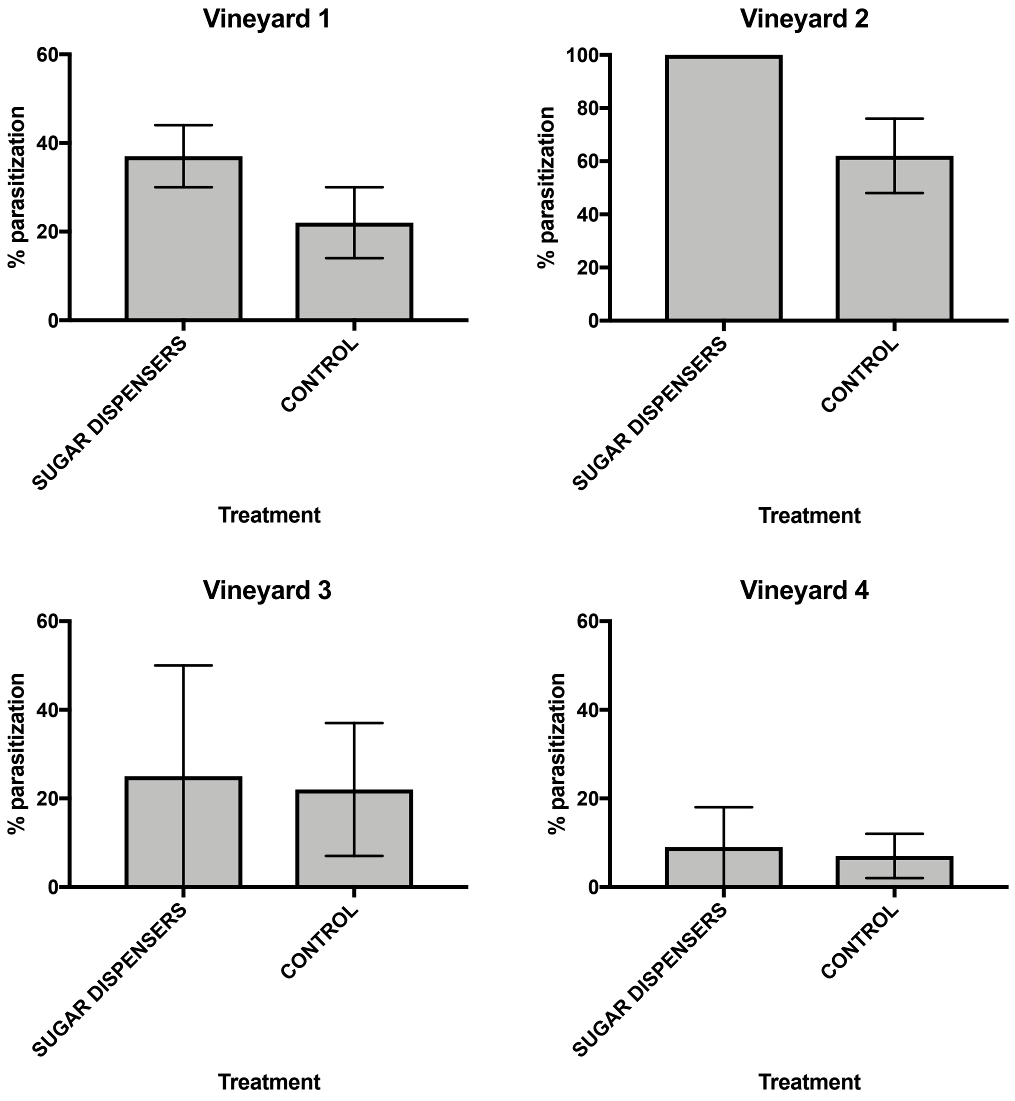

Figure 11. Percentage of parasitized mealybugs (±binomial SE) on randomly-collected bunches in sugar dispenser and control plots in each vineyard.

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Concerning *C. montrouzieri*, predated mealybugs were more frequently recorded among colonies in sugar dispenser treatment compared to control (Table 6, Figure 12a) (p<0.001). The greater activity of predators, most of which likely belonged to *C. montrouzieri,* was also confirmed by the mean number of *C. montrouzieri* larvae per bunch (n=2), which was higher in sugar dispenser colonies than in control ones (Figure 12b). Finally, a significant effect of vineyard on the percentage of predated mealybugs was recorded (p<0.001) (Table 6).

Table 6. Log linear results showing the effect of each factor (T=treatment; V=vineyard) on predation rate (Pr=predation rate).

Effect	df	Chi square (Partial association test).	P (Partial association test)	Chi square (Marginal association test)	P (Marginal association test)
T x Pr	1	12,46	<0,001	37,96	< 0,001
V x Pr	3	188,44	< 0,001	213,94	< 0,001

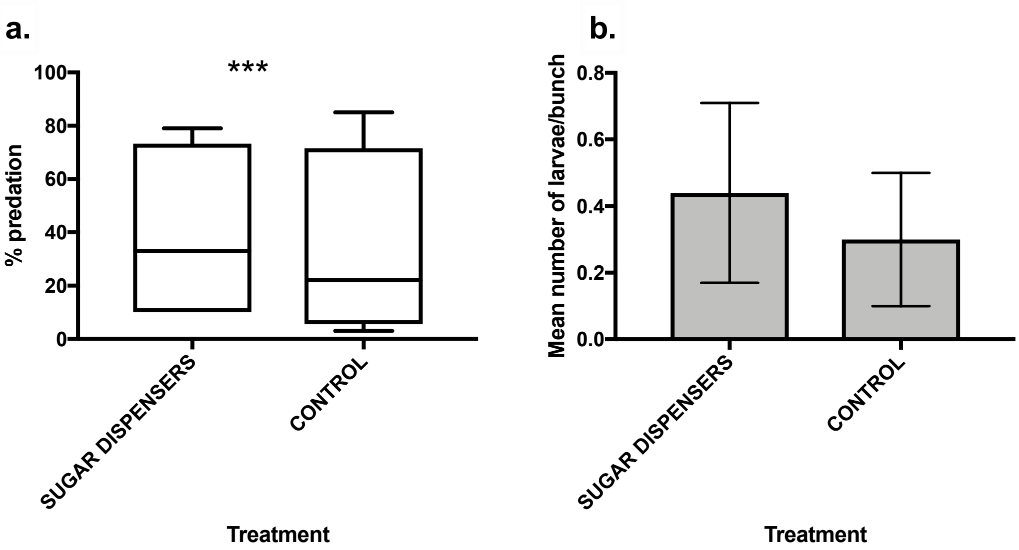

Figure 12. Average C. montrouzieri *response (n=4): mean percentage of predation (a) and mean* C. montrouzieri *larvae per bunch (±SE) (b). Box plots (a) indicate the median (solid line) and the range of dispersion (the lower and upper quartiles); the whiskers (vertical lines) represent the minimum and maximum predation rates observed. Log linear analysis showed a significant difference of predation rate between the two treatments (***=p<0.001).*

The percentage of predated mealybugs on colonies was very high and similar in sugar dispenser treatment and in control in vineyard 1, even if it was slightly higher in control plot (Figure 13). This evidence agrees with the higher density of the predator larvae per bunch in control compared to sucrose dispenser plot (Figure 14a). Vineyard 2 showed a similar level of predated mealybugs on colonies both in sugar dispenser plot and control one (Figure 13). Moreover, no *C. montrouzieri* larvae was collected in any plot in vineyard 2. Colony predation was significantly lower in control compared to sugar dispenser plot in vineyard 3 (χ^2=12.51; df=1; p<0.001) (Figure 13), but no predator larvae was found during sampling at harvest. Finally, the percentage of predated mealybugs was significantly higher in sugar dispenser plot than in control one in vineyard 4 (χ^2=6.23; df=1; p=0.01) (Figure 13); indeed, more *C. montrouzieri* larvae were collected where sugar dispensers were present, compared to control plot (Figure 14b).

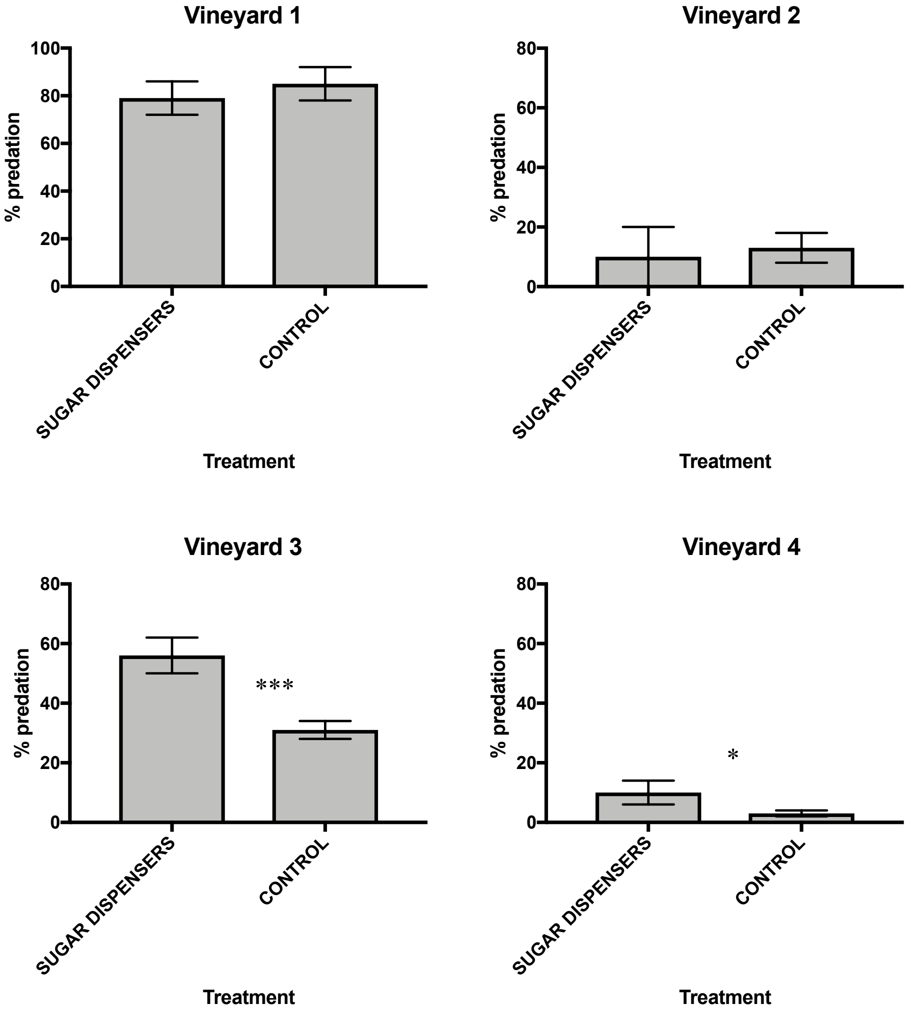

*Figure 13. Percentage of predated mealybugs on colonies (± binomial SE) in sugar dispenser and control treatments in each vineyard. Chi square test showed a significant difference of predation rate between the two treatments in vineyard 3 (***=p<0,001) and vineyard 4 (*=p<0,05).*

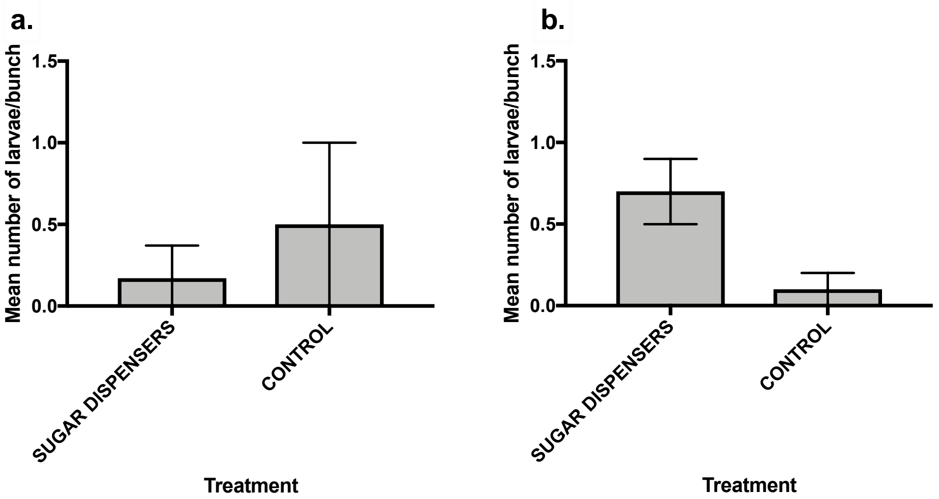

Figure 14. Mean C. montrouzieri *larvae per bunch (± SE) in bait and control treatment in vineyard 1 (a) and 4 (b).*

3.5 Discussion and conclusion

The use of liquid sucrose dispensers significantly enhanced ecosystem services in vineyard. This outcome is likely attributable to a reduction of ant activity, which was significantly lower in the presence of sugar dispensers. The percentage of infested bunches was significantly lower in sugar dispenser treatment compared to control plot. Also Beltrà et al. (2017) demonstrated that sugar dispensers reduced vineyard infestation in terms of vine mealybug abundance. The use of sugar dispensers with insecticides decreased fruit damage also in California vineyards (Daane et al., 2008). Despite a reduction of percentage of infested bunches in plot with sugar dispensers, the mean number of mealybugs per bunch detected by random bunch sampling was similar in sugar dispenser treatment and in control. This result seems to demonstrate that sugar dispensers act mostly decreasing mealybug spatial diffusion in the field, for example reducing colony formation, more than reducing colony size. Anyhow, the very high infestation of vineyard 1 contributed to level off the mean mealybug infestation in both treatments; notwithstanding, a lower population in sugar dispenser plot in comparison with control was recorded in two vineyards out of four. The decrement in the number of colonies would favour biological approaches for mealybug suppression, for example releasing *C. montrouzieri* only on plants where colonies occur. Moreover, at least in two vineyards out of four, it seems that a relationship between mealybug abundance and infested bunch rate was present. Growers and consultants could benefit from this relationship and use the number of infested bunches instead of counting the number of mealybugs as a decision-making tool to define the severity of mealybug infestation.

A significantly higher colony parasitisation in sugar dispenser plots in comparison with controls was observed. Similarly, Pérez-Rodríguez et al. (2021) found higher *Planococcus citri* (Risso) parasitism in citrus trees with sugar-feeders on the branches compared to control trees. In our trial, a tendency of higher mealybug parasitization was also found in dispenser treatment on randomly-collected bunches, but this difference was not significant. These different responses in parasitisation according to the types of bunch sampling (randomly-collected bunches *vs* colonies) were likely caused by the size of mealybug samples and ant behaviour. The higher number of mealybugs in colony than in randomly-collected bunches contributed to a more robust evaluation of this ecosystem service, leading to the significant effect of the treatments on colony parasitisation. Moreover, higher mealybug aggregation attracts more tending ants, which can benefit from higher amounts of honeydew. Sugar dispensers may "distract" ants more effectively from colonies, making mealybugs more susceptible to natural enemies. Also *C. montrouzieri* benefitted by the reduction of ant visits on colonies; indeed, average predation rate was significantly higher in sugar dispenser plot compared to control one.

Apart from enhancing beneficial activity, reduced ant-attendance might also have caused accumulation of honeydew on mealybug bodies, which could lead to a higher mortality, especially of first instar nymphs (Beltrà et al., 2017; Mani & Shivaraju, 2016).

A high variability was detected among vineyards both in terms of infestation and ecosystem services. Overall, the use of sugar dispensers reduced ant activity in most of the vineyards. Only vineyard 3 presented an inverted trend in some sampling dates. This outcome may be explained by an inhomogeneous mealybug density between treatment plots due to high spatial aggregation of this pest. Indeed, a low infestation was found in control plot of vineyard 3 during ant activity monitoring, whereas more mealybugs and tending ants were counted in sugar dispenser area. Nevertheless, there was no significant difference between the percentage of infested bunches in dispenser and control plots before harvest. Sugar dispensers likely decreased ant-attendance thus leading to non significant difference in damaged bunches between the two treatments at the end of the season.

Anagyrus vladimiri and *C. montrouzieri* showed a complementary action, which was enhanced by provisioning sugar dispensers. The highest parasitization rates were observed in vineyard 2 and 4, where bunch damages were caused principally by *P. ficus*. Anyhow, in our trial, a significant parasitization on *P. comstocki* was recorded. Our results are in agreement with a recent study reporting that *A. vladimiri* successfully parasitized both *P. ficus* and *P. comstocki* (Ricciardi et al., 2021). The highest predation pressures on mealybugs were detected in vineyard 1 and 3, that were infested by *P. comstocki*. The high abundance of *C. montrouzieri* larvae in control plot of vineyard 1 was likely due to the high mealybug density in control plot, thereby confirming the strongly density-dependent behaviour of the predator. Overall, the use of sugar dispensers showed a tendency to increase biological control in each field site, in terms of parasitization or predation rate. Moreover, this tactic demonstrated to be efficient both in vineyards infested by *P. ficus* and *P. comstocki*.

Parasitized mealybugs may have been overestimated since only adult and females with ovisac were used to calculate parasitization rate. On the other hand, if nymphal stages had been considered, parasitized mealybugs would have been underestimated, disguising *A. vladimiri* potential in controlling mealybug population. Before harvest, bunches are primarily infested by juvenile stages, on which it is extremely demanding to visually distinguish their three instars and detect parasitization signs.

The performances of natural enemies of mealybugs as well as their infestations were likely influenced by ant species. A high diversity of ants was observed in this trial, highlighting clear differences in species assemblages among vineyards. This underlines how different the disrupting activity of ant individuals against natural enemies could be, depending on their behavioural characteristics and species. The highest number of species was found in vineyard 1; three of them (*L. niger*, *Tetramorium immigrans* Santschi and *Tetramorium* cfr. *caespitum* (Linnaeus)) are considered very aggressive (Lebas et al., 2019). The most abundant ant species in vineyard 1 was *Messor ibericus* Santschi. Species belonging to the genus *Messor* have been already found in vineyards, even if this genus encompasses mainly seed harvester ants (Masoni et al., 2017). *Lasius niger* was also the most abundant species recorded in vineyard 3. This ant is known for protecting *P. comstocki* mealybugs building shelters made by earth grains (Campos et al., 2006). *Lasius paralienus* Seifert and *Plagiolepis pygmaea* (Latreille) were the most abundant ant species in vineyard 2 and 4, respectively. The genus *Plagiolepis* was already found foraging on vines, by Beltrà et al. (2017). Both *L. paralienus* and *P. pygmaea* species are considered less aggressive than the species found in vineyard 1 and 3; thus, it would seem that colony parasitisation and sugar dispenser efficacy were higher in the vineyards attended by these less aggressive ants (vineyard 2 and 4). Moreover, the coexistence of several aggressive ant species in the same site may have amplified their disrupting activity against released beneficials. Sugar dispensers may not have been able to compensate ant-attendance and consequently enhance natural enemy performance. Overall, sugar dispensers may be more effective in vineyards characterized by more harmless ants compared to those where aggressive ants are common.

Just few individuals of *T. nigerrimum* were detected in our study although this ant species has been reported as one of the most common ant species associated with vine and citrus mealybugs in the Mediterranean areas (Mansour et al., 2012).

Sugar dispenser density in our experiment (about 120/ha) provided a reduction of tending-ant population. Nelson and Daane (2007) showed that in their experiment there was not an optimal ant dispenser density maximising ant population control. They concluded that deploying more dispensers could provide higher ant and mealybug suppression. Moreover, in order to maximize the impact of this tactic on ant population, dispensers should be set up in the field starting from spring, in accordance with Nelson and Daane (2007).

Insecticides, such as boric acid or neonicotinoids, may be added to sucrose liquid. The addition of pesticides could provide a suppression of ant population, acting on ant brood which is usually present in spring. The use of insecticides within sucrose solution should be suspended when inoculative releases of natural enemies are carried out and during flowering of ground cover plants, which could be intensively visited by pollinators. In this way, side effects on pollinators and other beneficials might be avoided, even though Cooper et al. (2008) and Tay et al. (2017) stated that the low quantity of insecticide deployed in ant baits should have a minimal impact on non-target insects. However, the use of pesticide in sugar dispenser seems to pose some risks that should be avoided for a true ecological management of vineyard. Furthermore, insecticides should be legally authorized for this particular use.

Dispenser provisioning should be adopted continuously for some consecutive years to optimize the efficacy of sugar dispensers against ant population. There are evidences that ant activity and mealybug infestations were reduced more strongly in the second year of bait program, especially when ant populations were high (Nelson & Daane, 2007).

Future trials should focus on figuring out also alternative delivering methods of sucrose liquids. Installation and maintenance of the sugar dispensers described in our experiment are labour-intensive to be adopted by growers. Recently, new methods have been studied to overcome conventional liquid baiting drawbacks. For example, Tay et al. (2017) demonstrated that alginate hydrogel provided an efficient delivery system for liquid baits to control Argentine ant *L. humil*e.

Beyond the use of ant dispensers to reduce mealybug infestation, other strategies could be adopted and integrated with ant management techniques. Lowering nitrogen fertilisation of plants may help reducing the infestation of sap sucking feeders. Altieri and Nicholls (2003) and Kumar (2017) highlighted that a higher nitrogen content in organically farmed crops resulted in a lower plant resistance against herbivore and piercing insects. Also Cocco et al. (2018) demonstrated that high nitrogen regimes boosted *P. ficus* reproduction on grapevines.

In conclusion, the potential efficacy of sugar provision to reduce ant activity has been demonstrated in accordance with previous studies (Beltrà et al., 2017; Carabalí-Banguero et al., 2013). Our field test proved that managing ant-attendance can enhance the biological control provided by *A. vladimiri* and *C. montrouzieri*. Several studies showed examples of ant deterring parasitoid and predators of mealybugs, as reviewed by Mani & Shivaraju (2016); our test quantifies for the first time the impact of sugar dispensers on released natural enemy ecosystem services, such as parasitization and predation, in vineyard field conditions. This tactic, if confirmed by a multi-year evaluation and in variable condition scenarios, could be adopted within mealybug management. Thus, ant attendance disruption could be integrated with inoculative releases of beneficials in vineyards to boost natural enemy activity in a sustainable and effective way.

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Chapter 4 The influence of ground cover management on infestation and parasitization rate of first generation of *Lobesia botrana*

4.1 Abstract

Habitat management has been frequently adopted to reduce pest infestation in several crop systems, improving natural enemy recruitment and fitness. Only a few studies have investigated the impact of interrow spontaneous vegetation on ecosystem services in vineyard.

The aim of this work was to explore if conservative ground cover management could reduce infestation and enhance parasitization of the first generation of *Lobesia botrana*, one of the most damaging pests of European vineyards. Three-year field trials were carried out in vineyards of Northern Italy. In each vineyard, two plots were defined to compare two different management of interrow ground cover: conservative ground cover where the growth and the flowering were allowed; intensive ground cover where frequent mowing maintained the vegetation close to the ground. Infested inflorescences were estimated by a visual sampling, whereas inflorescences with living larvae inside the glomerulae were collected to assess the parasitization in laboratory. Parasioid diversity and trophic relation complexity was also estimated.

Results showed that spontaneous vegetation was not sufficient to significantly reduce *L. botrana* infestation and boost parasitization, but conservative ground cover management increased beneficial diversity.

Further studies should examine more focused habitat management strategies by means of the use of selected flower strips.

4.2 Introduction

The European grapevine moth (EGVM), *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae), is one of the most detrimental pests of vineyard worldwide. This pest completes three generations per year: the first one which is antophagous, and two consecutive carpophagous generations, which correspond with the second and third generation. Its damages can occur both on inflorescences and bunches and are caused by larvae feeding activity. Moreover, *L. botrana* infestation induces the formation of sour rot and botrytis, which can definitively compromise the production (Ioriatti et al., 2011; Lucchi et al., 2018; Pavan et al., 2018).

For EGVM control, regular insecticide sprays are required, especially for the second and third generations. In order to promote the adoption of low impact strategies, new tools need to be tested. Sustainable methods have been available for several years, such as mating disruption, which is particularly effective (Larsson Herrera et al., 2020; Lucchi et al., 2018), but still limited conservation biological control techniques have been attempted against EGVM.

It is well known that habitat management strategies provide important resources for beneficials, such as shelter, nectar, alternative hosts/preys and pollen (SNAP) (Gurr et al., 2017; Snyder, 2019), which increase their longevity and fecundity. For this reason, some flowering species prove to be particularly attractive towards natural enemies. In the mites-phytoseiids system, pollen availability on the vegetation constitutes a source of extra food for predators when their prey is absent or at a low density (Duso et al., 2010, 2012). Burgio et al. (2016) demonstrated that alyssum, buckwheat and “Vetch and Oat” mixture attract some Hymenoptera parasitoid family in Northern Italy.

Besides increasing beneficial fitness, associated ecosystem functions, such as parasitization and predation, can be enhanced by means of habitat management practices. For example, Begum et al. (2006) and Berndt et al. (2006) showed that tortricid parasitism increases when flowering plants, such as buckwheat and alyssum, are present.

In addition to selected flower strips, endemic plants, which naturally occur in the interrow of vineyard ecosystem, can strengthen biological control of pests. Flowers developed by ground cover plants may have a critical impact on endemic parasitoid activity and need to be further investigated. Shapira et al. (2017) highlighted that local flowering annual plots present more parasitoids and generalist predators compared to plots sprayed with herbicide in Israeli vineyard.

In this context, habitat management could be exploited to increase biological control of the first generation of *L. botrana*. It would be interesting to assess the parasitization impact on the EGVM antophagous generation in order to clarify if the use of insecticides represents a real necessity in that stage. Nowadays, some growers of Emilia-Romagna region continue to carry out sprays against *L. botrana* first generation, in clear contrast with the regional regulations of Integrated Pest Management which do not allow the use of pesticides against this generation.

The goal of this paper is to evaluate if the conservative ground cover management increases the parasitization of *L. botrana* individuals of the first generation compared to intensive ground cover management in Reggio Emilia Province (Italy). This is a well-known viticulture area home to Lambrusco, which is exported worldwide.

Conservative ground cover management is characterized by a low mowing frequency in order to allow local plant flowering. Conversely, intensive ground cover management, which is typical in Reggio Emilia vineyards, requires regular mowing as conventional grass surface practices are usually carried out (plants very close to the ground).

To our knowledge, there are no studies about the impact of ground cover management on *L. botrana* parasitization in Italy. Most of the researches concern only the systematic aspect of

EGVM parasitoid, mating disruption and the effect of cover crops on *L. botrana* infestation (Lucchi et al., 2018; Scaramozzino et al., 2017, 2018; Serra et al., 2006).

Finally, this study aims to assessing EGVM parasitoid diversity in commercial vineyards of Lambrusco production area.

4.3 Material and methods

4.3.1 Field trials

The trials were carried out in 7 vineyards of Reggio Emilia Province in 2018, 10 vineyards in 2019 and 6 vineyards in 2020 (Table 1).

Table 1. Details of the vineyard sites used for the field trial. Location (Province, Longitude, Latitude), year of the trial, vine variety and pest management are showed. (C)= conservative ground cover management; (I)= intensive ground cover management.

Site	Province	Longitude	Latitude	Year	Variety	Pest management
1	Reggio nell'Emilia	10°48'14.90" E	44°43'59.32" N	2018 2019 2020	Lambrusco Salamino	IPM with prophylactic use of insecticides
2	Reggio nell'Emilia	10°45'12.52" E	44°51'15.68" N	2018 2019	Ancellotta (C); Lambrusco Marani (I)	IPM
3	Reggio nell'Emilia	10°41'54.55" E	44°41'00.81" N	2018	Lambrusco Grasparossa (C); Lambrusco Maestri (I)	IPM
4	Reggio nell'Emilia	10°45'53.50" E	44°47'47.87" N	2018 2019	Ancellotta	IPM with prophylactic use of insecticides
5	Reggio nell'Emilia	10°47'23.77" E	44°51'50.52" N	2018 2019 2020	Lambrusco Salamino	IPM
6	Reggio nell'Emilia	10°48'33.85" E	44°44'05.19" N	2018 2019	Lambrusco Salamino (C); Ancellotta (I)	IPM with prophylactic use of insecticides
7	Reggio nell'Emilia	10°40'16.07" E	44°40'16.07" N	2018 2019 2020	Ancellotta	IPM with prophylactic use of insecticides
8	Reggio nell'Emilia	10°48'30.61" E	44°46'00.56" N	2019 2020	Lambrusco Salamino	IPM
9	Reggio nell'Emilia	10°30'34.45" E	44°49'26.93" N	2019	Ancellotta	IPM
10	Reggio nell'Emilia	10°38'46.06" E	44°43'33.32" N	2019 2020	Ancellotta	IPM with prophylactic use of insecticides
11	Reggio nell'Emilia	10°46'35.60" E	44°48'41.17" N	2019 2020	Lambrusco Oliva	IPM with prophylactic use of insecticides

The trial was carried out in approximately 1 hectare inside each vineyard. Two treatment plots were selected to compare two thesis: conservative and intensive ground cover management. In the first one, the ground cover was not mowed for approximately two months before *L. botrana* infestation and parasitization sampling to allow the growth and flowering of the cover (Figure 1a); in intensive ground cover, the herbaceous cover was regularly mowed so as to maintain it close to the ground and avoid flower development (Figure 1b).

The two plots were at a minimum distance of 25 meters away from each other. This buffer zone was regularly mowed as intensive ground cover treatment.

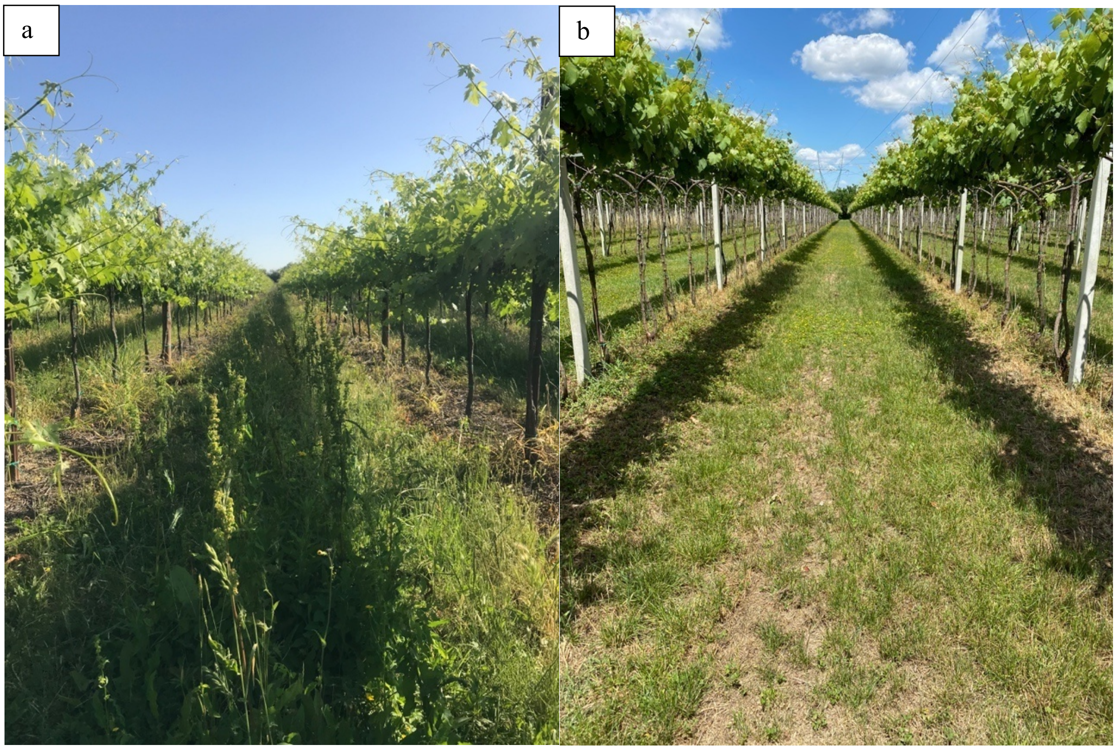

Figure 1. Different ground cover management, conservative (a) and intensive (b), was tested to investigate the impact of spontaneous vegetation on infestation and parasitization of the first generation of L. botrana.

4.3.2 Inter-row ground cover plant species

All vineyards were characterized by similar inter-row grass species. The most common plant species were: *Taraxacum spp.* Wiggers, 1780 (Compositae), *Veronica spp*. L. (Plantaginaceae), *Bellis spp.* L. (Compositae) and gramineous plants such as *Hordeum spp.* Moreover, *Bromus spp*. L. (Poaceae) and *Plantago spp.* L. (Plantaginaceae) were found out in vineyard 2 and 5 and *Avena spp*. (Poaceae) was observed in vineyard 8. Inter-row flora of vineyard 2 was also characterized by *Geranium spp.* L. (Geraniaceae) e *Trifolium spp.* L. (Fabaceae), which was found also in vineyard 1, whereas *Malva sylvestris* L. (Malvaceae) was present in vineyard 5, 6, 7 and 10. *Trifolium spp.*, *Papaver rhoeas* L. (Papaveraceae), *Potentilla reptans* L. (Rosaceae)*,* *Plantago lanceolata* L. (Plantaginaceae) e *Convonvuls arvensis* L. (Convolvulaceae) were observed in vineyard 4. Finally, the weed species *Potentilla reptans* L. (Rosaceae) was also found in vineyards 8 and 11.

4.3.3 Infestation

To assess the infestation, 200 inflorescences were randomly selected in each treatment plot, counting the infested ones. Glomerulae with alive larvae inside them were considered as infested inflorescences (Figure 2). This visual sampling was carried out directly in the field.

Figure 2. Vine inflorescence with glomerulae.

4.3.4 Parasitization

Infested inflorescences per treatment were collected in plastic boxes to evaluate the percentage of parasitization. In most vineyard a minimum number of 50 inflorescense was established; less than 50 infested organs were collected in vineyards characterized by a low percentage of inflorescence infestation. After inflorescence collection, this disruptive sampling continued in the laboratory of the Department of Agricultural and Food Sciences (University of Bologna), to rear EGVM and its parasitoids (Figure 3a). Emerged EGVM adults and its natural enemies were counted, to estimate the parasitization rate (Figure 3b).

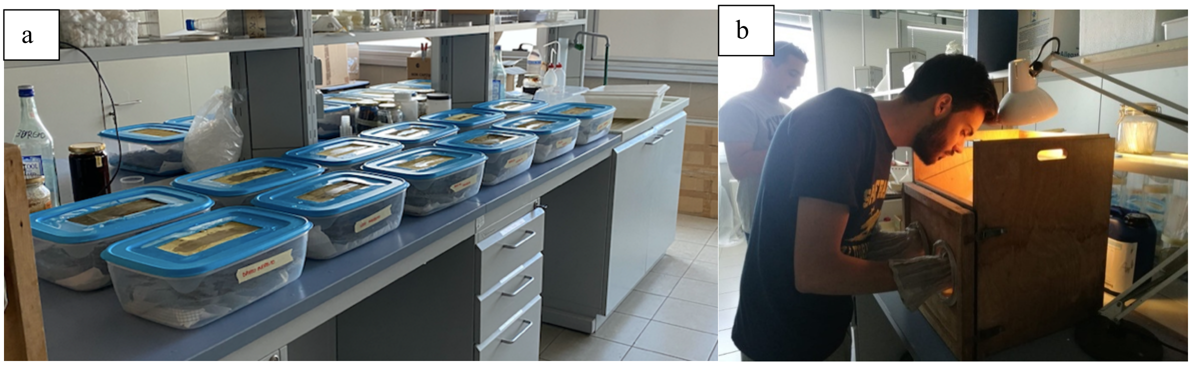

Figure 3. Lobesia botrana *rearing (a) and box check to collect* L. botrana *adults and/or parasitoids (b).*

To prevent mould formation, metallic nets were used to lift up the inflorescences and allow the air to circulate through them. Moreover, paper towels were positioned under the nets to provide *L. botrana* larvae with a proper substrate to pupate and to create a perfect climate for its development. The boxes were wetted two-three times a week to maintain the optimal moisture condition for EGVM development and checked weekly to detect and count grapevine moth adults and/or parasitoids. Natural enemies were refrigerated and stored in test tubes until their identification. The complexity of trophic relations was also defined by means of beneficial identification (Figure 4).

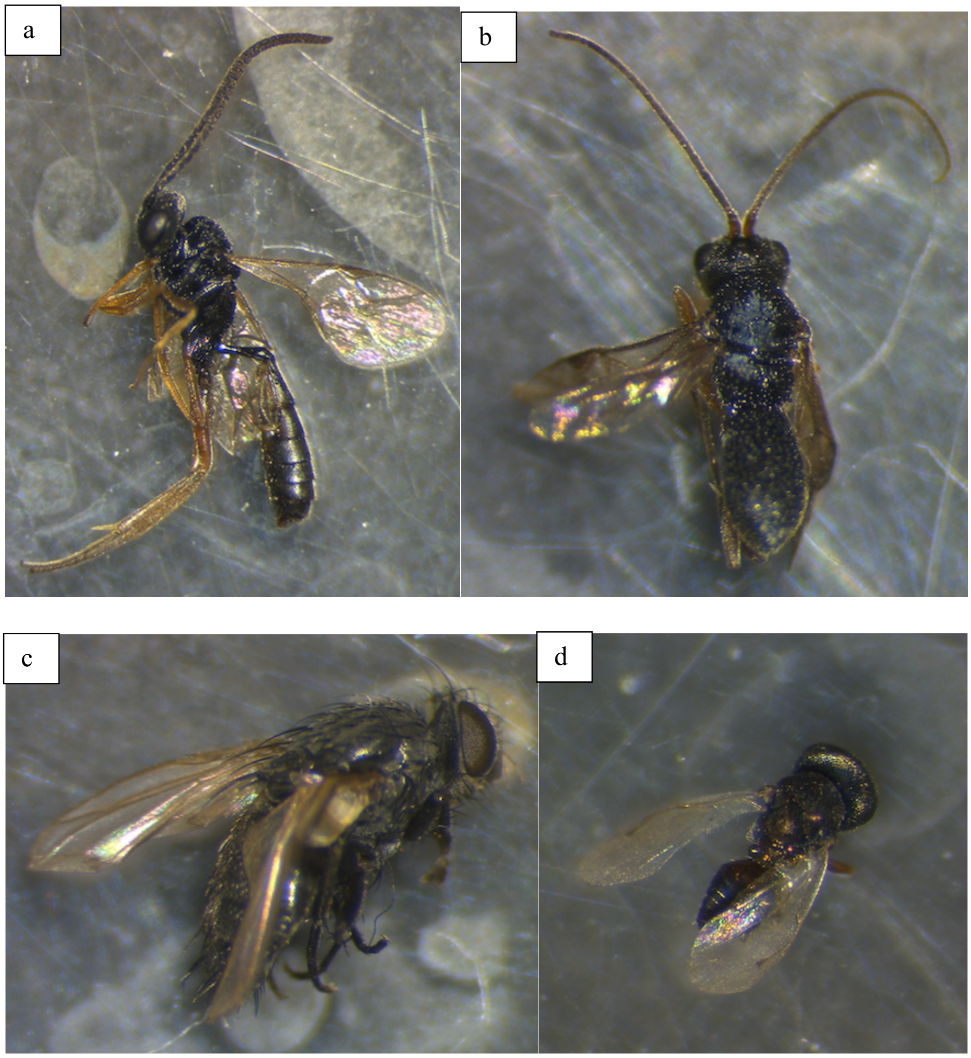

Figure 4. Some of L. botrana *parasitoids collected:* Campoplex capitator *(a),* Ascogaster quadridentata *(b),* Phytomyptera nigrina *(c) and* Dybrachis affinins *(d).*

4.3.5 Statistical analysis

Log linear analysis was used to carry out a preliminary analysis to evaluate the effect of the treatments (conservative, intensive), year (2018, 2019, 2020) and vineyards (2018: n=7; 2019: n=10; 2020: n=5) on the ratio of infested inflorescences and parasitism.

Two-way ANOVA was carried out in order to assess the effect of the different ground cover management on *L. botrana* infestation and parasitization in each year (2018, 2019, 2020). Arcsin transformation of data was carried out to correct non-normality of data.

Log linear analysis and two-way ANOVA were performed using Statistica version 10 software (StatSoft^{TM}, Tulsa, OK, USA).

To evaluate the diversity in *L. botrana* parasitoid community, Shannon-Weaver index (H') was calculated. The Jack-knife technique was used to improve the estimate of H' and calculate the standard error of Shannon-Weaver index. This method is recommended when a number of samples (in this case the different vineyards) are analyzed (Burgio, 2007; Maia et al., 2000).

4.4 Results

Log linear analysis showed a significant difference on the infested inflorescences between conservative and intensive ground cover management. Also a significant effect of vineyard and year on the infestation was detected (Table 2).

Table 2. Log linear results showing the effect of each factor (T=treatment; V=vineyard; Y=year) and their interaction on infestation rate (I=infestation).

Effect	df	Chi square (Partial association test).	P (Partial Association test)	Chi square (Marginal association test)	P (Marginal association test)
T x I	1	29,25	< 0,001	27,51	< 0,001
V x I	10	403,27	< 0,001	207,57	< 0,001
Y x I	2	336,72	< 0,001	141,32	< 0,001
T x V x I	10	51,39	< 0,001	47,99	< 0,001
T x Y x I	2	6,64	0,04	6,27	0,04
V x Y x I	20	145,12	< 0,001	140,97	< 0,001

Treatment did not significantly affect *L. botrana* parasitization, whereas statistical analysis highlighted a significant effect of vineyard and year (Table 3).

Table 3. Log linear results showing the effect of each factor (T=treatment; V=vineyard; Y=year) and their interactions on parasitization rate (P=parasitization).

Effect	df	Chi square (Partial association test).	P (Partial Association test)	Chi square (Marginal association test)	P (Marginal association test)
T x P	1	0,002	>0,05	0,03	>0,05
V x P	10	37,90	< 0,001	34,03	<0,001
Y x P	2	18,07	<0,001	14,06	<0,001
T x V x P	10	8,08	> 0,05	11,53	> 0,05
T x Y x P	2	0,32	> 0,05	0,19	> 0,05
V x Y x P	20	45,38	<0,001	45,16	0,001

4.4.1 Year 2018

4.4.1.1 Total infestation and parasitization

Significant difference of infested inflorescences was detected between the two ground cover managements (df=1; F=9,97; p=0,03) (Figure 5a). Statistical analysis also showed a significant vineyard effect on infestation (df=4; F=12,03; p=0,02).

Even though there was a higher percentage of parasitized individuals in the conservative ground cover management compared to the intensive one, statistical analysis highligheted no significant difference between the two treatments (df=1; F=1,07; p>0,05) (Figure 5b).

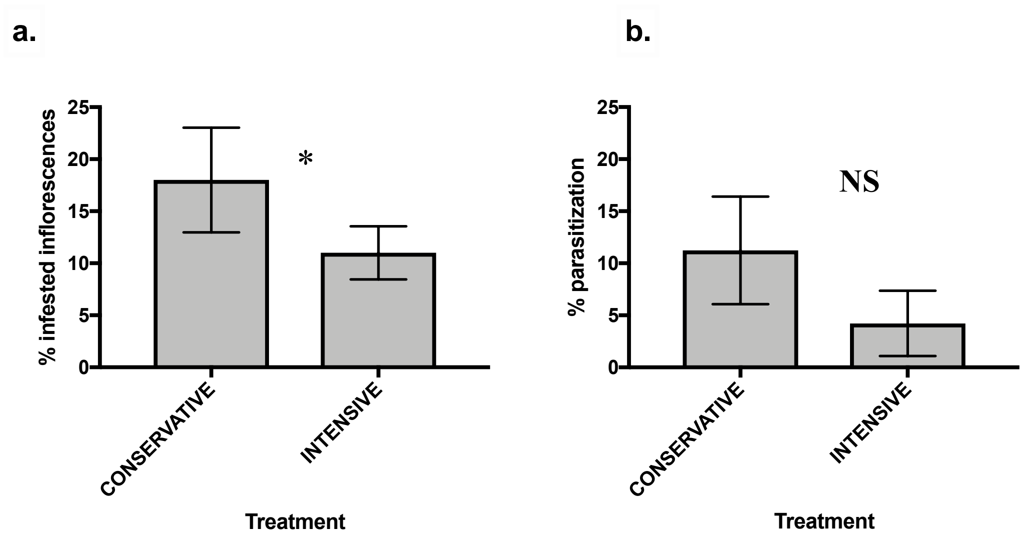

Figure 5. Average rate of infested inflorescences (± SE) (n=7) (a) and average percentage of L. botrana *parasitization (±SE) (n=5) (b) in conservative and intensive ground cover management in 2018. Two-way ANOVA was used to compare the two treatments: * p<0,05; NS= not significant.*

4.4.1.2 Parasitoid species and trophic relations

In 2018, six parasitoid species emerged from *L. botrana* individuals: *Campoplex capitator* Aubert (Hymenoptera: Ichneumonidae), *Ascogaster quadridentata* Wesmael (Hymenoptera: Braconidae), *Dibrachys affinis* Masi (Hymenoptera: Pteromalidae), *Phytomyptera nigrina* (Meigen) (Diptera: Tachinidae), *Elasmus steffani* (Viggiani) (Hymenoptera: Elsamidae) and *Eutetrastichus amethystinus* (Ratzeburg) (Hymenoptera: Eulophidae).

A higher number of parasitoids emerged from conservative cover management inflorescences compared to the intensive ground cover management. Four parasitoids and two hyperparasitoids were collected in the conservative treatment, whereas only two parasitoids and one hyperparasitoid were found in intensive management (Figure 6).

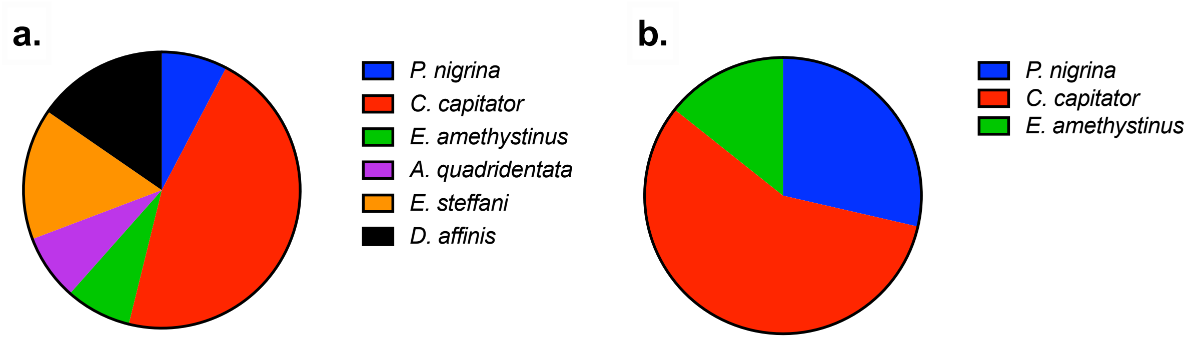

Figure 6. Parasitoid species complexity in conservative (a) and intensive (b) ground cover management in 2018.

Also Shannon index showed a higher value in conservative ground cover management than intensive one (Figure 7).

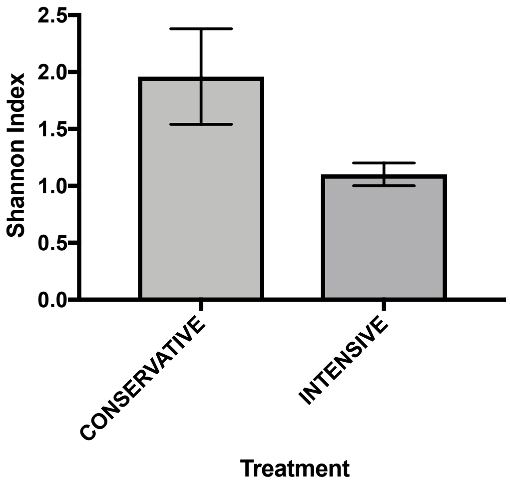

Figure 7. Shannon-Weaver index (± SE) in conservative and intensive ground cover management in 2018.

The most frequent parasitoid was *C. capitator*, which represented the 46% of *L. botrana* parasitoids in the conservative ground cover management and 57% in the intensive ground cover management.

Parasitoids of different trophic levels were found in the conservative ground cover management. Indeed, *E. steffani* parasitizes larvae of *C. capitator*, while *E. amethystinus* is responsible for *E. steffani* parasitization (Figure 6a).

On the other hand, only the tachinid parasitoid *P. nigrina*, *C. capitator* and *E. amethystinus* were collected in the intensive ground cover management (Figure 6b).

4.4.2 Year 2019

4.4.2.1 Total infestation and parasitization

About infestation, no significant difference was detected between the two treatments in 2019 (df=1; F= 3,61; p>0,05) (Figure 8a), whereas vineyard effect showed statistical difference (df=9; F=8,20; p=0,002).

No significant difference was found between parasitization in conservative and intensive ground cover management (df=1; F=0,10; p>0,05) (Figure 8b), while there was a significant vineyard effect (df=6; F=8,24; p=0,01).

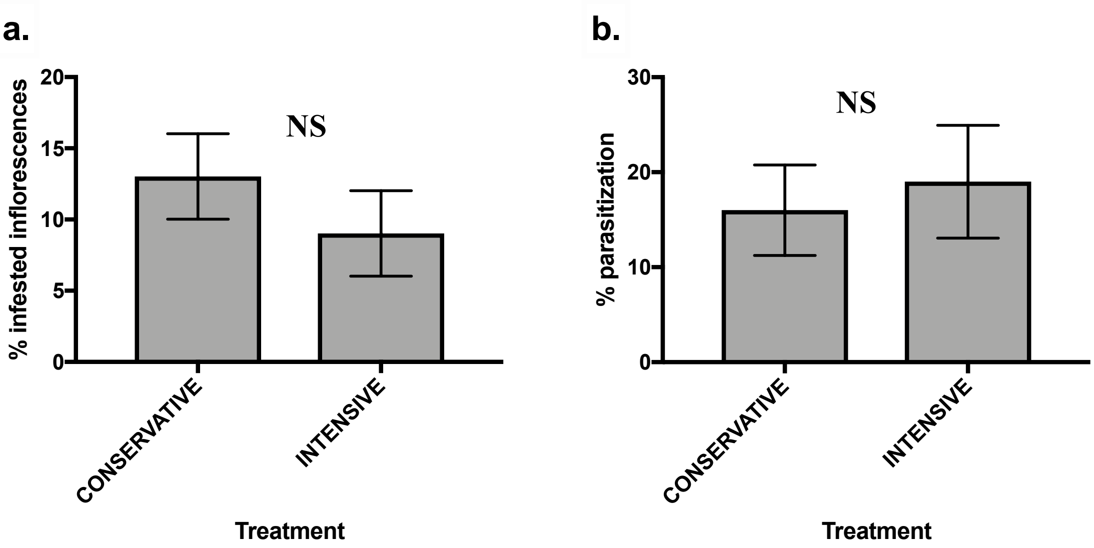

Figure 8. Average rate of infested inflorescences (± SE) (n=10) (a) and average percentage of L. botrana *parasitization (±SE) (n=7) (b) in conservative and intensive ground cover management in 2019. Two-way ANOVA was used to compare the two treatments: NS=not significant.*

4.4.2.2 Parasitoid species and trophic relations

In 2019, five parasitoid species emerged from *L. botrana* individuals: *C. capitator*, *A. quadridentata, P. nigrina*, *E. steffani* and *Tranosemella prerogator* (Linnaeus) (Hymenoptera: Ichenumonidae).

Figure 9 shows the parasitoid species emerged from infested inflorescences in each ground cover management.

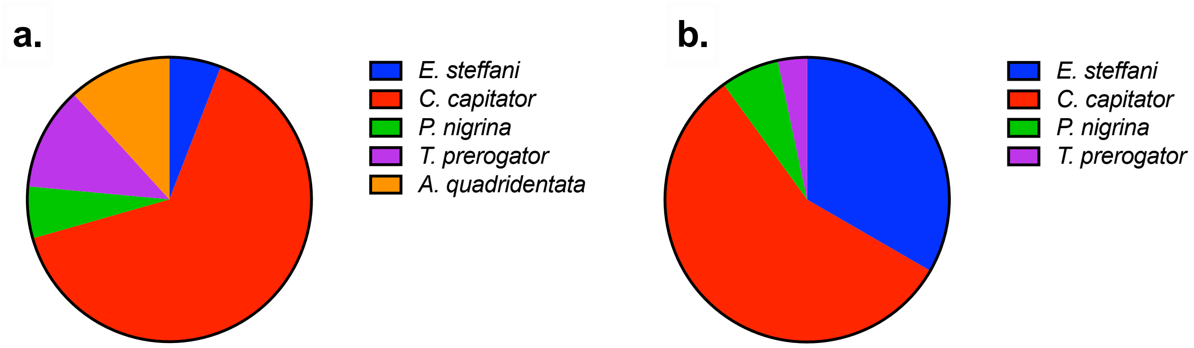

Figure 9. Parasitoid relative abundances of species emerged from inflorescences in the conservative (a) and intensive (b) ground cover management in 2019.

As in 2018 season, *C. capitator* was the most abundant parasitoid of *L. botrana* in 2019. This Ichneumonidae parasitoid represented 65% and 57% of the emerged natural enemies of the conservative and intensive ground cover treatment, respectively.

Figure 9 shows how the conservative ground cover management presented a richer parasitoid complex (4) compared to the intensive treatment. *E. steffani* emerged from inflorescences of both treatments.

The higher beneficial richness can be evinced from Shannon index, which was slightly higher in the conservative treatment compared to the intensive one (Figure 10).

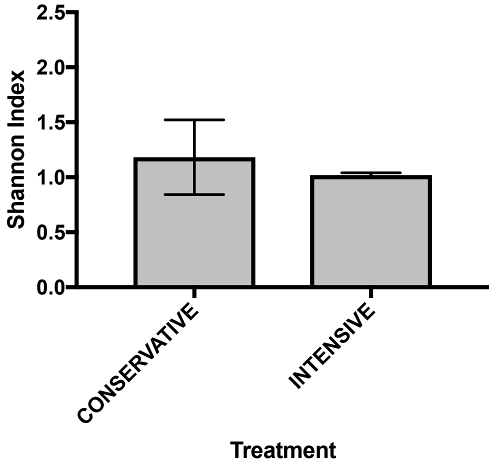

Figure 10. Shannon-Weaver index (± SE) in conservative and intensive ground cover management in 2019.

4.4.3 Year 2020

4.4.3.1 Total infestation and parasitization

The number of damaged inflorescences was lower in 2020 compared to previous years (2018, 2019) and no significant difference was detected between the two ground cover treatments (df=1; F= 0,09; p>0,05) (Figure 11a). Moreover, there was no statistical difference between the parasitization in the conservative ground cover management and in the intensive one (df=4; F= 1,96; p>0,05) (Figure 11b).

No significant vineyard effect was found out both on infestation and parasitization.

Vineyard 11 was not included in the total infestation and parasitization because of the absence of *L. botrana* in 2020.

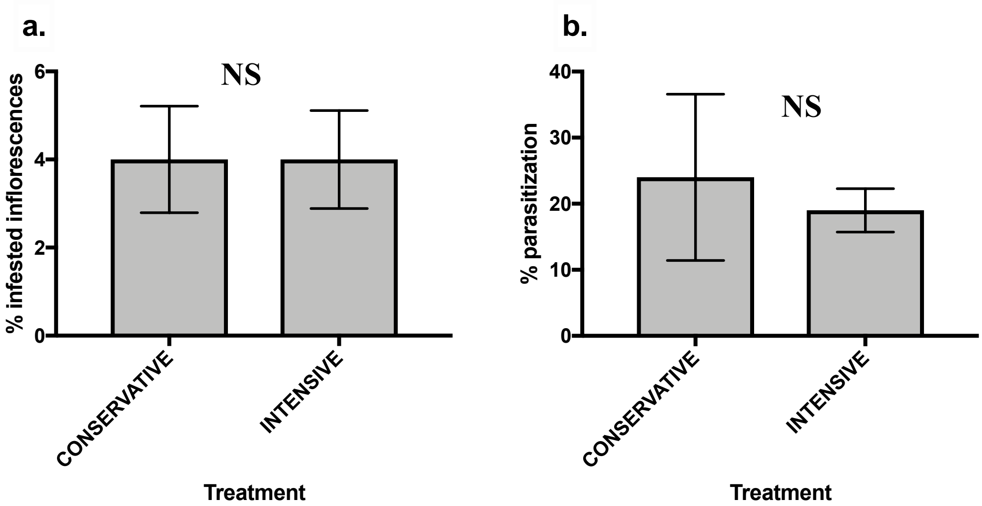

Figure 11. Average rate of infested inflorescences (± SE) (a) and average percentage of L. botrana *parasitization (± SE) (b) in conservative and intensive ground cover management (n=5) in 2020. Two-way ANOVA was used to compare the two treatments: NS=not significant.*

4.4.3.2 Parasitoid species and trophic relations

In 2020, only four parasitoids were collected: *C. capitator*, *T. prerogator*, *P. nigrina* and *Exochus* sp. (Hymenoptera: Ichneumonidae) (Figure 12). The most prevalent parasitoid remained *C. capitator*. 86% of emerged *L. botrana* parasitoids was represented by *C. capitator* in the conservative treatment (Figure 12a), whereas 93% was the percentage of *C. capitator* parasitization in the intensive ground cover management (Figure 12b). A new species of *L. botrana* parasitoid, *Exochus* sp., was collected, which was not recorded in 2018 and 2019.

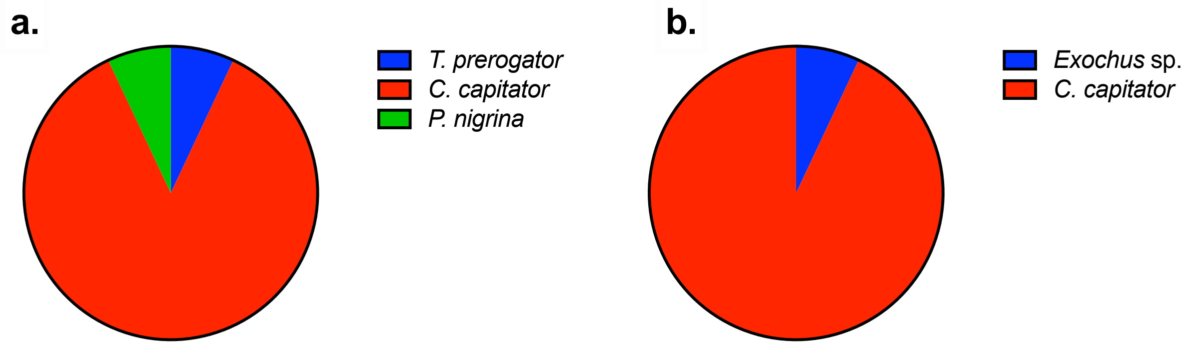

Figure 12. Parasitoid relative abundance of species emerged from inflorescences in the conservative (a) and intensive (b) ground cover management in 2020.

A higher diversity of species was observed in conservative ground cover management compared to intensive one. Indeed, Shannon index confirmed this result, showing a higher value in conservative treatment compared to intensive ground cover management (Figure 13).

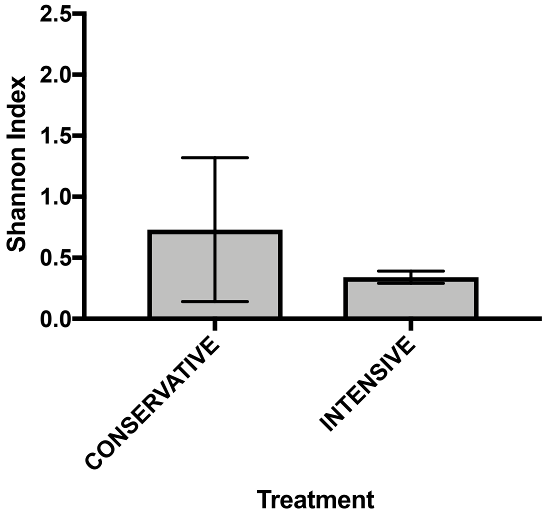

Figure 13. Shannon-Weaver index (±SE) in conservative and intensive ground cover management in 2020.

4.5 Discussion and conclusion

During this three-year trial, conservative ground cover management did not reduce infestation caused by *L. botrana* first generation as well as did not enhance significantly parasitization rate. Regarding infestation, our result is not in line with Rusch et al. (2017) work, which demonstrated that grape moth occurrence decreased in vineyards with full compared to partial grass cover. In our study, inflorescence infestation rate was significantly higher in the conservative treatment compared to the intensive one in the first year. Similar results were obtained by Zanettin (2018), who showed that the presence of non-mowed spontaneous grass in vineyard inter-rows boosted grapevine leafhoppers. The ground cover may have acted as refuge for the pest (Shields et al., 2016) and increase the fitness of herbivorous insects and, consequently, infestation (Deguine et al., 2019). Daane et al. (2018) highlighted that native grasses reduced the number of vineyard leafhoppers by means of water and soil competition with the perennial crop. In our study, it is possible that ground cover did not compete enough to reduce vigory of vines and, consequently, host quality for *L. botrana*. Markheiser et al. (2017) highlighted that green colour of leaves, peduncles and buds, which characterizes vigorous plants, represented a basic signal for any oviposition site selection of *L. botrana*.

Concerning EGVM parasitization, our results were in line with Rusch et al. (2017) who showed that no significant effect of local vegetation management was found on parasitization rate of the pest. Tscharntke et al. (2016) highlighted that, despite the well-known role of natural habitat to sustaining pest biological control, variability is high and reverse effects can occur. Indeed, in this study the authors reported five hypothesis which may justify no, or even negative, effect of natural habitats on pest suppression. In particular, interrow ground cover plants and their flowering may not be sufficient, both in amount and composition, to provide a great biological control by beneficials. Moreover, the use of insecticides in the surrounding fields may disrupt natural enemy control against pests. Viticultural areas of Lambrusco are characterized by highly intensive monoculture, where most of the growers adopt chemical control to face pest infestation. They generally carry out insecticide treatment against the first generation of EGVM, even if the antophagous generation does not usually cause yield losses (Ioriatti & Lucchi, 2016).

It is well known that flower morphology and apparency and pollen and nectar composition highly influence the suitability of flowering plants as parasitoid food sources. Wäckers and Van Rijn (2012) underlined how natural enemy increase is not fostered by simply enhancing agroecosystem diversification but requires specific knowledge of plant-parasitoid interaction and necessitates the right flowering plant. Araj et al. (2019) showed the potentiality of weed flower resources, such as shepherd's purse and white rocket, to increase biological control of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) on cauliflower plants in laboratory conditions. Weed plants can be adopted in climates that are less suitable for commonly used selected flower plants and can provide critical elements for natural enemy fitness, such as shelter. Also Daane et al. (2018) affirmed that native perennial ground cover may be more compatible with crop management than exotic plant. Although, ground cover plants were not likely sufficient to improve *L. botrana* control and its parasitization in this field trial. Thus, more focused habitat management strategies should be implemented in the future and alternative flower resources could complement insectary plants. Previous studies demonstrated how buckwheat plays a critical role for biological control enhancement. Jado et al. (2018) showed that *Aphidius colemani* Viereck (Hymenoptera: Braconidae) had longer longevity and parasitized more aphids when wasp had access to buckwheat compared to control. Moreover, buckwheat increased the mid bug *Apolygus lucorum* (Meyer-Dür) (Hemiptera: Miridae) parasitism in cotton fields in China (Li et al., 2019). Finally, Hoffmann et al. (2017) demonstrated that egg predation of *L. botrana* was enhanced by greencover crops.

About *L. botrana* parasitoid diversity, *C. capitator* represented the most collected parasitoid, This result confirms previous studies, which defined this ichneumonid parasitoid as the best candidate for future EGVM biological control programs (Ioriatti et al., 2011; Scaramozzino et al., 2017, 2018; Xuéreb & Thiéry, 2006). Nevertheless, *C. capitator* releases have not carried out yet because of the difficulties linked to its mass-rearing (Ioriatti et al., 2012; Scaramozzino et al., 2017). The emergence of *T. prerogator*, *P. nigrina* and *A. quadridentata* from *L. botrana* individuals are in accordance with Scaramozzino et al. (2107, 2018) studies, which previously highlighted the presence of these parasitoid species in Italian regions. Moreover, our field trial represents the first recording of *T. prerogator* and *A. quadridentata* in Emilia-Romagna region. Shannon Index showed a higher richness in conservative ground cover management compared to intensive one. Therefore, conservative ground cover showed a positive effect on the preservation of EGVM parasitoids, acting as buffer zone. Likely, ground cover vegetation represented an important ecological corridor which increased connectivity in a highly fragmented ecosystem as Lambrusco viticultural area. Indeed, ecological theory affirms that isolated habitat patches will be characterized by greater rates of species loss compared to less isolated habitats (Collinge, 2000).

Among the several parasitoid species observed in this trial, some hyperparasitoids were found. Agroecological manipulations may provide food also for the fourth trophic level, creating possible ecosystem disservices. In future trials, these seconday and unwanted effects may occur, but they surely remain less damaging than insecticides. Even though the disruption of biological control by hyperparasitoids is feasible, unmanaged habitat in the landscape has not yet been directly responsible for such negative effects (Gillespie & Wratten, 2017).

The different level of parasitoid diversity in the three years of the experiment may be explained by the fact that different vineyards were sampled in 2018, 2019 and 2020. Moreover, *L. botrana* infestation was characterized by high variability among years which may have influenced trophic relation complexity as well as parasitoid abundance and diversity. Log linear analysis confirmed the significant effect of year and vineyard on infestation rate and parasitization, as reported in previous studies (Bagnoli et al., 2019).

In 2020, few wasp species were observed due to low rainfall during spring which impeded ground cover growth and flowering. Moreover, some growers did not always pay attention on maintaining vegetation very close to the ground in the intensive ground cover management, thereby causing imperceptible differences between the two treatments and compromising the experiment.

To conclude, habitat strategy by means of ground cover management did not show to enhance EGVM parasitization, though it resulted in increasing diversity in the trophic guild of hymenoptera parasitoids. This study represents one of the first work on the effect of ground cover management on parasitism and infestation of EGVM first generation in Italy. More focused strategies need to be implemented and investigated. Apart from biological control, agro-ecological manipulations provide a wider range of ecosystem derived benefits, such as the reduction of soil erosion, the enhancement of soil biodiversity and fertility and the conservation of organic matter content (Daane et al., 2018; Garcia et al., 2018; Shields et al., 2016; Winter et al., 2018), making them an effective multifunctional strategy in crop systems.

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Chapter 5 The efficacy of silica gel as elicitor to enhance the attraction of vineyard natural enemies

5.1 Abstract

Apart from direct defence methods, plants can face insect attack by means of volatile production. Studies on the effect of elicitors both on the second and third trophic level have increased in recent years. In particular, silicon-derived compounds have shown promising results.

The aim of this work was to test silica gel as a resistance inducer to increase the recruitment of Hymenoptera parasitoids in vineyard. Field trials were carried out in three vineyards of Northern Italy. Two plots were used to compare silica gel treated plants with untreated plants (control). Two silica gel treatments were carried out, each followed by two insect samplings. Sticky traps were placed in vine canopy, collected after one week and analysed in laboratory to assess Hymenoptera parasitoid captured.

Results highlighted a significant higher attraction of treated plants towards two important parasitoid families of vineyard, Mymaridae and Encyrtidae, in respect to untreated control plants.

Silica gel showed a great potentiality in recruiting parasitoid insects, even though a high variability was observed among vineyards. Further studies should focus on integrating this strategy with other biological control methods, testing “Attract and Reward” approach or complementing chemical ecology with natural enemy augmentation.

5.2 Introduction

Multiple defence mechanisms can be used by plants to withstand insect attack. Indeed, both morphological and biochemical strategies affect herbivore growth, development, fecundity and behaviour as well as natural enemy attraction (de Oliveira et al., 2020; Luyckx et al., 2017; Rashid War et al., 2018).

Herbivore-Induced Plant Volatiles (HIPVs) play a critical role in tritrophic relations between plants, pests and their natural enemies (Turlings & Erb, 2018). These volatiles represent an indirect plant defence strategy by which first trophic level individuals communicate herbivore attack to natural enemies. Chemical ecology application, such as the use of synthetic volatiles or HIPVs-eliciting compounds (elicitors), has considerably risen during the last 30-40 years (Furlong et al., 2018), especially to enhance plant indirect resistance against pests (Coppola et al., 2017; Lucchi et al., 2017; Rostás & Turlings, 2008; Simpson et al., 2011a; Simpson et al., 2011b; Sobhy et al., 2014; James & Price, 2004).

Among the several elicitors studied to repel herbivores or boost natural enemy attraction, silicon has shown very promising results. Apart its well-recognized properties of increasing plant tissue abrasiveness and rigidity (Alhousari & Greger, 2018; Alvarenga et al., 2017; Yang et al., 2018), silicon elicits the production of phytohormones, thereby representing an important element for triggering plant chemical defences. In particular, a strong interaction has been observed between silicon and jasmonic acid, a precursor of HIPVs (Alhousari & Greger, 2018; Dicke, 2009; Parrilli et al., 2019; Reynolds et al., 2016; Ye et al., 2013). In particular, chewing insects are mostly responsible for jasmonic acid production, whereas phloem-feeding herbivores cause the activation of salicylic or jasmonic acid pathways (Alhousari & Greger, 2018; Leroy et al., 2019).

Several silicon-derived substances have been used to increase plant resistance against herbivores in recent years. Assis et al. (2015) highlighted the inhibition of *Chlosyne lacinia* *saundersii* Doubleday and Hewitson (Lepidoptera: Nymphalidae) development. Moreover, potassium silicate provided an increase of *Zinnia elegans* Jacq. defence level against *Myzus persicae* (Sulzer) (Rhyncota: Aphididae) (Ranger et al., 2009). Finally, Alvarenga et al. (2017) showed fecundity decrease of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) adult derived from larvae fed on plants treated with silicic acid.

Some researchers have carried out studies on the attraction of silicon treated plants towards beneficial insects. Most of works were conducted in laboratory by means of Y-tube olfactometer. For example, Kvedaras et al. (2009) demonstrated that plants treated with potassium silicate and infested with *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae) became more attractive to *Dicranolaius bellulus* Guèrin-Mèneville (Coleoptera: Melyridae) compared to untreated and infested plants. Furthermore, de Oliveira et al. (2020) highlighted that *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae) was attracted to uninfested wheat plants treated with silicon and not from untreated and uninfested plants.

On the other hand, few plant volatile studies have been carried out in field conditions (Kaplan, 2012).

The goal of this work was to study in deep silica gel treatments in attracting Hymenoptera parasitoids in vineyard system. A previous study (Parrilli et al., 2019) showed great potentiality of silicon-derived compound in eliciting attraction towards Mymaridae (Hymenoptera). Two different vine varieties, old and traditional, were treated with silica gel in order to detect potential differences in plant defence response.

5.3 Material and Methods

5.3.1 Field sites

Field trials were carried out in three vineyards of Bologna Province in 2018 and 2019 (Table 1).

Table 1. Details of the vineyard sites. Location (Province, Longitude, Latitude),vine variety and pest management are showed.

Site	Province	Longitude	Latitude	Variety	Pest management
1	Bologna	11°26’37.53” E	44°23’47.47” N	Pignoletto	IPM
2	Bologna	11°06’01.98” E	44°29’19.03” N	Pignoletto	Organic
3	Bologna	11°07’28.03” E	44°33’34.98” N	Pignoletto; Alionza	IPM

Regarding Pignoletto variety, two areas of 648-2160 m^2 (one for silica gel treatment and one control) were selected in each field site, keeping 25-30 meters away from each other.

Concerning Alionza variety, the trial was carried out in a row characterized by 30 old vine plants (more or less 40 years old) in vineyard 3, keeping 18 meters as buffer zone between treated and untreated plants.

5.3.2 Silicon treatment

Silica gel (Siqur Salute, Vigonza, Italy) was used by a foliar application carried out at 0.12 g l^{-1} acqueous solution of micronized powder at the stage of ten leaves per shoot (Figure 1).

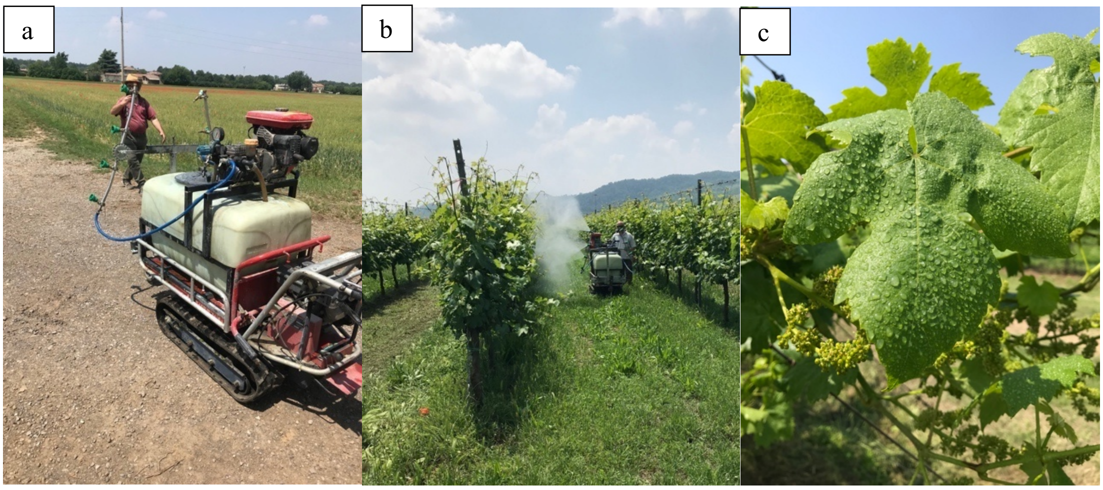

Figure 1. Silica gel treatment was carried out by means of bar sprayers (a, b), paying attention to evenly distribute the solution on foliage (c).

Two silicon treatments were performed to guarantee the presence of silica gel on the new shoots appeared after the first treatment and also to ensure active ingredient action on plant immune system over time. Table 2 shows silica gel treatment dates per field site.

Table 2. Silica gel treatment dates in 2018 and 2019.

Field sites	Silica gel treatments 2018		Silica gel treatments 2019	
	1° treatment	2° treatment	1° treatment	2° treatment
1	28/05/18	09/07/18	03/06/19	11/07/19
2	25/05/18	06/07/18	30/05/19	16/07/19
3	25/05/18	06/07/18	30/05/19	16/07/19

5.3.3 Natural enemy sampling and identification

Natural enemy samplings were carried out by using transparent sticky traps (12x15 cm^2) (Figure 2).

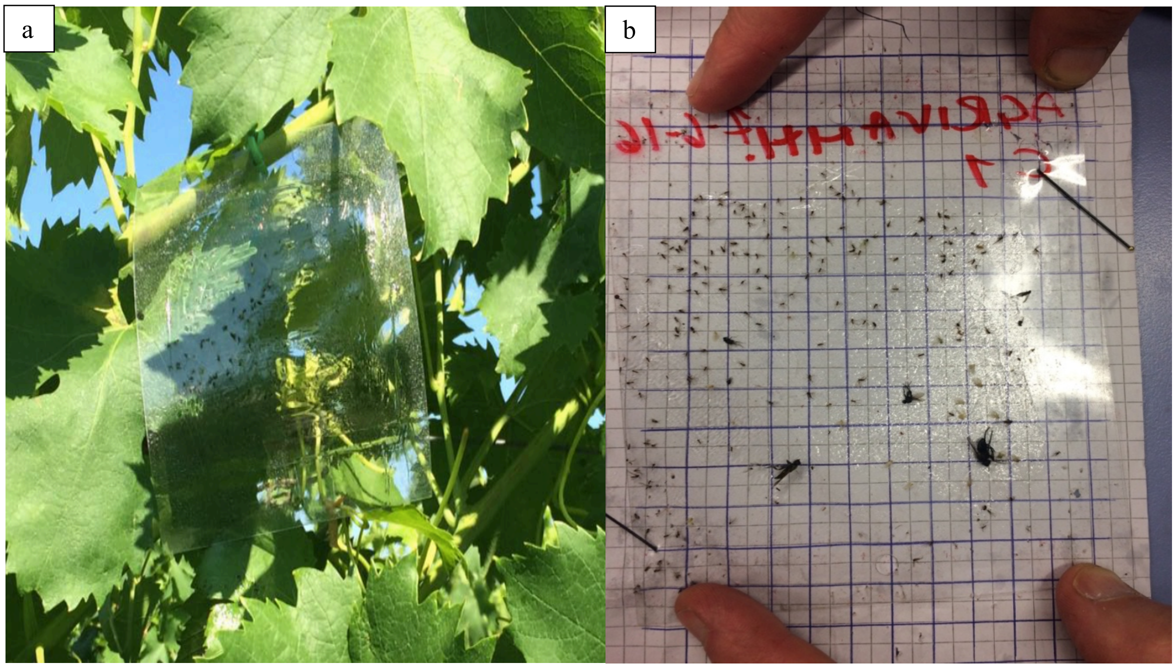

Figure 2. Sticky traps on vine canopy (a) and insects collected by sticky traps after 1 week in field (b).

Two sticky traps were used for each replicate, which were 5 per treatment in the traditional vine variety (vineyard 1, 2 and 3) and 4 in the old variety (vineyard 3). Each replicate was minimum 20 meters away from others in Pignoletto variety and 9-12 meters in Alionza variety. Sticky traps were placed in the field two times after each silicon treatment and collected after one week. Table 3 shows sticky traps collection dates after each silica gel treatment.

Table 3. Dates of sticky trap collection both in 2018 and 2019 after each silica gel treatment.

Sites	Samplings 2018				Samplings 2019			
	1° treatment		2° treatment		1° treatment		2° treatment	
1	15/06/18	22/06/18	26/07/18	02/08/18	21/06/19	28/06/19	29/07/19	05/08/19
2	12/06/18	19/06/18	24/07/18	31/07/18	19/06/19	25/06/19	01/08/19	09/08/19
3	12/06/18	19/06/18	24/07/18	31/07/18	19/06/19	25/06/19	01/08/19	09/08/19

A total of 368 sticky traps were collected each year and examined in the laboratory (Department of Agricultural and Food Sciences, University of Bologna) at a stereomicroscope to identify Hymenoptera parasitoid families.

5.3.4 Statistical analysis

Relative abundances of each Hymenoptera parasitoid family were calculated to evaluate the different level of biodiversity in each vineyard.

A repeated measure analysis of variance (ANOVA) was used to test the effect of the treatments on the abundance (mean individuals/sticky trap) of four most important Hymenoptera parasitoid families in vineyard ecosystem (Mymaridae, Encyrtidae, Ichneumonidae, Braconidae) (Parrilli et al., 2019) (Figure 3). Sampling dates were used as repetead measure. Log transformation of data was carried out when homogeneity of variance did not occur.

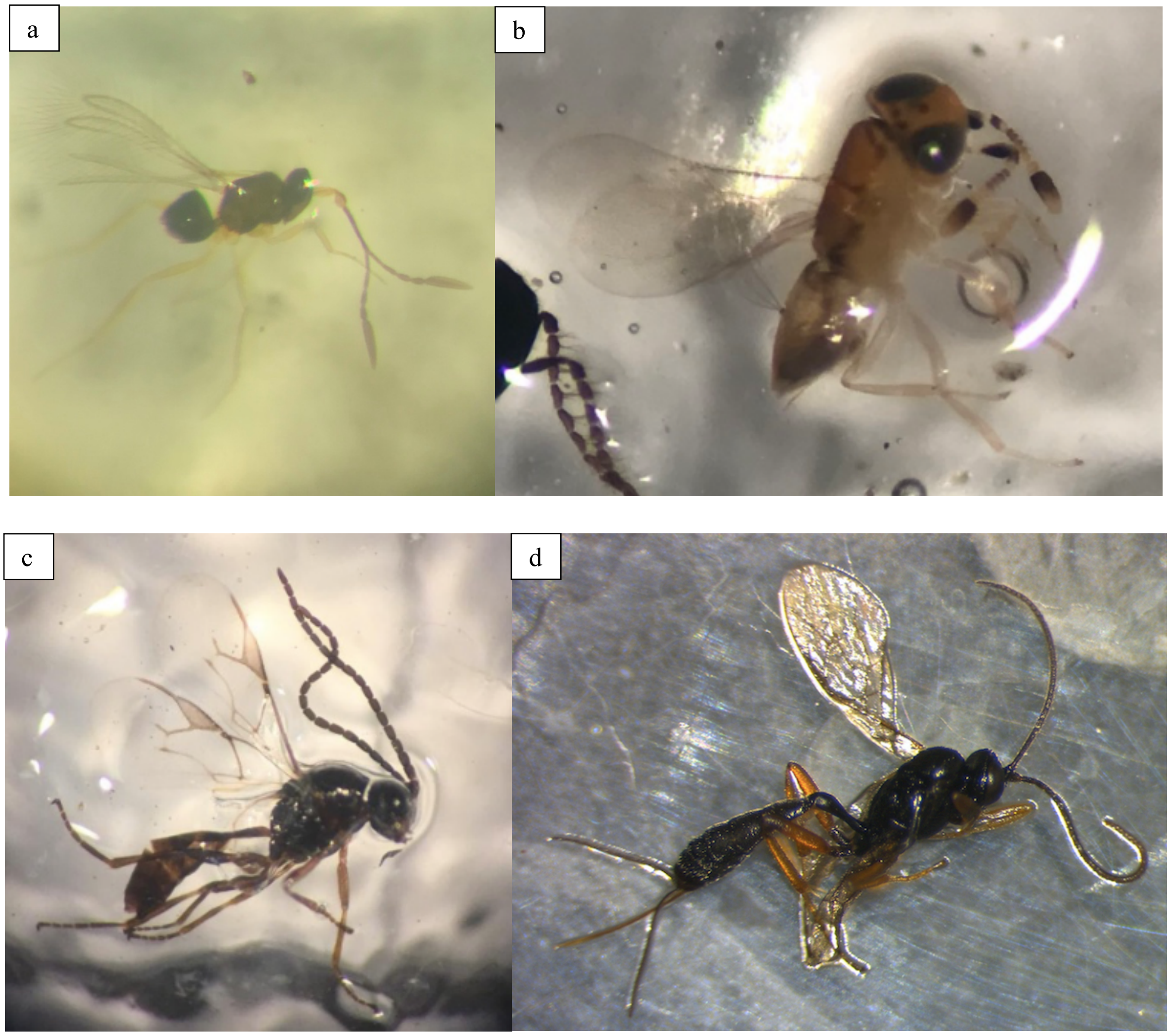

Figure 3. Four individual examples collected of the most important parasitoid of vineyard pests: Mymaridae (a), Encyrtidae (b), Braconidae (c) and Ichneumonidae (d).

5.4 Results

5.4.1 Year 2018

In 2018 4417 Hymenoptera parasitoids were collected. Figure 4 shows parasitoid families identified in each vineyard. More Hymenoptera parasitoid families were identified in vineyard 1 and 2 compared to vineyard 3. Mymaridae and Encyrtidae were among the most collected families in all field sites.

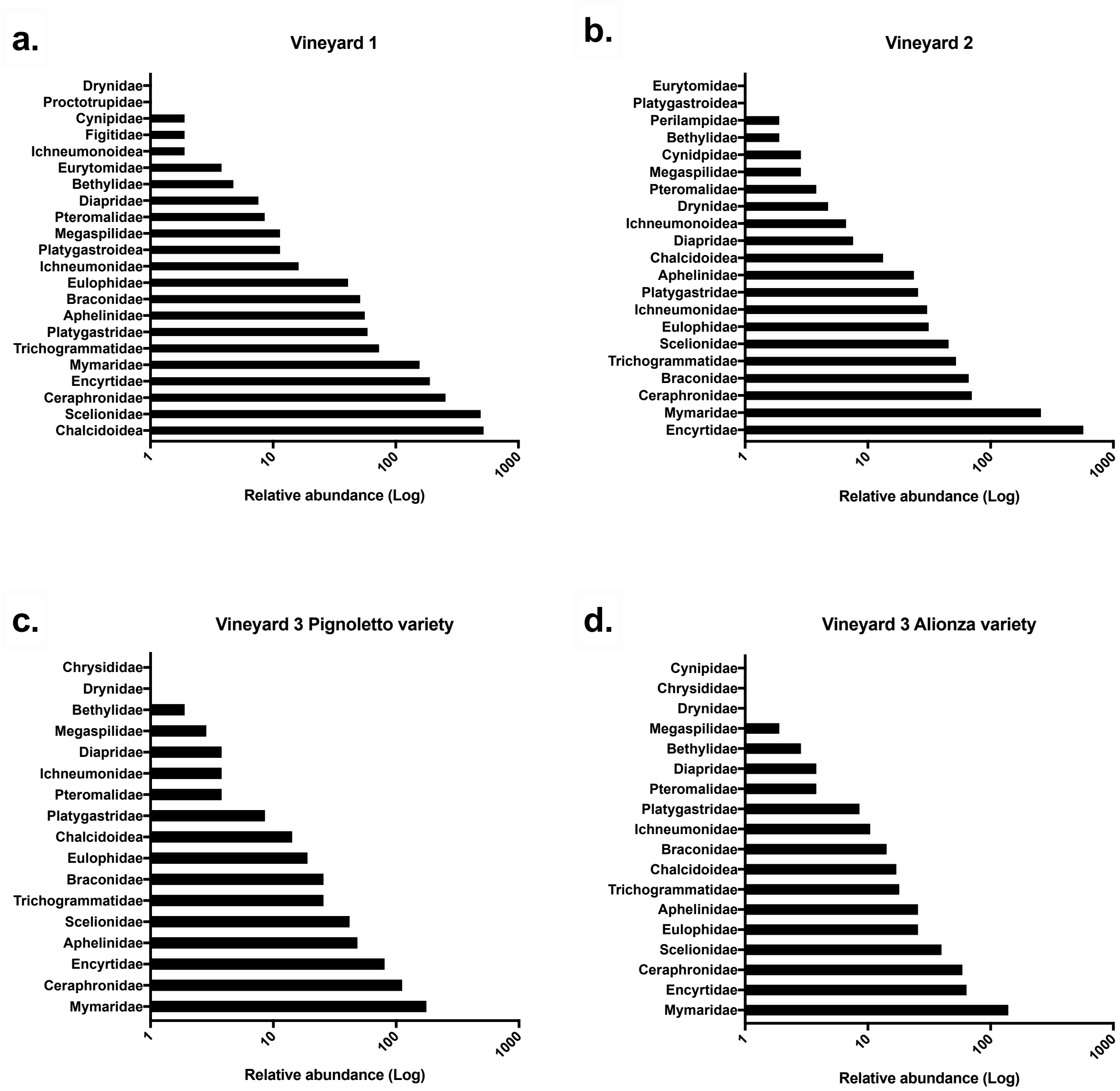

Figure 4. Relative abundance of Hymenoptera parasitoid families collected in each vineyard in 2018.

5.4.1.1 Vineyard 1

Starting with Chalcidoidea, Mymaridae was collected in higher significant quantity in control plot compared to silica gel treatment (Table 4), especially in the second sampling date (Figure 5a). The significant attraction of the control area towards Mymaridae can be evinced by the main effect, which compares the mean captures between silica gel and control treatment. Indeed, there were 5,15 ± 0,49 (SE) Mymaridae in control treatment, whereas only 3,1 ± 0,64 (SE) Mymaridae in silica gel treatment. Silica gel did not display any significant attraction towards Encyrtidae individuals (Figure 5b, Table 4), as well as towards Ichneumonidae and Braconidae families (Figure 5c and 5d, Table 4). However, time showed a significant effect on Braconidae and Encyrtidae (Table 4).

Finally, the interaction between silica gel and time was not significant for all Hymenoptera families (Table 4).

Table 4. Repeated measure ANOVA results for each parasitoid family of vineyard 1 in 2018. Effect: T (treatment), D (date=time), T x D (treatment x date=treatment x time). p in bold shows a significant effect.

Effect	Mymaridae			Encyrtidae			Ichneumonidae			Braconidae		
	df	F	p	df	F	p	df	F	p	df	F	p
T	1	7,45	**0,03**	1	0,40	0,54	1	0,04	0,85	1	0,01	0,91
D	3	0,10	0,96	3	9,67	**<0,001**	3	1,75	0,18	3	15,51	**<0,001**
T x D	3	1,83	0,17	3	0,32	0,81	3	1,23	0,32	3	0,88	0,46

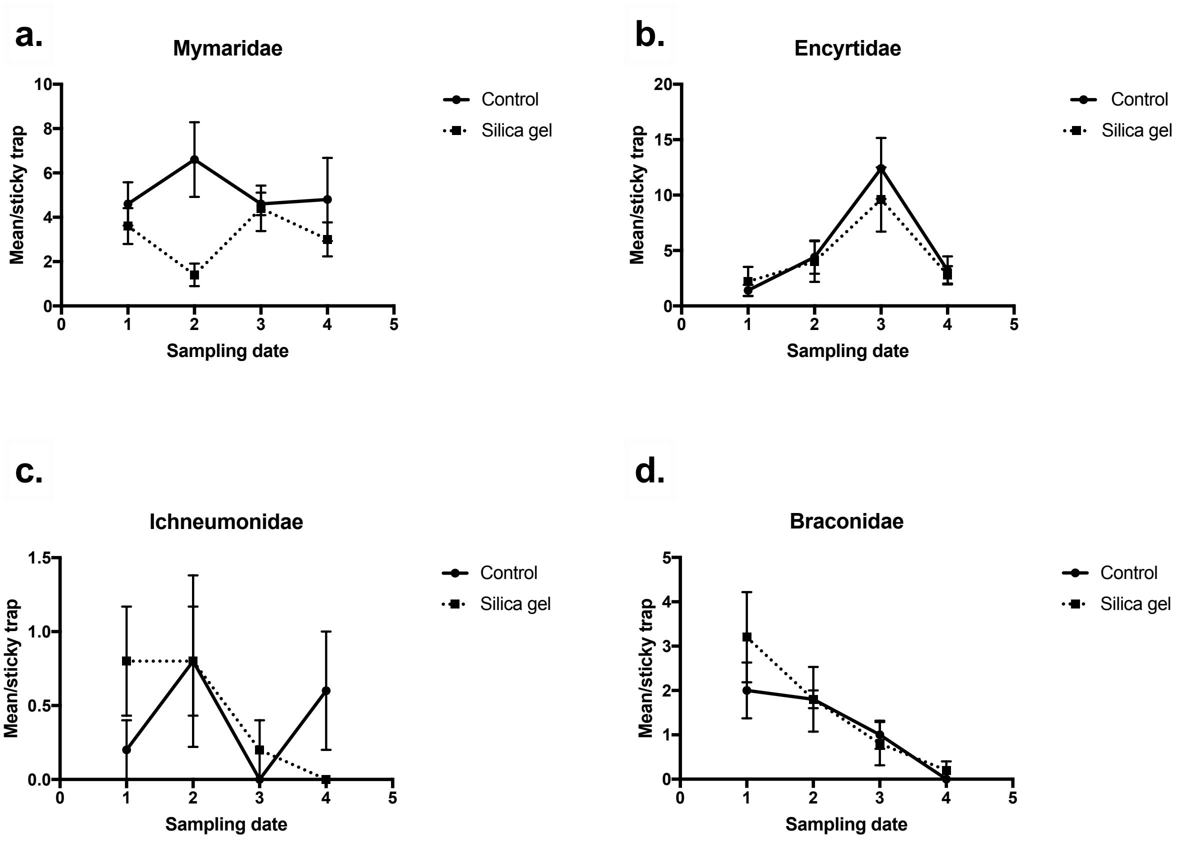

Figure 5. Mean number of parasitoids per sticky trap (± SE) of Mymaridae (a), Encyrtidae (b), Ichneumonidae (c) and Braconidae (d) families in each sampling date of vineyard 1. Mymaridae: T (p<0,05).

5.4.1.2 Vineyard 2

Silica gel and control did not present any significant difference on Mymaridae, Encyrtidae, Ichneumonidae and Braconidae captures (Figure 6, Table 5). Although, Braconidae individuals tended to be more collected in silica gel compared to control (Figure 6d).

Time displayed a significant effect on parasitoids of Mymaridae, Encyrtidae and Braconidae families (Table 5).

The interaction between treatment and time was significant only for Braconidae (Table 5).

Table 5. Repeated measure ANOVA results for each parasitoid family of vineyard 2 in 2018. Effect: T (treatment), D (date=time), T x D (treatment x date=treatment x time). p in bold shows a significant effect.

Effect	Mymaridae			Encyrtidae			Ichneumonidae			Braconidae		
	df	F	p	df	F	p	df	F	p	df	F	p
T	1	0,55	0,48	1	0,05	0,84	1	0,58	0,47	1	0,20	0,67
D	3	13,60	**<0,001**	3	95,37	**<0,001**	3	0,39	0,76	3	3,76	**0,02**
T x D	3	1,04	0,39	3	1,70	0,19	3	0,39	0,76	3	3,13	**0,04**

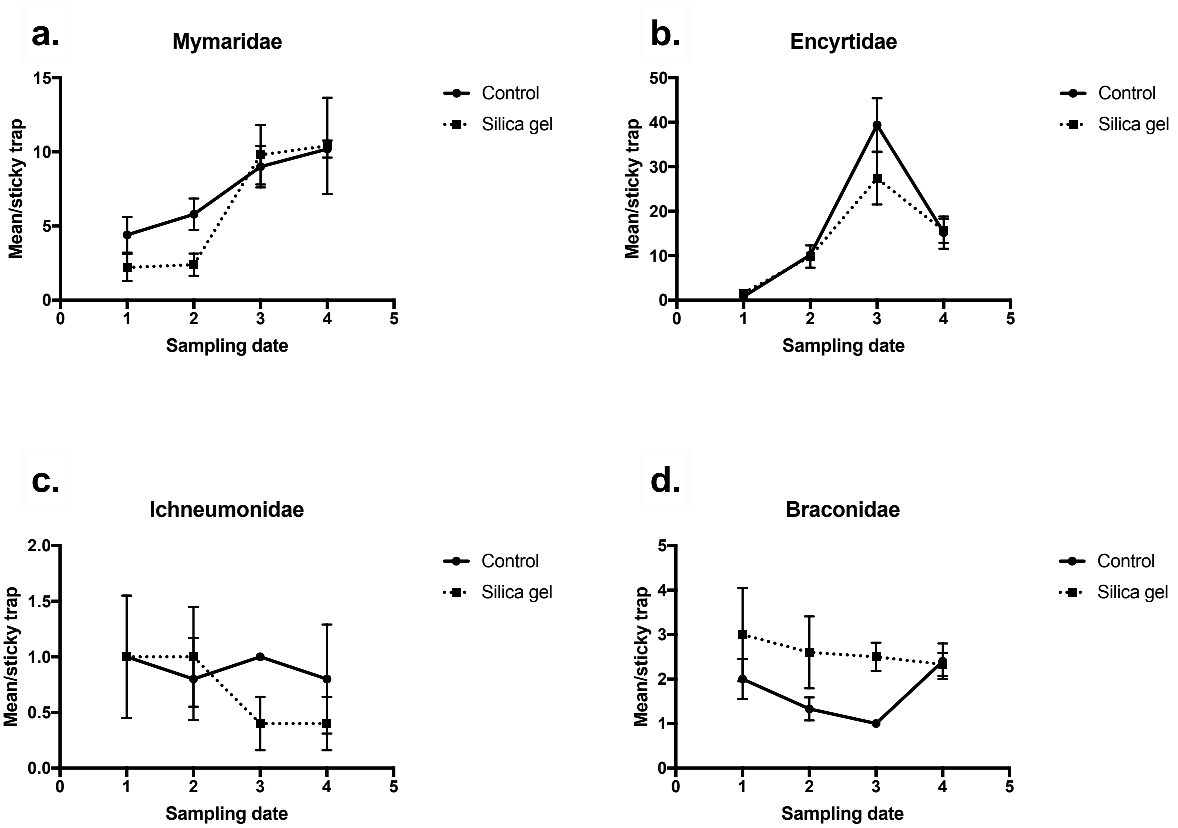

Figure 6. Mean number of parasitoids per sticky trap (± SE) of Mymaridae (a), Encyrtidae (b), Ichneumonidae (c) and Braconidae (d) families in each sampling date of vineyard 2. Braconidae: T x D (p<0,05).

5.4.1.3 Vineyard 3

On the traditional variety, silica gel treated area attracted a significant higher number of Braconidae parasitoids than control (Table 6), especially in the second date (Figure 7d). The significant attraction of silica gel towards Braconidae can be evinced by the main effect; indeed, there were 0,90 ± 0,17 (SE) Braconidae in silica gel treatment, whereas only 0,45 ± 0,15 (SE) Braconidae in control treatment.

Silica gel treatment showed a marginal effect (p=0,08), close to the significance level, towards Encyrtidae individuals (Figure 7b, Table 6); a significant "time x treatment" effect was also detected on this important family (Table 6), explained by the progressive increase of captures during the time in the plot treated with this elicitor. The marginal effect of silica gel towards Encyrtidae can be also evinced by the main effect; indeed, there were 2,60 ± 0,94 (SE)

Encyrtidae in silica gel treatment, whereas only 1,65 ± 0,22 (SE) Encyrtidae in control treatment.

Statistical analysis on Ichneumonidae family was not performed due to very low captures (Figure 7c). No significant attraction by silica gel was observed on Mymaridae (Figure 7a), though time displayed a significant effect on this family (Table 6), as well as on Encyrtidae (Table 6).

Table 6. Repeated measure ANOVA results for each parasitoid family of vineyard 3 Pignoletto variety in 2018. Effect: T (treatment), D (date=time), T x D (treatment x date=treatment x time). p in bold shows a significant effect. – indicates that statistical analysis was not performed due to low captures.

Effect	Mymaridae			Encyrtidae			Ichneumonidae			Braconidae		
	df	F	p	df	F	p	df	F	p	df	F	p
T	1	1,74	0,22	1	4,03	0,08	-	-	-	1	14,91	**0,005**
D	3	16,53	**<0,001**	3	4,98	**0,008**	-	-	-	3	0,53	0,66
T x D	3	0,27	0,85	3	2,99	**0,05**	-	-	-	3	0,22	0,88

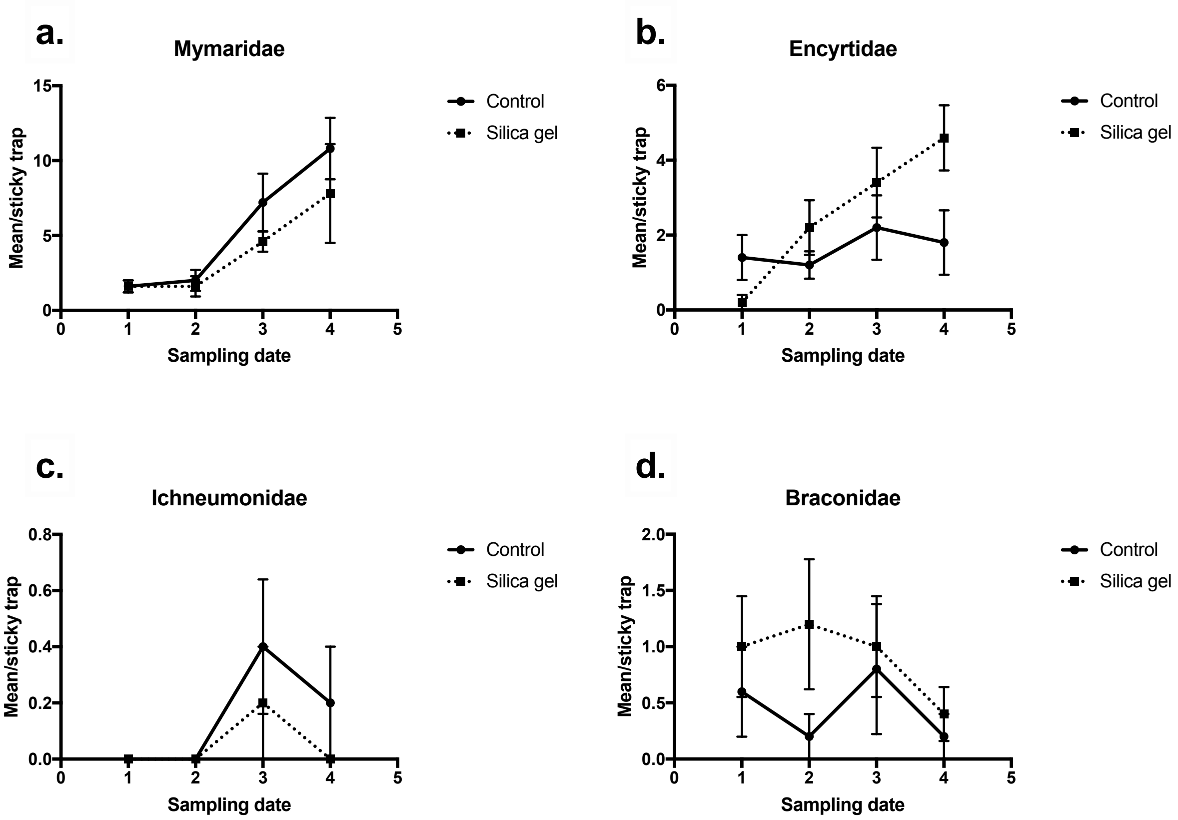

Figure 7. Mean number of parasitoids per sticky trap (± SE) of Mymaridae (a), Encyrtidae (b), Ichneumonidae (c) and Braconidae (d) families in each sampling date of vineyard 3 Pignoletto variety. Encyrtidae: T (p=0,08); T x D (p=0,05). Braconidae: T (p<0,01).

On the Alionza variety, no significant difference was detected between silica gel and control on all Hymenoptera families, as well as no silica gel x time significant effect (Figure 8, Table 7). Time displayed a significant effect on Encyrtidae and Mymaridae parasitoids (Table 7).

Table 7. Repeated measure ANOVA results for each parasitoid family of vineyard 3 Alionza variety in 2018. Effect: T (treatment), D (date=time), T x D (treatment x date=treatment x time). p in bold shows a significant effect.

Effect	Mymaridae			Encyrtidae			Ichneumonidae			Braconidae		
	df	F	p	df	F	p	df	F	p	df	F	p
T	1	1,04	0,35	1	0,20	0,67	1	2,14	0,19	1	3,10	0,13
D	3	10,13	**<0,001**	3	3,65	**0,03**	3	0,20	0,90	3	0,60	0,62
T x D	3	1,73	0,20	3	2,41	0,10	3	0,76	0,53	3	1,60	0,22

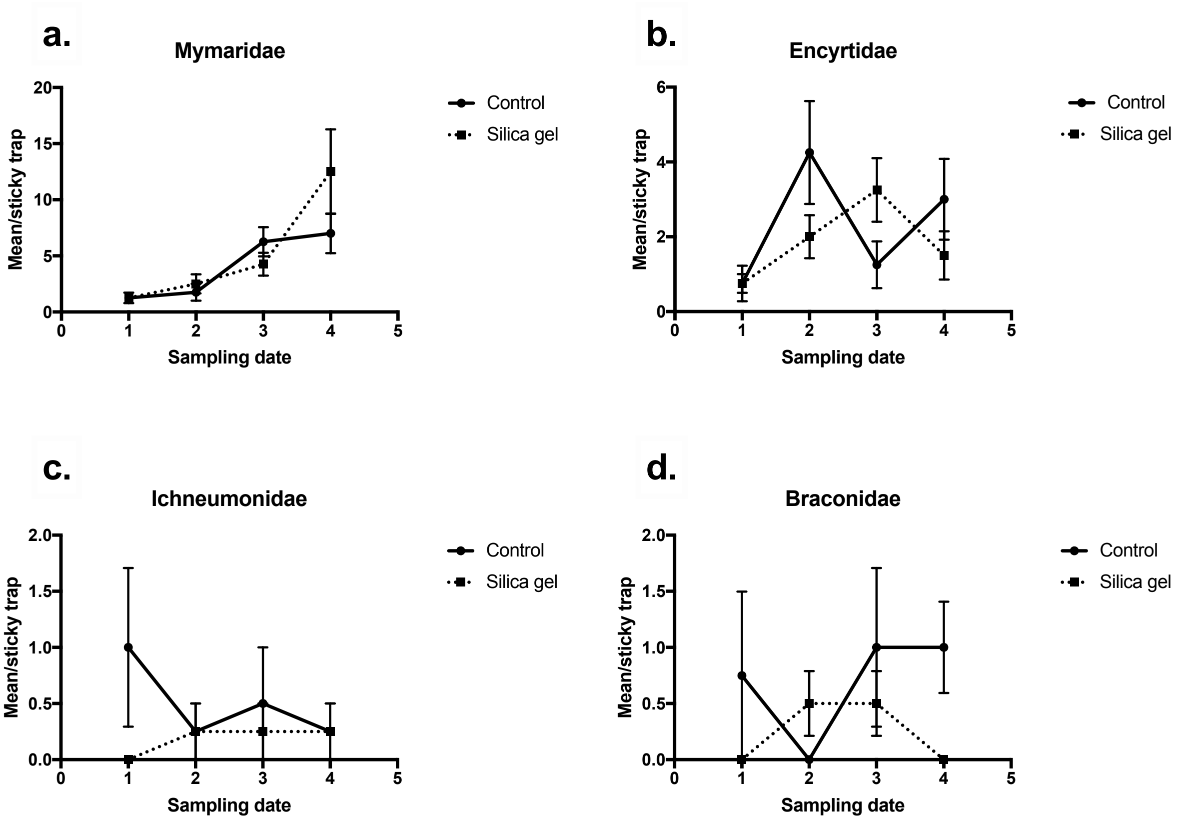

Figure 8. Mean number of parasitoids per sticky trap (± SE) of Mymaridae (a), Encyrtidae (b), Ichneumonidae (c) and Braconidae (d) families in each sampling date of vineyard 3 Alionza variety.

5.4.2 Year 2019

A total of 4213 Hymenoptera parasitoids were collected in 2019. Figure 9 shows parasitoid families identified in each field site. As in 2018, Mymaridae and Encyrtidae, represented the most collected families. Indeed, they assume a critical role in controlling the most damaging vineyard pests, such as mealybugs and leafhoppers.

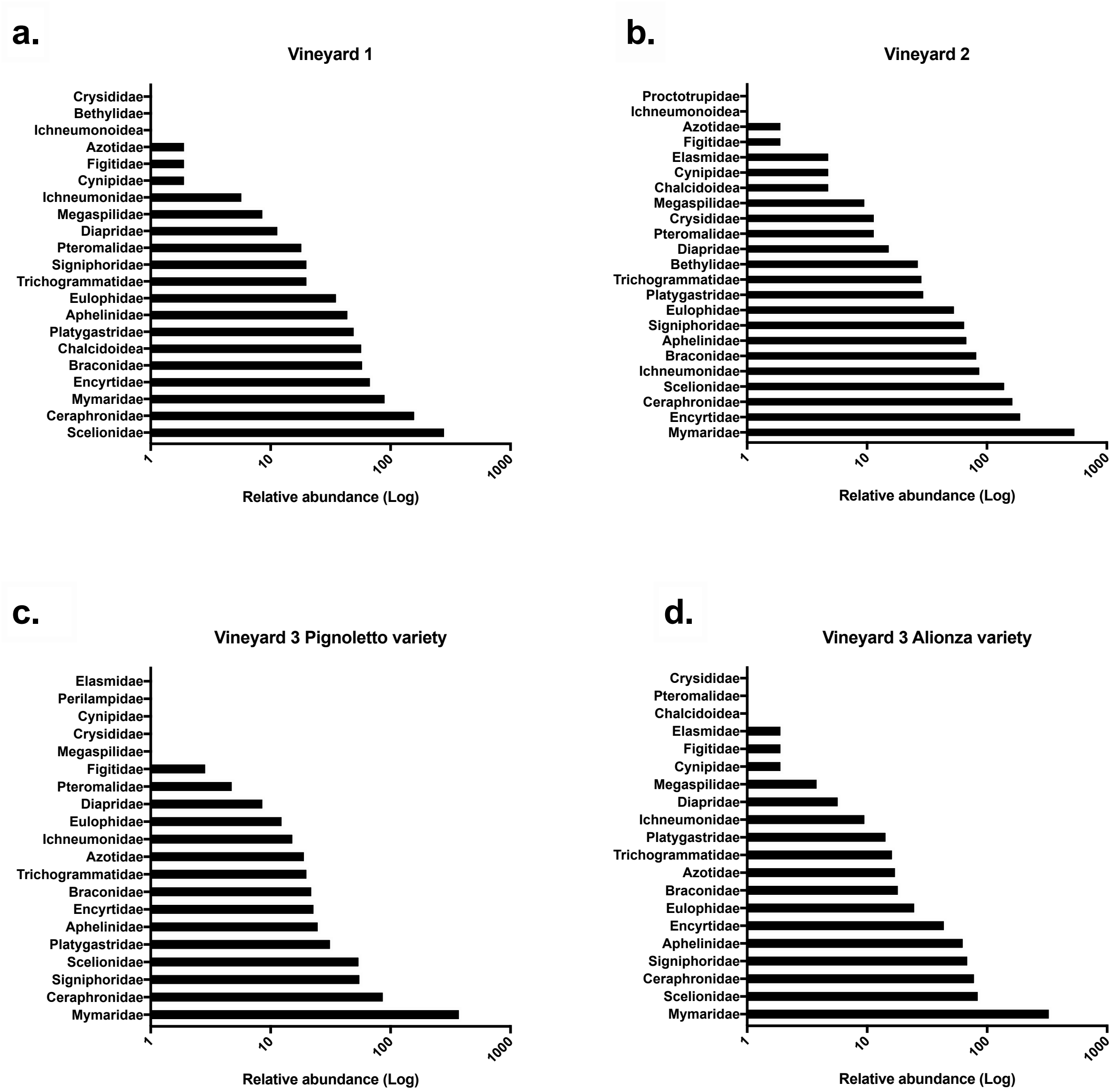

Figure 9. Relative abundance of Hymenoptera parasitoid families collected in each vineyard in 2019.

5.4.2.1 Vineyard 1

Figure 10 shows the mean captures of the four most representative families in vineyard 1. Silica gel treated area did not display any significant effect on both Chalcidoidea and Braconidae (Table 8). There was a significant effect of time on Braconidae captures (Table 8), whereas time showed a marginal effect on Encyrtidae (Table 8).

Statistical analysis on Ichneumonidae family was not carried out due to very low captures.

Table 8. Repeated measure ANOVA results for each parasitoid family of vineyard 1 in 2019. Effect: T (treatment), D (date=time), T x D (treatment x date=treatment x time). p in bold shows a significant effect. – indicates that statistical analysis was not performed due to low captures.

Effect	Mymaridae			Encyrtidae			Ichneumonidae			Braconidae		
	df	F	p	df	F	p	df	F	p	df	F	p
T	1	0,85	0,38	1	2,02	0,19	-	-	-	1	0,009	0,93
D	3	0,61	0,62	3	2,47	0,09	-	-	-	3	7,64	**<0,001**
T x D	3	0,25	0,86	3	1,82	0,17	-	-	-	3	0,87	0,47

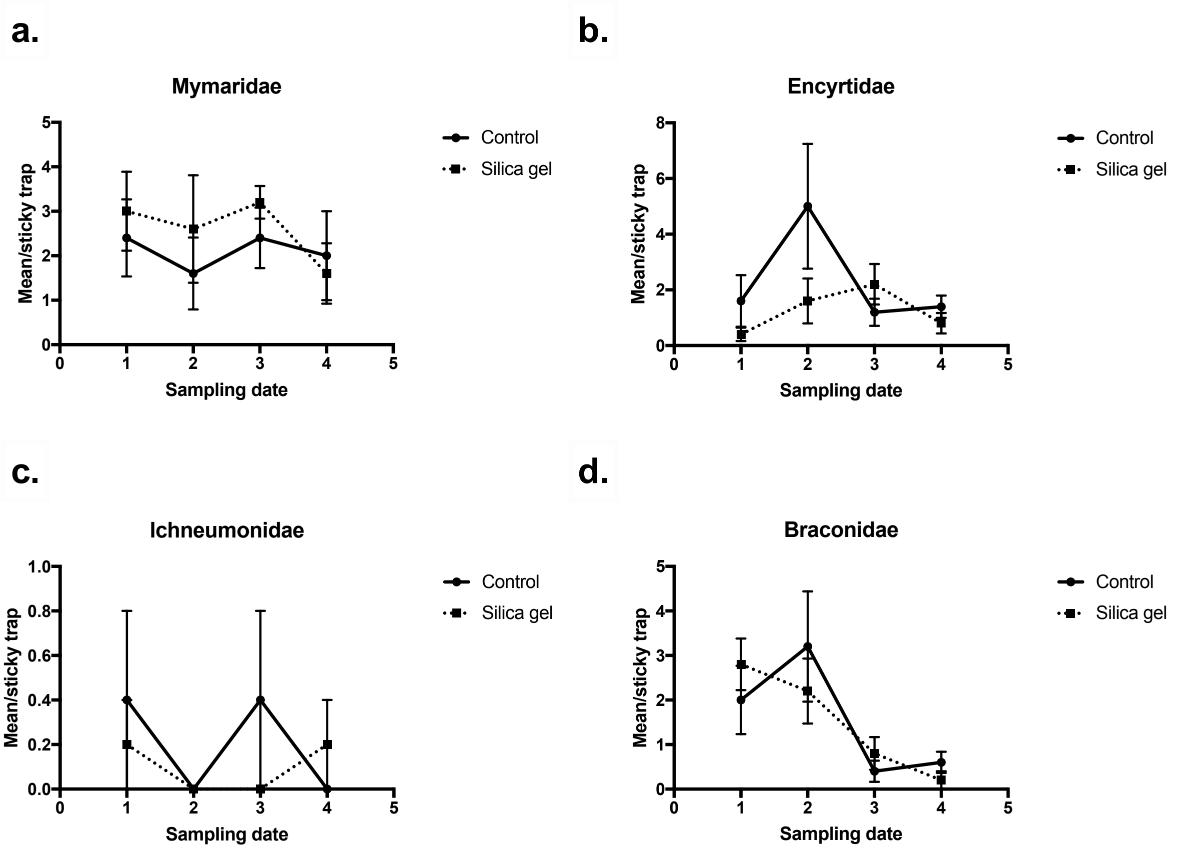

Figure 10. Mean number of parasitoids per sticky trap (± SE) of Mymaridae (a), Encyrtidae (b), Ichneumonidae (c) and Braconidae (d) families in each sampling date of vineyard 1.

5.4.2.2 Vineyard 2

Silica gel treated plants showed a significant attraction towards Encyrtidae and Mymaridae in vineyard 2 (Table 9). Mymaridae captures were higher than control in all sampling dates, especially in the first and second one (Figure 11a), whereas the number of Encyrtidae captured were similar in the first two sampling dates and became higher in silica gel compared to control in the last two dates (Figure 11b). In particular, Encyrtidae showed an exponential increase of capture in the consecutive samplings. Also time and the interaction between time and silica gel displayed a significant effect on these Chalcidoidea families (Table 9). The significant attraction of silica gel towards Mymaridae and Encyrtidae can be evinced by the main effect. Indeed, there were 18,65 ± 4,97 (SE) Mymaridae in silica gel treatment, whereas only 9,70 ± 0,52 (SE) Mymaridae in control treatment. There were 6,45 ± 3,12 (SE) Encyrtidae in silica gel treatment, while only 3,55 ± 1,36 (SE) in control treatment.

There was a significant effect of time on Ichneumonidae and Braconidae, whereas no significant increase of captures in silica gel treatment compared to control occurred (Figure 11c and 11d, Table 9).

Table 9. Repeated measure ANOVA results for each parasitoid family of vineyard 2 in 2019. Effect: T (treatment), D (date=time), T x D (treatment x date=treatment x time). p in bold shows a significant effect.

Effect	Mymaridae			Encyrtidae			Ichneumonidae			Braconidae		
	df	F	p	df	F	p	df	F	p	df	F	p
T	1	5,88	**0,04**	1	6,57	**0,03**	1	0,13	0,73	1	0,44	0,53
D	3	5,43	**0,005**	3	13,27	**<0,001**	3	15,46	**<0,001**	3	6,38	**0,002**
T x D	3	3,30	**0,04**	3	3,26	**0,04**	3	0,34	0,80	3	0,16	0,92

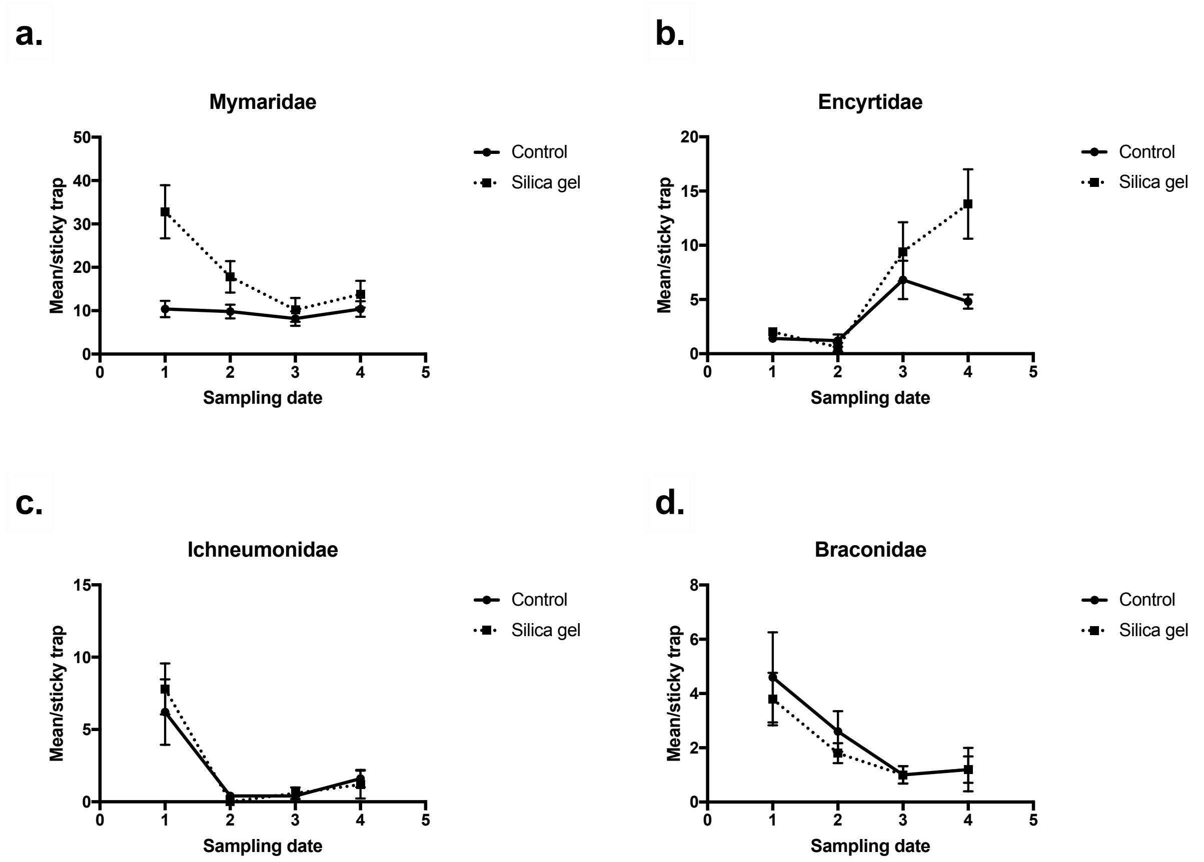

Figure 11. Mean number of parasitoids per sticky trap (± SE) of Mymaridae (a), Encyrtidae (b), Ichneumonidae (c) and Braconidae (d) families in each sampling date of vineyard 2. Mymaridae: T ($p<0,05$); T x D ($p<0,05$). Encyrtidae: T ($p<0,05$); T x D ($p<0,05$).

5.4.2.3 Vineyard 3

Silica gel treated area did not display any effect on the collection of Hymenoptera parasitoids in Pignoletto variety (Figure 12, Table 10). Nevertheless, there was a significant effect of time on Mymaridae, Encyrtidae, and Braconidae and a marginal effect on Ichneumonidae (Table 10). Finally, the interaction between silica gel and time was not significant for all parasitoid families (Table 10).

Table 10. Repeated measure ANOVA results for each parasitoid family of vineyard 3 Pignoletto variety in 2019. Effect: T (treatment), D (date=time), T x D (treatment x date=treatment x time). p in bold shows a significant effect.

Effect	Mymaridae			Encyrtidae			Ichneumonidae			Braconidae		
	df	F	p	df	F	p	df	F	p	df	F	p
T	1	0,02	0,90	1	0,07	0,79	1	0,38	0,55	1	0,02	0,88
D	3	21,36	**<0,001**	3	7,54	**0,001**	3	2,83	0,06	3	7,91	**<0,001**
T x D	3	1,86	0,16	3	1,03	0,40	3	0,34	0,80	3	0,74	0,54

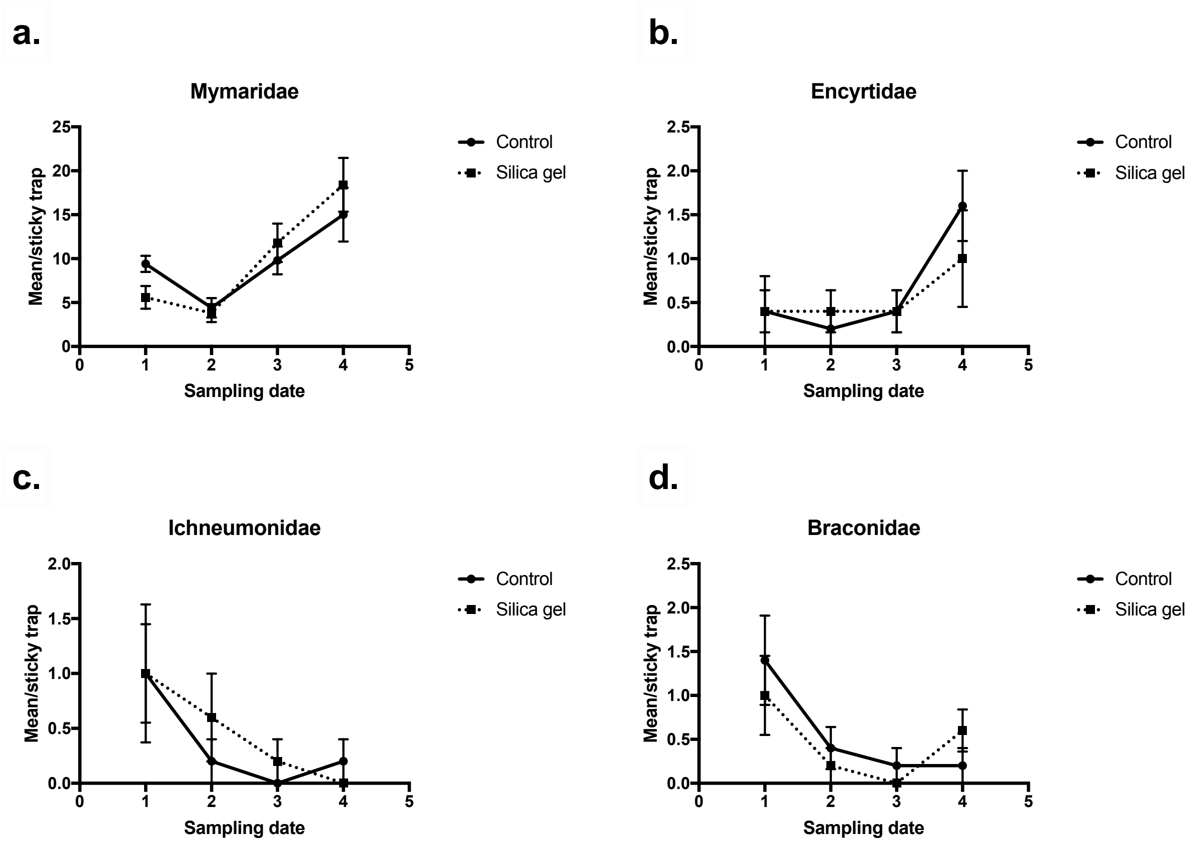

Figure 12. Chalcidoidea and Ichneumonoidea mean per sticky trap (±SE) in Pignoletto variety of vineyard 3.

On Alionza variety, both silica gel and time did not display any significant effect on parasitoid captures, apart from Mymaridae, on which time had a significant effect (Figure 13, Table 11). It was not possible to perform statistical analysis on Ichneumonidae family due to very low captures.

Table 11. Repeated measure ANOVA results for each parasitoid family of vineyard 3 Alionza variety in 2019. Effect: T (treatment), D (date=time), T x D (treatment x date=treatment x time). p in bold shows a significant effect. – indicates that statistical analysis was not performed due to low captures.

Effect	Mymaridae			Encyrtidae			Ichneumonidae			Braconidae		
	df	F	p	df	F	p	df	F	p	df	F	p
T	1	0,93	0,37	1	0,90	0,38	-	-	-	1	0,05	0,82
D	3	6,39	**0,004**	3	1,96	0,16	-	-	-	3	0,19	0,90
T x D	3	0,36	0,78	3	0,08	0,97	-	-	-	3	0,61	0,62

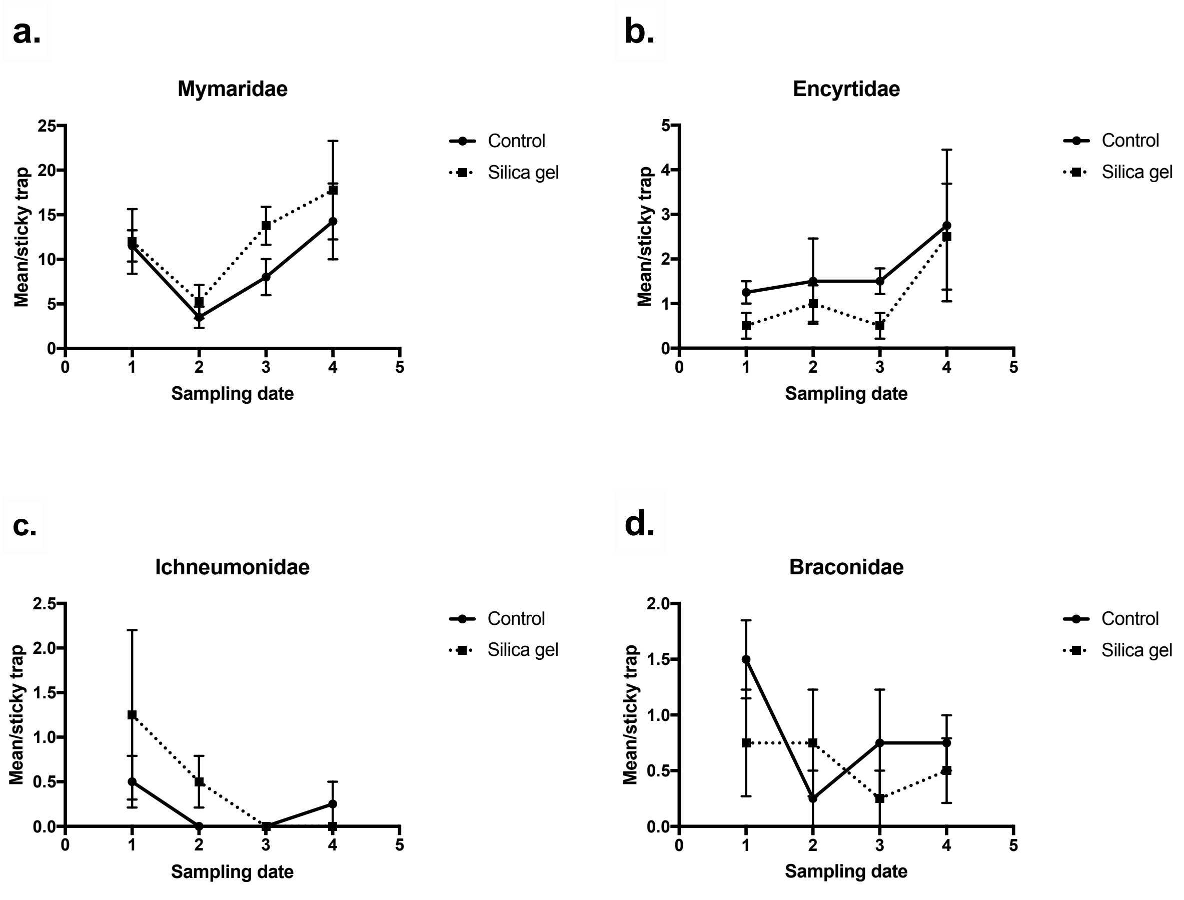

Figure 13. Chalcidoidea and Ichneumonoidea mean per sticky trap (±SE) in Alionza variety of vineyard 3.

5.5 Discussion and conclusion

Plants treated with silica gel elicited a significant attraction towards parasitoids in three cases; in particular, silica gel attracted Encyrtidae, Mymaridae and Braconidae. Moreover, a significant time x treatment was detected in four cases. On the other hand, control increased attraction of parasitoids only in one case.

In 2018, the field study showed a significant attraction of silica gel treated plots towards Braconidae in Pignoletto variety of vineyard 3, whereas more Encyrtidae and Mymaridae parasitoids were collected in silica gel compared to control in vineyard 2 in 2019.

A high variability was recorded among vineyards and years, but data confirm results of previous studies. Indeed, the significant attraction towards Mymaridae family in vineyard 2 in 2019 corroborates Parrilli et al. (2019) result, where a higher number of leafhopper parasitoids in silica gel treatment compared to untreated plants was detected. Moreover, Parrilli et al. (2019) showed that downy mildew infection stimulated JA production in silica gel treated plants, especially on the first and third day after pathogen inoculation. *Plasmopara viticola* infection was detected in out test in vineyard 2 on 14th of June 2019, exactly during the week of the first sampling (12th-19th of June), in which Mymaridae captures were significantly higher in silica gel treatment compared to control.

A significant higher attraction of Encyrtidae in 2019 occurred also in 2018, when a silicon marginal effect (p=0,08) was detected in Pignoletto variety of vineyard 3. In vineyard 2 in 2019, no downy mildew infection was observed during the sampling in which a significant higher attraction of Encyrtidae was detected. Parrilli et al. (2019) highlighted that a high level of JA production was also detected twenty days after silica gel treatment and before *P. viticola* infection. The second silica gel treatment was carried out on 16th of July 2019. A significant higher attraction of Encyrtidae was observed in the fourth sampling (1st-9th of August), exactly 20 days after silica gel treatment.

Only in vineyard 1 in 2018, a higher number of Mymaridae was collected in control treatment compared to silicon treated plants, although the capture increase was evident just in the second sampling. The opposite response of Mymaridae towards silica gel in vineyard 1 in 2018 and in vineyard 2 in 2019 and the significant time x silica gel interactions may be attributed to a taxonomic artifact. Insect family constitutes a complex of several species, which vary in their phenologies and may have different responses to lures (Kaplan, 2012). Consequently, dissimilarities in silica gel attraction towards Mymaridae may be justified by the presence of different species complex in vineyard 1 and 2. Pests, potential hosts for parasitoid, were not observed during our trial, but the higher Mymaridae captures in control plot than in silica gel one may be explained by different levels of leafhopper infestation between the two treatments. Probably, a scarcer level of parasitoid hosts in silica gel than in control plot led to few Mymaridae captures in treated plants. This interpretation may also justify the few Ichneumonidae and Braconidae captures in the same two years in most of the vineyards. These two families are important parasitoids of *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae), which is one of the most important vineyard pests in Italy. The absence of European grapevine moth infestation may explain the lack of beneficial presence in these fields. Kaplan (2012) highlighted that the indiscriminate use of lures, such as deploying volatiles in pest-free crop, can lead natural enemies to ignore or even perceive HIPVs as repellents. Moreover, background odors can camouflage hosts and plant volatiles and may hinder parasitoid foraging activity (Dicke, 2009; Kaplan, 2012). Only vineyard 3 presented significantly more Braconidae individuals in silica gel treatment compared to control in Pignoletto variety in 2018, even though the captures were very low.

Non-attractive sticky traps used in this study can be considered an effective trapping method to sample parasitoid taxa, as demonstrated in the experiments carried out in Australia (Simpson et al., 2011a; Simpson et al., 2011b).

Studies demonstrated that size of the insects captured by sticky traps was significantly smaller than other kind of sampling, such as glass-barrier, Malaise traps and rotary (Juillet, 1963); this may further explain the low response of Ichneumonoidea superfamily to inductors in our trials.

Finally, the greater presence of local vegetation compared to higher spatial scale resources may have influenced the type of Hymenoptera parasitoids captured. Indeed, larger individuals, such as Ichneumonoidea, need vaster undisturbed non-crop areas to be retained in agricultural areas (Parrilli et al., 2019; Smith et al., 2015; Thomson & Hoffmann, 2010).

Finally, no difference was observed between traditional and old vine varieties in terms of Hymenoptera parasitoid captures. RNA-seq analysis is still ongoing to detect which genes are involved in defence response of these two vine varieties (PSR project Viteambiente).

In our field experiment, silica gel showed a great potentiality in increasing plant defence and in boosting natural enemy recruitment as previous studies on silicon-derived compounds demonstrated (Alvarenga et al., 2017; Assis et al., 2015; Kvedaras et al., 2010). To further strengthen elicitor/plant volatile research field, the "attract and reward" approach should be adopted. Flower strips or companion plants together with attractants would represent the proper strategy to lure natural enemies and promote their retention in crop systems (Rodriguez-Saona et al., 2012; Simpson et al., 2011a). Deploying volatiles in field areas where preys/hosts might be absent may ruin rather than enhance conservation biological control. Consequently, some researchers, such as Furlong et al. (2018), suggested to use volatiles or HIPVs-eliciting compounds as retention tool to supplement inoculative or inundative biological control.

Apart from testing other promising elicitors (Coppola et al., 2017; Sobhy et al., 2014), it would be interesting to investigate the potential attraction of silica gel towards natural enemies deployed for inoculative releases in vineyards. For example, *Anagyrus vladimiri* Triapitsyn (Hymenoptera: Encyrtidae) and *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) performance may benefit from volatiles released by silica gel treated plants, which may increase the attraction of these mealybug natural enemies. Beneficial responses to volatiles are extremely specific and vary depending on plant and herbivore species (Dicke, 2009; Heil, 2008), thus it would be necessary to test this silicon-derived compound in defined natural enemy-plant context.

The increasing interest in chemical ecology and the positive results that have been shown by recent studies on this topic make elicitors a promising tool that requires further investigations, especially in the field. The potential to integrate resistance inductors with other biological control approaches represents an innovative way to implement sustainable and ecological plant defense strategies.

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Chapter 6 Summarising discussion and conclusion

The goal of this thesis was to investigate new sustainable control strategies in vineyard ecosystem.

In chapter 2, the combined release of *A. vladmiri* and *C. montrouzieri* was tested to suppress *P. ficus* and *P. comstocki* populations. My data showed that the augmentation technique was efficient in controlling mealybug infestation. In particular, the predator demonstrated a huge potential in suppressing both mealybug species, especially in highly infested vineyards.

Both Pseudococcidae species were parasitized by the Encyrtid wasp, even though the parasitoid showed a higher efficacy on *P. ficus*. Further studies should examine more in depth *A. vladimiri* - *P. comstocki* association and optimise the timing of parasitoid and predator releases to increase augmentation efficacy.

Chapter 3 focused on the trophobiosis between attending ants and mealybugs, which is often responsible for the variability of augmentation efficacy, and tested a control method to reduce this strong association. Deploying sugar dispensers to manage ant attendance proved to be very effective in reducing ant activity and mealybug infestation as well as enhancing ecosystem services in vineyard. The regular use of this strategy would increase the efficacy of inoculative releases against mealybugs and could also foster augmentation approach among farmers. More efficient releasing method of sucrose solution should be elaborated and tested in future trials.

In chapter 4, the maintainance of spontaneous grass vegetation was investigated as a habitat management strategy to reduce the population of the first generation of *L. botrana* and increase EGVM parasitization. Results showed that conservative ground cover management did not significantly reduce *L. botrana* infestation and did not enhance the parasitization of this moth. Conversely, Shannon-Weaver index showed an increase of parasitoid diversity in non-mowed interrow plot compared to intensive ground cover management. Future trials should examine more focused habitat management strategies, such as a mixture of sown flower resources, to boost the control of *L. botrana* first generation.

In chapter 5, the use of elicitors as new biotechnic control strategy was investigated. In particular, silicon-derived compound was tested to increase parasitoids' population. Results showed that silica gel significantly boosted the attraction of Mymaridae and Encyrtidae, two of the most important Hymenoptera parasitoids of vineyard ecosystem.

Altogether, the result of this thesis highlight the great potential of these new sustainable control strategies in vineyard ecosystem. Although it is well known that pesticides lead to the development of resistance in the targeted insect pests and cause severe environmental and human health problems, sustainable control approaches are still undervalued. Lack of knowledge, scarce confidence in effectiveness of ecological methods and communication gap among stakeholders hinder the adoption of sustainable control strategies (Lucchi & Benelli, 2018).

Researchers together with consultants of plant defense extension services should cooperate to foster the dissemination of good integrated control practices. The use of chemicals often represents the first control method chosen by farmers (Dara, 2019) due to the common belief of insecticides to be more effective and cheaper. Benefits from biological control approaches are usually visible after multi-year adoption, whereas farmers and consultants are used to perceive and detect pest suppression immediately by using chemical control. Even if sustainable and ecological strategies may initially require more economic resources, they can provide efficient pest control and turn out to be cheaper than insecticides after 2-3 years of application. An example is BIOCONVITO project, in which biological control costs were extremely lower compared to conventional insecticides after 2 years of natural enemy releases against mealybugs. Indeed, augmentation became unnecessary in the third year since beneficials settled autonomously and reproduced in the meantime, increasing their population (Lucchi et al., 2018).

Cooperation among stakeholders is fundamental to spread knowledge, promote advances in crop protection and increase awareness among growers about pest management options, which do not always have to rely on pesticides. The adoption and the integration of eco-friendly control methods, such as those presented in my thesis, may provide a satisfactory control against vineyard pests by means of their synergistic and complementary effect. However, to guarantee the success of such approaches, accurate knowledge of pest-natural enemy-vineyard system, pest monitoring and precise treatment timing are required.

"Attract and Reward" represents a good example of strategy complementarity, in which elicitors and habitat management are used together to enhance biological control. Moreover, resistance inductors should also be used with natural enemy augmentation to boost the permanence of released beneficials in vineyard. Finally, sugar dispensers as ant distraction from mealybug honeydew constitutes a sustainable strategy which exploits multitrophic relation phenomenon and complements released natural enemies, increasing their activity and so the control on the pest.

To conclude, the combination of environmentally friendly practices may really reduce and even avoid insecticide treatments in some vineyard context, providing a successful suppression of targeted pests.

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