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STUDY ON THE EFFECT OF CALCIUM CARBONATE NANOPARTICLES AS A NEW STRATEGY TO CONTROL THE OLIVE FRUIT FLY *Bactrocera oleae* (Rossi) IN OLIVE ORCHARDS

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STUDY ON THE EFFECT OF CALCIUM CARBONATE NANOPARTICLES AS A NEW STRATEGY TO CONTROL THE OLIVE FRUIT FLY *Bactrocera oleae* (Rossi) IN OLIVE ORCHARDS

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Dedication

Every challenging work needs self efforts as well as guidance of persons especially those who were very close to our heart.
My humble effort I dedicate to my wounded country IRAQ and to my beloved family

My parents & brothers

The warmth of their support was strongly felt. Their love, encouragement and prayers of day and night make me able to get such success and be the best to reach my dreams!

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Summary

Nanotechnology has received significant attention in crop protection over the last decade, in the form of nanoparticles. This research aimed at studying the effects of application of Calcium carbonate nanoparticles $CaCO_3$ NPs on the control of the olive fruit fly *Bactrocera oleae* in the field, and on the development of all immature stages of the target insect in the laboratory. Similarly, another formula; colloidal calcium CaCol was tested for comparison purposes. The field experiment was carried out in an olive orchard in Viterbo province (Lazio-Italy), yellow traps were used for pest monitoring at the onset of the invasion, and the number of captured adults was evaluated. Infestation level was assessed based on the number of insects detected in field-collected fruits. The orchard was divided into three blocks that were subjected to the following treatments: (A) tap water as a control; (B) CaCol; (C) $CaCO_3$ NPs. Additionally, in another part of the orchard, some olive branches were covered before the presence of the pest and were treated intensively with the above-mentioned suspensions. Thereafter, treated fruits were collected randomly and incubated to evaluate the effect of applied materials on insect development. The monitoring results showed that the highest peak of the fly was on October 16th and the optimal temperature for the fly vitality was (21±5 C°). Infestation level reached the higher number of detected individuals on 26th of September. The field tests have shown that Calcium carbonate NPs interacts negatively with the fly emergence better than colloidal calcium, by averting the insect development during early life stages. Laboratory bioassays were conducted in order to ascertain the results obtained in the field. *B. oleae* were reared from infested olives collected in different orchards. Afterwards, three cages were established containing an equal number of healthy olive fruits and each cage was provided with the same number of *B. oleae*. Each cage received a different treatment; $CaCO_3$ NPs, CaCol and tap water (control) respectively. Adult emergence per treatment was recorded after 21 days and statistically analyzed. The results showed that insects exhibit similar lifespan, even though treated with different materials, and no insect mortality was observed. There was a reduction in *B. oleae* adult's emergence using $CaCO_3$ NPs, however, no significant effect was observed when comparing Calcium carbonate suspensions with control. The ultimate objective of this research was to study the effect of the used materials on the viability of *B. oleae*, female's productivity, as well as on the interaction between *B. oleae* and its bacterial endosymbiont

Summary

"*Candidatus* Erwinia dacicola". Several studies have documented that this bacterium plays an important role in the *B. oleae* life affecting its fitness, larval development, oviposition and mating behavior. To this end, *B. oleae* rearing was carried out under laboratory conditions and three trials were performed and replicated three times. Treatments were applied by exposing the olive fruit flies in each cage to a different component ($CaCO_3$ NPs, CaCol and tap water) mixed with artificial diets. Thereafter, equal numbers of treated flies were collected from each cage at different intervals; after 1, 2 and 3 weeks of treatment, then killed and used for *Ca*. E. dacicola DNA extraction. Detection and quantification of the bacterial DNA in insect samples was carried out by qPCR targeting the 16S rRNA gene, with EdF1 and EdEn primers in order to quantify the abundance of the bacterial endosymbiont associated with *B. oleae*, based on the number of cycles (Ct) values. The findings indicate that the two formulas of Calcium carbonate incorporated into the insect diets, reduced egg production in female flies compared to control, and that $CaCO_3$ NPs reduced significantly egg production than CaCol. The qPCR analyses revealed that no significant effect was observed on the bacterial abundance in treated flies, and insect samples exposed to Calcium carbonate NPs and colloidal calcium illustrated approximately an equal amount of bacterial DNA compared to control throughout the trial period. Overall, our results encourage further research on Nano-calcium carbonate, in order to use this inorganic nanomaterial for the control of the olive fruit fly in the framework of an integrated pest management program.

Keywords: *Olea europea*, *Tephritidae, Dacinae*, nanotechnology, pest control, colloidal calcium, symbiosis, *Erwinia dacicola.*

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I. LITERATURE REVIEW

I.1 History and distribution of the olive

Olive (*Olea europaea L.*, *Oleaceae*) is among the oldest known cultivated plant species in the world. It was domesticated between 8,000 and 6,000 years ago in the eastern Mediterranean from where it spread widely in Northern Africa, the Iberian Peninsula, and the rest of southern Europe by civilizations that successively occupied the Region. Its cultivation spread very early to all the Mediterranean countries and later in many other areas of the American continent suited to its cultivation. In more modern times, the olive growing has also been introduced in other countries without an earlier tradition of olive oil production or consumption. Nowadays, it is farmed in Southern Africa, Australia, Japan and China (Cimato and Attilio, 2011; Efe et al., 2011) (Figure I.1).

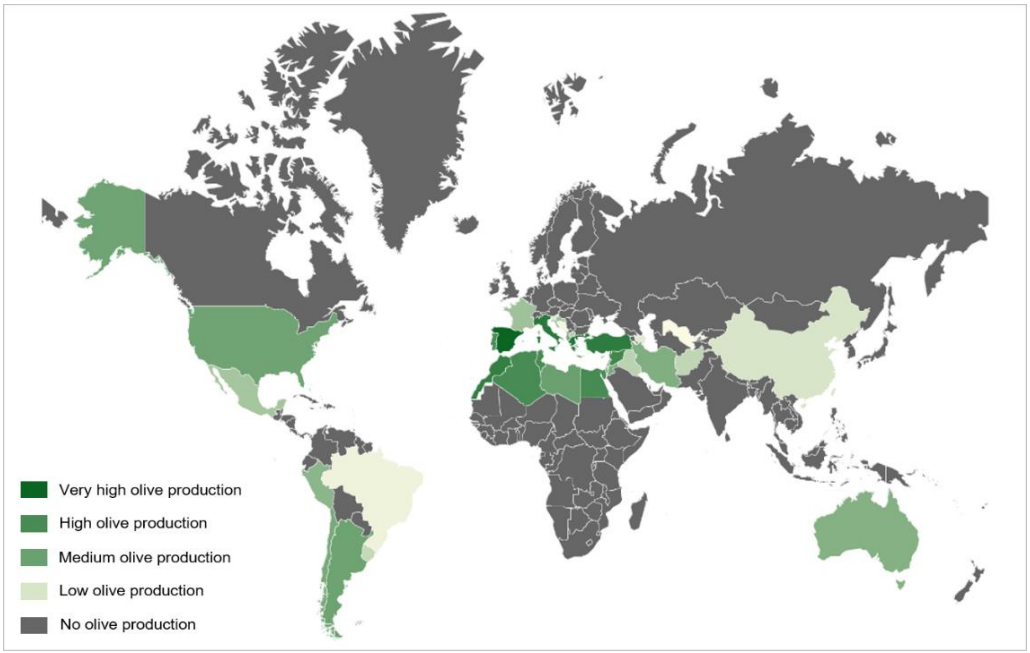

Figure I.1 World Olive producing countries.
Source: (Atlas Big, 2020)

I.2 Olive production and importance

The Olive tree is considered one of the most important long-lived fruit species in the Mediterranean area (Zohary et al., 2012). According to the International Olive Oil Council (IOC) data, over 10 million hectares are cultivated with olive groves globally, of which 95% are located in the Mediterranean basin (IOC, 2016). The global production of table olives was about 2.6 million tons, while the global yield of olive oil reached 3.13 million tons in 2018/19 season. Over 90% of the world's olive production is produced in the Mediterranean region with Spain, Greece, Italy, Turkey and Morocco as the top five producing countries (IOC, 2019).

The olive is a functional plant in the agricultural system of many countries and has acquired a huge socioeconomic importance. It is widely cultivated for the production of both table olives and oil and very significant because of its commercial value. Olives and olive oil, as traditional food products with thousands of years of history, are the essential components of the Mediterranean diet and are largely consumed in the world. Beside of their economical contribution to national economy, these are an important food in terms of their nutritional value (Uylaşer and Yildiz, 2014). The great commercial value of the olive and the recognition of olive oil dietetic properties have led to an increase in its production and consumption worldwide (Veloso et al., 2018).

Today, Italian olive production covers approximately 1.1 million ha with a production of 175,000 tons of olive oil delivered in the 2018/19 harvest season (FAOSTAT, 2018; IOC, 2019). In fact, olive growing is an important agricultural activity in Italy, generating 3 151 million euros in the olive oil sector, and accounting for 2.4% of the value of agri-food production. In terms of olive yields, The Apulia region is the leader and accounts for 45% of the total olive growing area, followed by Calabria (19%); Sicily (10%); Campania (7%), Lazio (5%), Tuscany and Sardinia (3% respectively), Basilicata and Umbria (2% respectively); with the rest of the surface area found in Molise, Liguria, Marche and Veneto (IOC, 2017). These areas have relatively warm temperatures, suitable for olive cultivation. In Italy, there are over 500 varieties of olive trees and more than 90% of oil production is obtained from 45 varieties (Caliskan, 2012; Fontanazza, 2005). Eighty percent of the production of olive oil is centered

around three regions: Apulia, Calabria and Sicily. These three regions account for more than 60 percent of Italian olive production (IOC, 2017).

I.3 Overview of the olive cultivation in Iraq

Iraq is an ancient agricultural country producing mainly wheat, barley, dates, rice, vegetables, and cotton. Although Iraq is now a minor producer of olives, the olive cultivation is beginning to grow and prosper recording production of 34,501 tons in 2019 and the average production rate of a tree in Iraq is 25.72 kg (CSO, 2019). Most of the producing areas are located in Northern Iraq, the vast majority of these groves containing indigenous olive table varieties such as Pa'shiqi and Ashrassi.

Unfortunately, in the past era, the Iraqi Ministry of Agriculture (MoA) has never considered olives as a strategic crop in the developmental plan for Iraqi Agriculture. However, since the 2000s, Iraq seems to have the potential to expand and greatly improve its production of olives for oil as well as table olives. Indeed, the Iraqi Ministry of Agriculture recognized the potential for olive oil production in Iraq, and several projects are underway with the main olive producing countries as new development patterns in agriculture in Iraq. The initiated programs aimed to establish nurseries to produce olive tree saplings. More recently, in 2005 and 2006, the Agricultural Reconstruction and Development Program for Iraq (ARDI) worked with the MoA to establish sixteen demonstration and research groves in eight governorates (Salah ad Din, Diyala, Wasit, Babylon, Qadissiyah, Muthanna, Dhi Qar and Basrah); Figure I.2 illustrates the olive growing localities throughout Iraq. Various species have been planted in these olive orchards, including local species as Arbequina, Manzanilla, and Picual (Harbi et al., 2012).

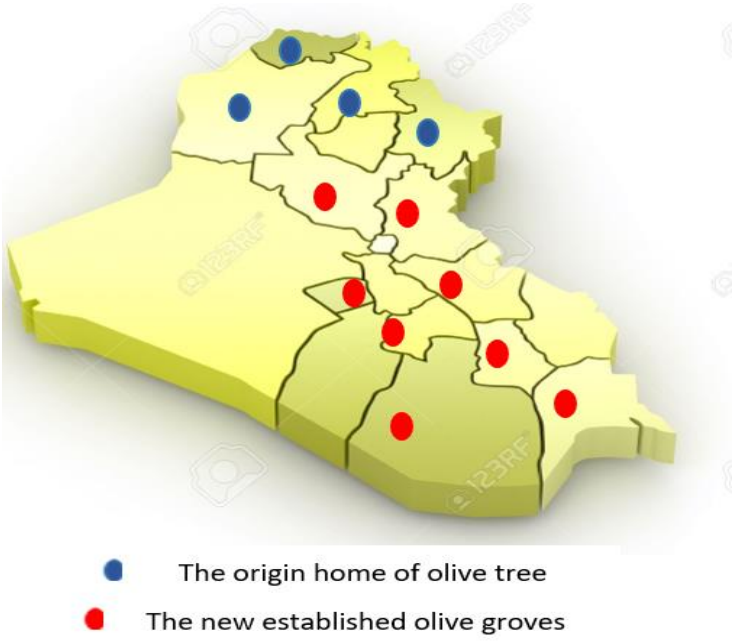

Figure I.2. Olive distribution in Iraq.

There are many reasons for choosing olive tree cultivation in Iraq including:

1- The olive tree is an evergreen tree and thus have an important role in improving the ecosystem in the region avoiding desertification.

2- Due to its economic importance, it contributes to the improvement of the country's economy and food security through the direct consumption of fruits or olive oil extraction, as well as its use in human nutrition and in certain industries such as soaps and cosmetics.

3- Olive is a major tree crop in the Mediterranean region and is moderately salt tolerant.

4- The climate in Iraq is generally suitable for olive cultivation success in terms of the temperatures required for flowering and fruit development stages.

I.3.1 Olive varieties in Iraq

Various varieties of olive are cultivated in Iraq, many of which are local, and others are imported from neighbouring countries such as Turkey, Jordan, Palestine, or imported from foreign countries such as Spain and Italy.

❖ **Local varieties**

The local olive cultivar Ashasi or ‘Ashrassi’ shown in Figure I.3.A, is considered to be one of the best cultivars in Iraq for preparation of table olives. Other local cultivars are Dahkan (Figure I.3.B) and Baskika or ‘Pa’shiqi’ (Garrido-Fernández et al., 1997).

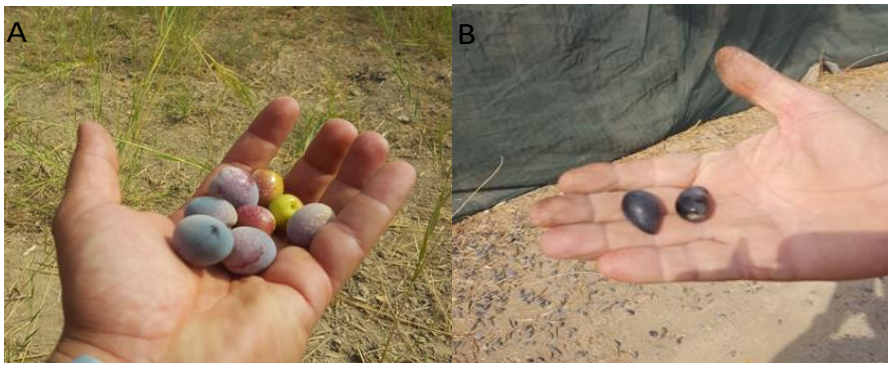

Figure I.3. Olive local cultivars. (A) Iraqi cultivar **Ashasi** and (B) Iraqi cultivar **Dahkan.**

❖ **Foreign varieties**

Despite the presence of local varieties, the need for new varieties with high production characteristics is relevant. For this purpose, many foreign varieties have been imported and cultivated for domestic production. The imported cultivars are (SO-RANI, KHODEIRI, KAISSY, SHAMI, JLOT) from Syria, (NABALI MUHASAN and K-18) from Palestine, (CHEMLALI) from Tunisia, (ARBEQUINA, MANZANILLO, GROSSA DI SPAGNA, GORDAL, PICUAL) from Spain, and (ASCOLANO, CORATINO, FRANTO-IO, SANTA CATRIN, GIARRAFFA) from Italy.

The cultivars were divided according to the purpose of use whether intended for pickling or oil extraction or for both oil and pickling (Harbi et al., 2012). The use of these olive cultivars based on oil content and other parameters are shown in the following (Table I).

Table I. Olive cultivars in Iraq, origin and other parameters (Harbi et al., 2012).

Co-de	Cultivar Name	Origin	Distribu-tion	Leaf shape	Fruit shape	Seed shape	% Oil cont.	Produ-ctivity	Salt tolerance	Pest resistance		
										Verticillium Wilt	Mites	Insects
1	Khodeiri	Syria	S,J,I	Spear	Oval	Sharp end with spine	26-28	High	Untolerant	Unknown	Unknown	Susceptible
2	Kaissy	Syria	S,J,I	Spear	Spherical	Oval with spine	18-21	High	Tolerant	Unknown	Unknown	Susceptible
3	Manzanilla	Spain	I,E,S,J,AS, AM,S	Spear to tapered ends	Oval to spherical	Oval smooth	16-20	High	Tolerant	Susceptible	Unknown	Susceptible
4	basskika	Local	North and middle of Iraq	Spear	Oval with end	Elongated with spine	19-21	High	Tolerant	Resistant	Susceptible	Susceptible
5	Arbequine	Spain	S,AS,S,I	Spear	Spherical	Small spherical smooth	17-22	High	Tolerant	Resistant	Susceptible	Unknown

Table I (continued). Olive cultivars in Iraq, origin and other parameters.

Co-de	Cultivar Name	Origin	Distribu-tion	Leaf shape	Fruit shape	Seed shape	% Oil cont.	Produ-ctivity	Salt tolerance	Pest resistance		
										Verticillium Wilt	Mites	Insects
6	Nabali	Jordania	P,J,I,S	Spear	Ova;	Oval to sharp ends	17-28	Mid	Tolerant	Resistant	Susceptible	Susceptible
7	JIot	Syria	S,I,J,P	Spear	Elongated oval	Elongated with spine	10-12	High	Tolerant	Susceptible	Susceptible	Susceptible
8	Dahkan	Local	North of Iraq	Spear to tapered ends	Large oval elongated	Elongated with spine	19-22	High	Tolerant	Resistant	Unknown	Susceptible
9	Sorani	Syria	S,I,J,P,L	Oval	Oval	Elongated	26-28	High	Tolerant	Susceptible	Unknown	Resistant
10	Shami	Syria	S,J,P,E,I	Spear	Spherical	Oval with spine	17-20	High	Tolerant	Unknown	Unknown	Susceptible

S: Syria, I: Iraq, J: Jordan, P: Palestine, L: Lebanon, E: Egypt, AM: America, AS: Australia, S: Spain.

I.3.2 The main olive pests and diseases in the country and their control

Keeping olive trees adequately watered and well-fed is the best initial defence against pests and diseases, as vigorous trees are more tolerant against pest's attack and less likely to suffer long-term damage ("Olive tree pests and diseases", 2014).

The situation of the country in the previous period like wars and crises, had led to the neglect of scientific research and pest control methods in the agricultural sector. Unfortunately, all of these conditions have led to the adoption of chemical methods as a major way of controlling pests and diseases.

The following section discusses the main pests and diseases that affect olives in Iraq.

I.3.2.1 Olive Pests

- **Olive–leaf midge, *Dasineura oleae* Angelini (Diptera: Cecidomyiidae)**

The damage is caused by the larval stage. Larvae feed on the leaves by sucking the nutrients, causing small galls, and therefore reduction in the yield. In addition, deformation and leaf curling may occur, as a result of larvae feeding on the leaves (Betta and Doganlar, 2020). For its control, chemical applications with Zolone 35%, (2 cm 3/L) from the first week of March are employed.

- **Olive Scale, *Parlatoria oleae* (Colvée) (Hemiptera : Diaspididae)**

The damage is caused by larvae and adult stages. All parts of the host plant, except the roots, are attacked by *Parlatoria oleae.* Infestations may cause purple discoloration of olives with losses resulting from this discoloration and distortion of the fruit, ("Olive scale", 2020). To control this pest, Dyazinon 60% (1.25 cm^3/L) is applied in March and June.

- **Olive Psyllid, *Euphyllura olivina* (Costa) (Hemiptera: Psyllidae)**

The damage is caused by nymphs and adult stages through feeding on buds, flowers, tender shoots, and small fruits; they produce a white, waxy secretion in spring which can cause premature flower drop, ("Olive Psyllid, *Euphyllura olivine*", n. d.). The most commonly used

insecticide to control this pest is Dyazinon 60% (1.25 cm^3/L), the treatment is recommended in March and April, also at the beginning of infestation and before flowering.

❑ **Olive Bud Mite, *Aceria oleae* (Nalepa) (Acari: Eriophyidae)**

Olive bud mite is considered to be one of the most important pests on olive shrub and trees in Iraq. This pest cause discoloration, bud distortion and leaf chlorosis, deformation and stunting of foliage and fruits; in severe infestations, shoots may be killed ("Olive Bud Mite", n.d.).

❑ **Olive Leaf Mite, *Oxycenus maxwelli* (Keifer)**

This mite species feed commonly on the upper surface of leaves, but at higher infestations level it could also feed on the lower surface, on buds, shoots, flowers and stems, causing the premature drop of flowers, silvering and distortion of leaves. When feeding on buds, they also cause shortening of internodes between young leaves, leading to the formation of overbudding (Shari and Jennifer, 2016).

❑ **Olive rust Mite, *Tegolophus hassani* (Keifer)**

This mite species was recorded in Iraq in 2009 and it is currently found in most olive growing regions. It was first known and named in Egypt by Hassan, who discovered and described it in 1934. Mites suck plants causing yellowish spots on the upper leaf surface and silvery parts on the lower surface. At heavy infestations, they can cause deformation and twisting of leaves; leading to misshapen fruits reducing its both quantity and quality (Abou-Awad et al., 2005).

All mite species mentioned above, can be controlled chemically using the following insecticides:

- ✓ Vertimec - Abamectin 1.8 % EC (764 gram /ha).
- ✓ Zolone 25% WP (1.5 gram/L).
- ✓ Torac 50% WP (1 gram/L).
- ✓ Mitac Amitraz 20% (1cm^3/ L).

- **Olive Bark Beetle, *Phloeotribus scarabaeoides* (Bern.) (Coleoptera, Curculionidae, Scolytinae)**

Damage is caused by both larvae and adult stages which drill holes in healthy branches that will eventually dry up and die. To control the olive bark beetle, beside the cultural practices, the application of Sevin 85% WP (6 g gallon water^{-1}) represents the most effective method, which is applied in April and May, and is repeated twice a month (Hastings et al., 2001).

I.3.2.2 Plant-parasitic nematodes

Four genera of nematodes are known on olives trees and seedlings in Iraq: Root-Knot Nematodes, *Meloidogyne* spp., Citrus Root Nematode, *Tylenchulus semipenetrans* Cobb., Reniform nematode, *Rotylenchus reniformis* and the Dagger nematode, *Xiphinema* spp. The last two genera were first reported on olives trees in 2018 (Ismail et al., 2018).

All these species impact the plant growth and yield, by directly attacking roots and subsequently predisposing them to secondary infections by bacteria and fungi. The impact of nematodes is much more significant in nurseries where irrigation favors the development of roots and nematodes multiplication. Direct damages are due to the trophic activity of second stage juveniles on roots, through the mechanical action of their stylet, causing alterations of root tissues which limit the uptake of nutrient solutions, leading to reduced both growth and fruit production. Indirect root damages occur due to infections by soil-borne pathogens like the fungal pathogen *Verticillium dahliae* (Verticillium wilt).

To control of nematodes in nurseries is recommended by the disinfestation of rootstocks by dipping the roots in suspensions of nematicides. Also, soil fumigation is recommended in new orchards before planting to reduce nematode populations. The use of resistant cultivars is potentially effective in reducing economic losses due to nematodes (Ali and Huda, 2007).

I.3.2.3 Olive Diseases

Despite the high temperature during the summer, the olive in Iraq can be affected by various pathogens. Here, we briefly describe one of the most important and devastating

diseases of olive tree in the country; Olive Wilt disease caused by *Verticillium dahliae* Kleb. The common symptoms on *Verticillium* infected trees appear as chlorosis of the leaves followed sometimes by defoliation, sudden wilting, leaf rolling, and dead brown leaves remain attached to the branches. On old trees, one or more branches suddenly wilt early in the growing season; this process becomes worse as the season progresses. On very young trees, the tree begins to look pale and stops growing, the leaves wilt and the tree may die. Darkening of xylem tissue, a key symptom for differentiating *Verticillium* wilt, is not apparent in olive wood as it does in many other crops (Nuria and Jesus, 2020).

This disease is difficult to manage and the most effective management strategies are those taken before planting. Inoculum levels can be reduced before planting by soil solarization and fumigation. Soil solarization is recommended to growers, it can be effective in reducing the degree of infection and led to suppression of the fungus.

I.4 Olive Fruit fly *(Bactrocera oleae)*

The olive fruit fly, *Bactrocera oleae* (Rossi 1790), Diptera Tephritidae is the main serious pest in most of the olive growing regions of the world. The monophagous pest feeds exclusively on wild precursors and cultivated olives (Economopoulos, 2002, Nardi et al., 2005, Daane and Johnson, 2010). However, in a previous study of host trees infested by *B.* oleae, Athar (2005) reported that olives were the preferred host, but trees in the *Rosaceae, Rutaceae, Anacardiaceae, Fabaceae, Lythraceae, and Malpighiaceae* families were also infested (Athar, 2005).

I.4.1 Distribution

The olive fruit fly is widespread throughout the world and is present in most areas where the olive is grown extensively, also in regions where wild olive trees are indigenous. The olive fruit fly is well known as an important pest and widespread in the Mediterranean basin, but it is also reported in various parts of the world, notably in the Near and the Middle East, California and Central America, South and Central Africa (Augustinos et al., 2002; Rice et al., 2003) (Figure I.4). As usual for most invasive species associated with the domesticated hosts

(plants and animals), the historical details of olive fruit fly populations are unclear. Despite the abundance and notoriety of the olive fruit fly in the Mediterranean region, it is also associated with wild varieties of olives in Africa from which the domesticated cultivars were derived (Zohary, 1993)

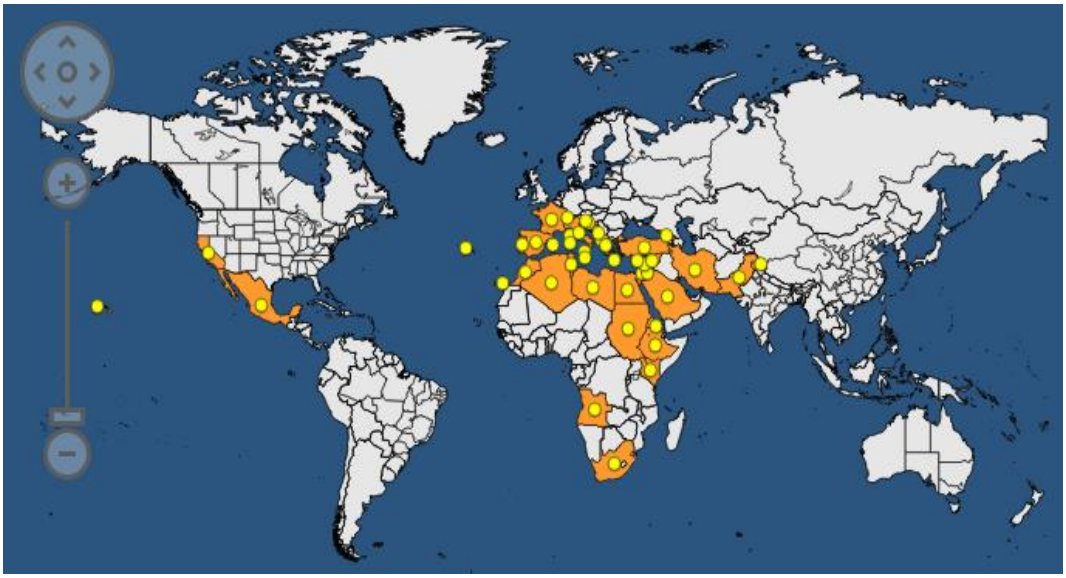

Figure I.4. Geographical distribution of the olive fruit fly.
Source: (EPPO, 2020)

I.4.2 Morphological features

Females can be distinguished from males by the presence of an ovipositor, a dark-coloured pointed structure at the end of the abdomen, that is used to pierce the fruits during oviposition (Perry and Elmore, 2006).

- ***Eggs***

The egg is not visible unless the fruit is opened, fruit fly deposits their eggs inside developing fruits. The females typically deposit one egg per olive on smaller fruits < 1 cm^3 (Yokoyama et al., 2006; Genç, 2014; Kounatidis et al., 2009). The colour of newly deposited eggs is opaque and creamy white (Figure I.5.A). Eggs size is about 0.74 mm long and 0.21 mm wide (Genc, 2014). The shape is typical of Tephritid fruit fly eggs- elongated and slightly

curved. They maintain this appearance until hatching when the first instar larva is visible through the chorion (the membrane surrounding the egg) (Genc, 2014).

- ***Larvae***

The larva emerges from the front end of the egg and it starts moving deeply within the mesocarp, i.e. the flesh or pulp, of the olive fruit to feed. Larvae colour is yellowish to white and they are typical Diptera legless (maggots) and the head is pointed (Figure I.5.B). The fruit fly maggots are small (5-6 mm long, 1.5 mm wide), elongated and slightly tapered at their ends (Phillips, 1946). After being fed for a while (about 20 days), they are easier to locate, especially when the fruit has started to rot (Perry and Elmore, 2006).

- ***Pupae***

Larval pupation depends on the time of year and the number of generations, it usually occurs in olive fruits or in the soil at the end of the season, in areas where there are many generations produced per year (Rice, 2000). The puparium is 3.5 to 4.5 mm long, and has a variable colour depending on the age of the pupa, it seems to be creamy white at the early age and later on turns to yellow-brown when it is dry (Figure I.5.C).

- ***Adults***

The adult fly is small, about 5 mm long with a wingspan of 10 mm (Weems, 1966), reddish-brown with large reddish eyes and small antennae. The body is long with large transparent wings containing dark brownish-black spot at the tip of each wing. The head, thorax, and abdomen of the adult fly are mostly dark-brown to black, with yellow-brown markings, and the thorax has several white or yellow patches on each side (Weems, 1966) (Figure I.5.D). The end of the male's abdomen is blunt, whereas females have a large black ovipositor at the end of their abdomen that is visible to the naked eye, which serves to pierce the skin of the fruit during oviposition (Daane and Johnson, 2010).

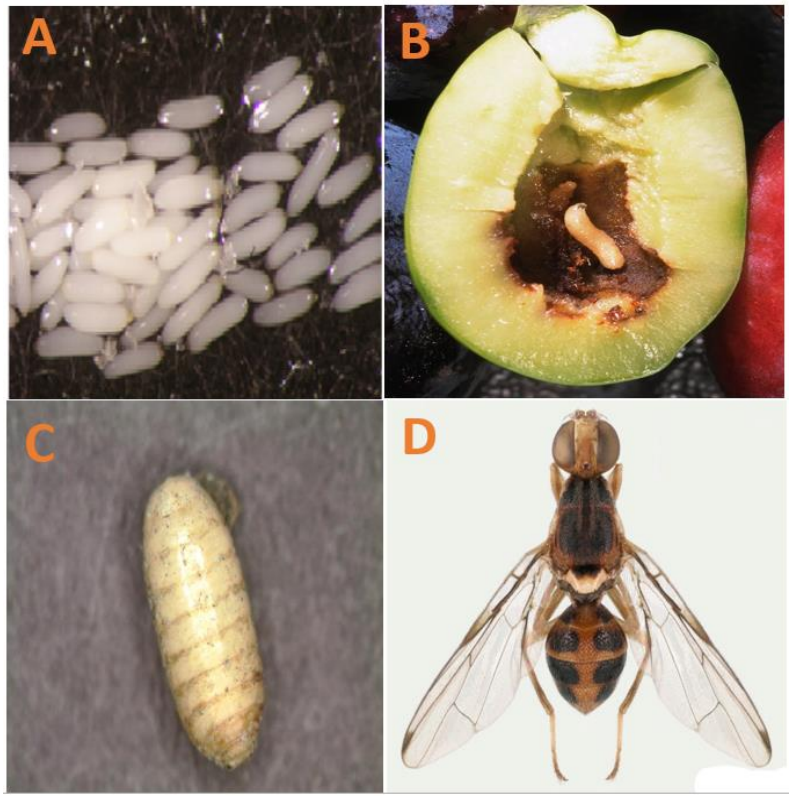

Figure I.5. (A) Eggs of olive fruit fly (Genç and Nation, 2008), (B) The larval stage (Tal, 2002), (C) pupa stage, and (D) Adult of olive fruit fly.

I.4.3 Life Cycle

The life cycle of the olive fruit fly is closely related to the seasonal development of the cultivated olive (*Olea europea*), and to the area climate. Adults remain active along the year through successive generations; therefore, eggs and maggots of olive fruit flies could be found during all year either in fruits left on the ground or on the trees. When female emergence occurs between March and May, they attack olive fruits that remain on the trees from the previous season, while those females that emerge in early summer (June), when both temperatures and daylength increase, do not lay eggs. Although few eggs are produced, adults remain active looking for new locations. By late June to the beginning of July as the new olive fructify develops, females are attracted to host fruit and start to lay eggs. Though eggs may be laid in small fruits, the larvae do not successfully develop till the ripening fruit grows to adequate size.

Eggs are laid just under the fruit skin, often creating a dimple or brown spot. When larvae development occurs during early to midsummer, they pupate into the fruit and emerge later in the season as adults. In contrast, when larvae development occurs during late fall they leave the host fruit to pupate into the soil for overwintering. However, some maggots could overwinter in host fruit left on trees and pupate later in spring. Multiple generations occur through the summer and autumn. In summer, the flies can complete a generation in about 30 to 35 days, at optimum temperatures range (from 20°C to 30°C) (Figure I.6) (Rust and Reierson, 2007; Byron et al., 2019).

Olive Fruit Fly Life Cycle

1- The life cycle of the olive fly begins with the new appearance of adults who reach sexual maturity and copulate occur.

2- Then, the females search for the suitable host fruit to oviposition.

3- Eggs lay alone under the skin of unripe olives and hatch in 2-3 days.

4- The larvae emerge and fed upon the olive pulp, developing through three larval instars. Larvae development occur between 10-15 days at optimum temperatures.

5- Pupation can occur either within the host fruits or into the soil.

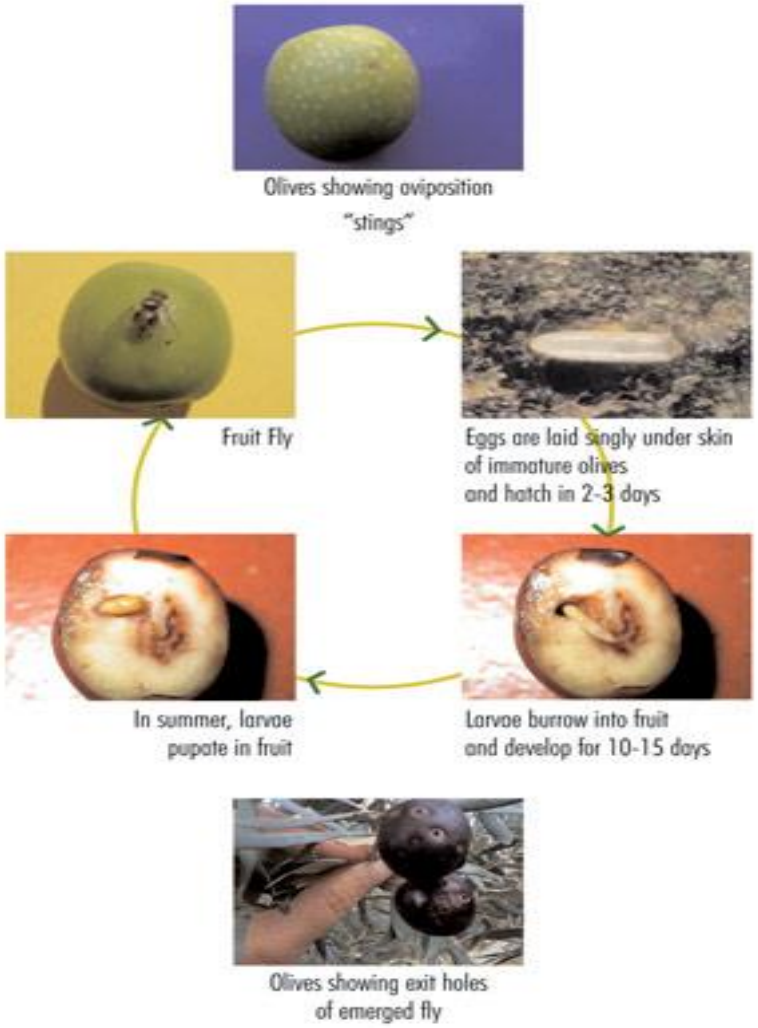

Figure I.6. Life cycle of olive fruit fly (*Bactrocera oleae*).
Source: (IDEASS, 2019)

I.4.4 Damages and Economic importance

Adult females sting and lay their eggs in developing olive fruits. After eggs hatching, newly emerged larvae start to feed upon olive pulp, resulting in important quantitative losses in the production of table olive (Agrios, 2008, Daane and Johnson, 2010) (Figure I.7). Quantitative damage is mainly due to fruit pulp destruction producing both lower oil production and a premature fall of the fruit, while qualitative damage is due to an increase in the acidity

of the olive fruits (Daane and Jhonson, 2010). The damage caused to harvested fruits by the olive fruit flies is estimated to be at least 5% of total olive production, resulting in economic losses of approximately 800 million US dollars per year (Weems and Nation, 1999, Nardi et al., 2005 Allmuça et al., 2013).

Figure I.7. Damage caused through feeding by the larvae of the fly.

I.4.5 Control and management

Application of integrated pest management techniques (IPM), is the best way to control this pest. These techniques are based on using various strategies together to reduce and keep the pest population at an acceptable level below the economic threshold level (ETL) (Daane and Johnson, 2010). These IPM control measures are applied on the basis of government legislation (laws) of each country.

Cultural control

Field sanitation is an important step in preventing the occurrence of the olive fruit fly (Han et al., 2011). The fruits that remain in the field, either in the soil or on the trees, could contain eggs or larvae, contributing to the population of olive fruit fly (Fletcher, 1987).

Another cultural technique against olive fruit fly is the application of bait sprays, in which attractant sprays combined with insecticides are applied in the field in concentrated areas. This strategy is effective to eliminate the female fruit flies, that are attracted to the (ammonia-protein) mixture when they are preparing for egg production (Varikou et al., 2015). Mass trapping is another common technique, the efficacy of this method has been demonstrated

in several researches (Martinez-Ferrer et al., 2012). This method also involves chemical attractants similar to those used in the spray method, but traps the adult females for removal (Varikou et al., 2015). Both techniques, bait spraying and mass trapping are effective in controlling olive fruit fly population, however the application of mass trapping might be preferred because this technique does not require a chemical application to the plants themselves (Baker, 2009).

Biological control

In order to control *Bactrocera oleae* using predators and parasitoids, many species of insects have been investigated. A small group of braconid wasps is recognized as parasitoids of the olive fruit fly (Daane and Johnson, 2010). Among braconid species that have previously shown parasitism on the olive fruit fly are include*: Psyttalia lounsburyi* (Silvestri, 1913), *Psyttalia concolor* (Szepligeti), *Psyttalia ponerophaga* (Silvestri, 1916), *Utetes africanus* (Szepligeti, 1910), and *Bracon celer* (Szépligeti, 1913), with variables effectiveness over different geographical locations (Nardi et al., 2003). Other common beneficial insects like ladybird beetles (*Coccinellidae* Latreille, 1807) and lacewings (*Chrysopidae* Schneider, 1851) are ineffective in controlling the olive fruit fly because these insects' prey upon the immature stages of pests, which in this case are sequestered within developing olives (Nardi et al., 2003).

Chemical control

Over the past four decades, the olive fruit fly management has been based on the use of organophosphate insecticides as cover sprays and bait sprays (e.g. dimethoate and fenthion). In recent years, the use of pyrethroids has increased to control this pest, and recently spinosad has been incorporated into a bait spray to increase the efficacy with lower active ingredients (Daane and Johnson, 2010).

Alternatives to conventional insecticides

The reduction of insecticide applications such as organophosphate and pyrethroids is an objective in many olive growing areas as a way to reduce environmental pollution, olive oil contamination and to conserve beneficial insects. In order to achieve this goal, several

alternatives can be used instead of the conventional insecticides such as mass trapping program, sterile insect techniques, and biological control agents (Allmuça et al., 2013). Currently, there is another environmentally friendly alternative to conventional insecticides such as plant-derived compounds. Essential oils (EOs) in several plant families have been found to be toxic to various pest species of insects through topical application, ingestion, and as fumigants. In a recent study, Oviedo et al. (2020) reported that the exposure of *Ceratitis capitata* (Wiedemann) and *Anastrepha fraterculus* (Wiedemann) sexually mature adults to volatiles and vapors of both *Bacharis dracunculifolia* DC. and *Pinus elliottii Emgelm* EOs resulted in lower longevity (half-life), survivorship, and female fecundity.

Trapping techniques

McPhail trap which is a type of trap commonly used to capture Tephritid flies by attracting them to an odour source such as Torula yeast, as well as Mass trapping using colored traps, food baits and /or sex pheromones as attractants of olive fruit flies to suppress pest population (Broumas et al., 2002, Allmuça et al., 2013).

Sterile Insect Technique

Several countries had begun researches on the use of sterile insect techniques against the olive fruit fly *B. oleae* such as the United States, Austria, Greece and Iran (Harris, 1975; Ahmadi et al., 2018; Ahmad et al., 2018). The effectiveness of sterile-insect techniques (SIT) was amply demonstrated and sterile insect releases have shown an effective and environmentally-friendly method to control olive fruit fly (Alphey et al., 2006). This technique involves the rearing and release of a large number of sterile male fruit flies. As soon as the sterile males mate with wild female flies, they will produce infertile eggs. Consequently, the next generation of olive flies is successfully reduced. Over time, this technique could eradicate the pest population. In addition, this technique has many advantages, it is species-specific, environmentally sound, and relatively speedy (Thomas et al., 2000).

I.5 Symbiosis

Symbiosis is defined as an interaction between two organisms living together in intimate association or even the merging of two dissimilar organisms. The symbiotic interactions between micro-organisms and insects are widespread (Buchner, 1965; Von Dohlen et al., 2001; Moran and Baumann, 2000). Symbiosis can occur between organisms (eukaryotes or prokaryotes), and often among those not cultured on common media in the laboratories (Smith and Douglas, 1987). In many cases, the symbionts are living inside the cells of the harbour hosts, such as the symbiosis among aphids and members of the genus *Buchnera* (Baumann et al. 1995). The other kind of symbiosis occur extracellularly, but the symbionts are relatively living close to their hosts, such as the gut-inhabiting micro-organisms of termites (Breznak and Brune, 1994). Also, the symbiosis can be a parasitic phenomenon, such as *Wolbachia pipientis* in a variety of arthropods (Werren et al., 1995; Werren and O'Neill, 1997). Several studies have shown that symbiotic micro-organisms can deliver a source of essential amino acids that are lacking in the diet of their hosts (Douglas, 1998).

I.5.1 The bacterial endosymbiont (*Candidatus* Erwinia dacicola)

The symbiotic bacterium "*Candidatus* Erwinia dacicola" (Enterobacteriaceae), is unculturable obligate symbiont and considered as a major symbiont of olive fruit fly *B. oleae*. This bacterium is one of the few non-pathogenic endosymbionts, that is localized and moves (intracellularly and extracellularly) during the different stages of host's life cycle (Ben-Yosef et al., 2014; Capuzzo et al., 2005; Estes et al., 2009, 2012; Kounatidis et al., 2009). The symbiosis between *B. oleae* (Tephritidae) and a bacterium "*Ca.* Erwinia dacicola" was first discovered at the beginning of the Twentieth century by Petri (1909), who described the possibility of hereditary symbiosis in the olive fruit fly *B. oleae* based on a microscopic examination. A recent study showed that the symbiotic bacterium is located within the esophageal bulb, midgut, and ovipositor of olive's fly *B. oleae* (Capuzzo et al., 2005). The existence of symbiont "*Ca.* Erwinia dacicola" in Italian natural populations was confirmed in 2008 by Sacchetti et al. (2008).

The symbiotic bacterium plays an essential role in many aspects of insect's life such as, ecology, biology, reproduction, behavior, and pest status, affecting plant nutrition, evolution, and immunity (Engel and Moran, 2013; Hosokawa et al., 2007; Wingfield et al., 2016). The bacterium "*Ca.* Erwinia dacicola" serves as a nutrient provider, particularly during juvenile development in immature olives, where it is crucial for larval survival (Ben-yosef et al., 2015; Hagen, 1966). According to previous studies, the presence of bacterial masses in the midgut is likely that these bacteria serve as a source for nitrogen fixation (Ben-Yosef et al., 2008; Drew, 2000). Other symbiont benefits to the insects have been identified, such as resistance to both changing of temperature (Montllor et al., 2002), parasitoids (Oliver et al., 2005), and production of aggregation pheromones (Dillon et al., 2002). Additionally, lack of endosymbionts in some insects strictly reduced their mating ability. Thus, the benefits of mutualistic bacteria are for both male and female reproductive success and eventually to fitness (Estes et al., 2014).

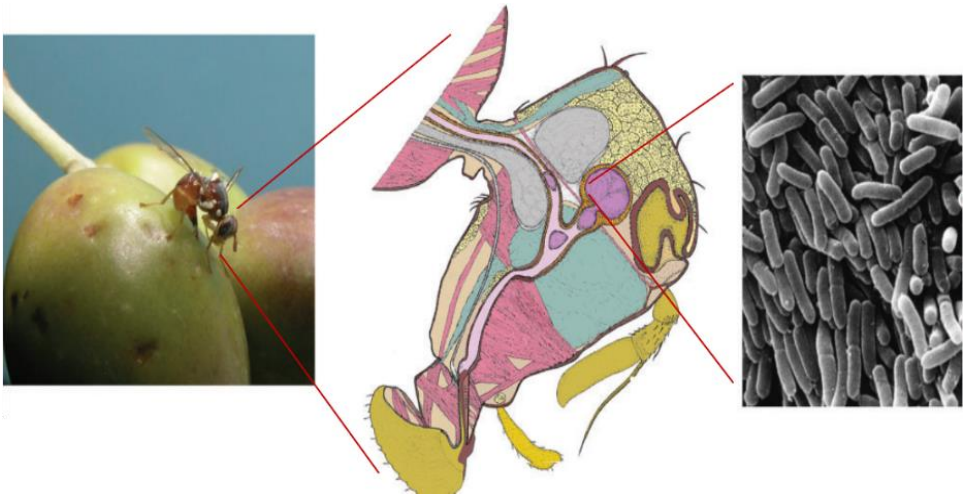

Figure I.8. The bacterial endosymbiont living within the olive fruit fly (Capuzzo et al., 2005).

I.5.2 Most common methods for bacterial detection in insect tissues

The presence of bacteria through the insect tissues, and the inability to both isolate and develop them in artificial media, made the use of molecular techniques the best way to discover and study them more clearly. Polymerase chain reaction (PCR) has been widely used to amplify the DNA of the symbiotic bacteria *Erwinia dacicola,* using universal primers in 16s rRNA

gene; forward primer 10F (5'-AGTTTGATCATGGCTCAGTTG-3') and a reverse primer 1507R (5'-TACCTTGTTACGACTTCACCCCAG-3'), to generate a PCR product of 1400 bp (Sandström et al., 2001). Moreover, a set of specific primers; EdF1 (5'-CTAATACCGCATAACGTCTTCG-'3) forward primer paired with 1507R (5'-TACCTTGTTACGACTTCACCCCAG-'3), have been used to generate an amplification of 1300 bp (Estes et al., 2009; Miyazaki et al., 2017).

I.6 Nano Materials

Nanomaterials are generally defined as "ingredients that contain particles that have been intentionally produced to have at least one dimension that measures between approximately 1 and 100 nanometres" (Jordan, 2010). Through the development of nanotechnology in combination with biotechnology, the application of nanomaterials has considerably expanded in diverse fields. A diversity of carbon-based materials and metal oxide-based dendrimers (non-sized polymers) and bio composite nanomaterials are being developed (Nair et al., 2010, Khot et al., 2012).

The nanotechnology is moving quickly from promise to reality; the rapid advance of these technologies has engaged diverse industry sectors, including food and agriculture, in what is essentially a regulatory gap (Paull, 2011). The application of nanotechnology technique in the agriculture sector is one of the important tools proposed for sustainable intensification. Small matter can behave differently from big and therefore nanoscale materials can exhibit novel and unpredicted properties. Therefore, reducing the size of the particle of a pesticide, through the use of nano-particles, could increase their effectiveness on target pests. The use of nano-metal oxides and nano-silicon to target soil pathogens increase water uptake efficiency in plants, adding that the development of a DNA-based nano-biosensor in a polymer to coat fertilizers would only release the amount of fertilizer required by plant root ionic signals (Suppan, 2013).

Moreover, nanomaterials include single-walled (SWCNT) and multi-walled carbon nanotubes (MWCNT), aluminium (Al), titanium dioxide (TiO2), copper (Cu), gold (Au), magnetized iron (Fe), silver (Ag), silica (Si), zinc (Zn) nanoparticles and zinc oxide (Zno),

Calcium carbonate ($CaCO_3$) nanomaterial, and cerium oxide (Ce2O3), etc. Generally, these materials have been used in wastewater treatment, water purification, food processing and packaging, medicine, environmental remediation, industrial and household purposes and smart sensor development (Qureshi et al., 2009, Lee et al., 2010, Zambrano-Zaragoza et al., 2011). Their applications have mainly focused on the importance of nanomaterials in improving efficiency and productivity. Nowadays, these materials are being applied in agriculture to improve production and crop protection (Emamifar et al., 2010).

I.6.1 Characteristics and applications of nanoparticles (NPs) in different fields

Nanomaterials and nanotechnology have occupied a prominent position in our life and environment, due to their novel characteristics such as chemical reactivity, increased strength, and high electrical conductivity. Consequently, this technology has become one of the most promising technologies in various sectors (Leiderer, 2008). Over the last two decades, several researches have been performed in nanotechnology focusing on their application in different domains such as energy, medicine, electronics, mechanics, and life sciences as well as plant sciences (Scrinis and Lyons, 2007; Nair et al., 2010). Nanotechnology has an inevitable impact in the field of agriculture, and a number of researches have proved their potential in improving agricultural and food systems through different methods that have shown an increase in agricultural yield and development of food products (Kole et al., 2016). The experiences gained in the above-mentioned fields simplify precision farming techniques, genetically modified crops, and plant protection chemicals. Application of nanomaterials in agriculture has led to great advances in agricultural researches, for instance the conversion of agriculture and food waste into energy, technology and reproductive sciences. In addition, other benefits come through the use of enzymatic nano-bioprocessing, disease anticipation and prevention and the use of nanocides for treatment of plant diseases and pests (Moraru et al., 2003; Nair et al., 2010).

Nanoparticles (NPs) were shown to have a significant effect on biotic stresses, and which is why scientists are working to increase the efficacy of agrochemicals including pesticides. Several nanoparticles have been used to control various fungal and bacterial

pathogens (Kole et al., 2016), e.g. the silver nanoparticles (Ag NPs) were found to be highly efficient to control pathogenic *Candida* species, and have shown more effectiveness against gram-negative bacteria compared to gram-positive bacteria (Nair et al., 2010; Singh et al., 2008). Moreover, other studies reported that the absorption of surface-modified hydrophobic Nano-silica on the cuticle of insects can cause damages to their protective wax layer, leading to insect death by dehydration (Nair et al., 2010). According to a study conducted in a cotton field infested with aphids to assess the effectiveness of NP compared to conventional suspension, it has been demonstrated that NP was better in terms of plant penetration, as well as in improving the systemicity of the active ingredient (Boehm et al., 2003).

I.6.2 The interaction between Nanoparticles and Diptera

The emergence of nanotechnology and the high potential of NPs for diverse applications explain the fact that NPs are likely to be more active compared to conventional-sized particles. Indeed, in a study by Chifiriuc et al. (2016) on the actions of NPs against the fruit fly *Drosophila melanogaster* (Meigen), and their effect during the different stages of the fly's life cycle, they indicated that nanomaterials can be incorporated into insect tissues as they showed an adequate capacity of adherence to the surface of insect's body. In fact, the superstructure and aggregation state of NPs influence its toxicity and their adhesion to the fly body surface, leading to the transport of NPs inside the insect body, impairing the grooming behavior and inducing increased mortality (Leeuw et al. 2007).

Several studies were conducted to investigate the uptake of a type of iron oxide nanomaterials (Fe_3O_4) by Diptera, and the results showed that these NPs materials significantly decrease the female's fecundity and delay insect development at the egg-pupae and pupae-adult (Chen et al.,2015). Adults exposed to nano-materials have shown disturbance of the oogenesis period, ovarian defects, defective egg chamber development, reduction in eggs length and number of nurse cells (Chen et al., 2015).

The application of NPs such as silver (Ag) and titanium dioxide (TiO2) have shown to induce a decrease in insect fecundity and survival rate, interrupts the development and the occurrence of various phenotypes. Along with all the above effects, some NPs showed reduced

adult longevity and decreased sperm competition in Diptera. In some cases, the ingested nanomaterials decreased the larval and pupal survival in addition to larval climbing ability (Jampílek and KráL'Ová, 2015; Posgai et al., 2011). Another interesting effect of an ingested NPs is the reduction of gut microbiota diversity of treated larvae, which make changes in fly microbiome due to the differential killing of NPs- susceptible gut bacteria (Silvia et al., 2015).

In general, the toxicity of different NPs could be the product of both chemical interactions between the nanoparticle and physical obstruction, and the biological targets (Das et al., 2012).

I.6.3 Calcium carbonate

Calcium carbonate $CaCO_3$, involves approximately 4% of the earth's crust and it is found all over the world. The most common natural forms of $CaCO_3$ are Chalk, limestone, and marble, the sedimentation of the shells of small fossilized shellfish, snails, and coral over millions of years. Although the three forms are comparable in chemical terms, they vary in several respects notably the purity, the whiteness, the homogeneity and the thickness. Calcium carbonate is one of the most useful and versatile materials known to man (Mathur et al., 2016).

In food production and personal health, calcium carbonate is used widely as an effective dietary calcium supplement, antacid, phosphate binder, or base material for medicinal tablets. It is also found on many grocery stores shelves in products such as baking powder, toothpaste, dry-mix dessert mixes, dough, and wine. Calcium carbonate is the active ingredient in agricultural lime and is used in animal feed. Calcium carbonate also benefits the environment through water and waste treatment (Mathur et al., 2016).

Moreover, calcium is known as a major essential plant element; it can strengthen plants against diseases or increase crop yields. In previous studies, calcium nutrition could reduce gray mold symptoms and the severity of postharvest *Botrytis* blight in rose flowers (Volpin and Elad, 1991). Likewise, Yamazaki (2001) demonstrated the effect of calcium nutrition on tomato bacterial wilt caused by *Ralstonia solanacearum* and indicated that the resistance of tomato to bacterial wilt is markedly affected by Ca nutrition of the host.

It has been reported that the foliar application of calcium on Egyptian cotton can increase yields of seed cotton and lint and also improve fiber properties (Sawan et al., 1997). Likewise, Xiumei et.al. (2005) compared the effectiveness of calcium carbonate with humic acid and organic fertilizer in groundnut, *Arachis hypogaea* L., and demonstrated that low concentrations of nano-calcium carbonate lead to an increase in the number of leaves and leaf area, soluble sugar, dry weight and peanut protein content. Recently, researchers have demonstrated the fertilization effects and control efficacy of nano-calcium carbonate on the infestations of Oriental fruit fly (*B. dorsalis*) and red scale insects *Aonidiella aurantii* (Maskell) (Hua et al., 2015).

I.7 Thesis aim and hypotheses

The olive, considered as one of the oldest domesticated fruit trees and the most widely grown crop in a lot of countries all over the world, has high economic value due to its high consumption. Unfortunately, in those countries where olive is a key crop, the olive fruit fly *Bactrocera oleae* is a major pest and known to cause significant yield loss. Therefore, the need to control this pest species increases over time, and find new techniques to improve the conventional methods for sustainable integrated pest management are necessary. As discussed previously, introducing the nanotechnology in plant protection has shown promising results to control several insect pests and pathogens attacking various crops. Moreover, most of the applied materials that have been used in nanotechnology are environmentally friendly, present in nature and have no side effects on the atmosphere as well as on the human health. Despite extensive research on *B. oleae* control methods, few studies have investigated the control effectiveness of nanoparticles on infestations of the olive fruit fly *B. oleae*. In the present study, we hypothesize that Calcium carbonate nanoparticles could decrease *B. oleae* populations by affecting the female's fertility and egg production; the development of immature stages and by reducing adult longevity. In this context, a comparative study using two formulas of Calcium carbonate ($CaCO_3$ NPs and CaCol) would give a better understanding of the effect of Calcium carbonate nanoparticles ($CaCO_3$ NPs), as an innovative strategy against *B. oleae* in olive groves. Also, we hypothesize that Calcium carbonate NPs have an antimicrobial effect, thus could interrupt the symbiosis interaction between the bacterial endosymbiont "*Candidatus*

Erwinia dacicola" and its host *B. oleae*. These hypotheses led us to design the study objective through a series of field and laboratory studies, and data set analyzes. More specifically, we aimed to:

1- Conduct a field assay to determine both the presence and infestation level of *B. oleae* in order to evaluate the efficacy of Calcium carbonate (NPs) and colloidal calcium carbonate treatments on them in olives orchards.

2- Evaluate, in laboratory conditions, the effect of $CaCO_3$ NPs and colloidal calcium treatments on the longevity of *B. oleae* adults and development of immature stages.

3- Detect and quantify the DNA of the bacterial endosymbiont "*Candidatus* Erwinia dacicola" in the body of target insect, and to evaluate the effects of Calcium carbonate NPs and colloidal calcium on the symbiotic bacterium, as well as on the egg production of *B. oleae* females.

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II. FIELD APPLICATION OF CALCIUM CARBONATE FORMULAS AGAINST THE OLIVE FRUIT FLY *BACTROCERA OLEAE*

II. 1. Introduction

The evergreen tree *Olea europaea* is one of the most widely grown tree-crop species in the Mediterranean basin, which has always been the authentic place for its cultivation and olive oil production since ancient times. It has been cultivated in Mediterranean basin around 6000 BC, where olive trees have played an important role in the rural and economic development of the Mediterranean's areas over the centuries (Efe, 2011).

Unfortunately, olive tree is subjected to be attacked by various pests, which affect its health, yield and its oil quality. In Italy, the olive fruit fly is considered as a major pest on olive trees, causing important quantitative losses primarily by pulp consumption and inducing premature fruit dropping. In addition, the pest affects negatively the quality of infested fruits and makes them unsuitable for direct consumption or processing, and the olive oil quality is sharply reduced due to the increase of fruit acidity and mould contamination (Mraicha et al.,2010).

In recent years, the application of nanoparticles in agriculture has gained enormous attention, by offering the alternative tactic to control and reduce the severity of various insect pests and pathogens. Several materials were introduced in nanotechnology and have been applied in plant protection processes; one of these materials is Calcium carbonate which is used as a fertilizer to improve plant health and vigour (Mazzaglia et al.,2017; Parisi et al.,2014).

Despite the studies that have been carried to investigate the effect of Nano-materials on insects, very few researches have been done on Nano-Calcium carbonate in plant protection. Those researches showed a significant result using Calcium carbonate nanoparticles (NPs) to control oriental fruit fly *Bactrocera dorsalis*, Diptera: tephritidae. In this context, the aim of the present study was to evaluate the effect of two calcium carbonate formulas (nanoparticles, and classical colloidal calcium) against the olive fruit fly, *B. oleae*, under field conditions.

II.2 Materials and methods

II.2.1 Experimental area

The field experiment was conducted in a typical olive orchard located at the center of Lazio region (Viterbo 01100 VT 42.410092N, 12.078059E) Central of Italy. The orchard's area, managed by Monegatti and Lanzi families, consisted on approximately five hectares that contains 525 olive trees belonging to three different cultivars (Canino, Frantoio, and Leccino) (Figure II.1; Olive trees had an average of 150 years of age).

Figure II.1. Field location (Viterbo, 01100 VT 42.410092, 12.078059).

II.2.2 Field experiment preparation

Olives are non-climacteric fruits (Kafkaletou and Tsantili, 2018) i.e. they can't ripen once removed from the plant. Since the larvae of olive fruit fly can't develop on hard unripe fruits and collected fruits tend to be rotted by fungus attack after a storage time, a different methodology in order to provide fresh olive fruit to *B. oleae* was used. For this purpose, several fruitful branches were wrapped and covered with special ‘‘muffs’’ of straw and tulle (Baratella, 2011) from late July until early August 2018 (as soon as the fruits reach both premature stages and became solid (Figure II.2) allowing to obtain healthy fruits throughout the year. The covered fruits were used to evaluate the effect of calcium carbonate formulas on *B. oleae* infestations after removing the muffs.

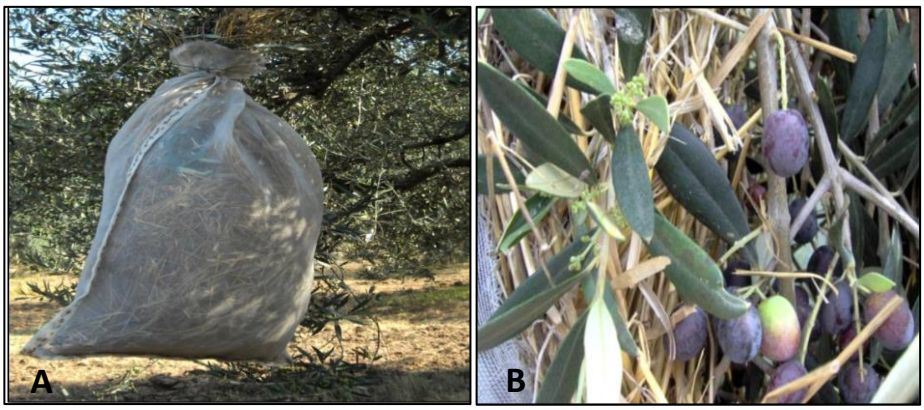

Figure II. 2. (A) the covered branches, and (B) the covered fruits after 6 months.

II.2.3 Field monitoring

The monitoring process of the olive orchard was started at the end of July 2018, to check the presence and density of *B. oleae* populations. Yellow coloured sticky traps were used to capture the adult flies of both sexes (male and female). Traps were placed in different positions in the olive orchard (Figure. II.3). They were hanged inside olive tree canopies, on fruitful branches, in open shade and within 8-10 inches from the foliage (Marchini et al., 2017; Sarwar, 2015). All traps were replaced weekly with new traps, and the old ones have been carried out to the laboratory of Dipartimento di scienze Agrarie e Forestali (DAFNE); University of Tuscia, Viterbo-Italy, in order to determine the presence of olive fruit fly's adults.

Figure II.3. The sticky yellow traps used for monitoring insects.

Temperature variations in the field-area were obtained from local meteorological station (Viterbo). The number of captured adults was recorded for both sexes (male and

female), collected in each trap per week from the mid of July till the end of October also the sampling dates, in order to evaluate the population dynamics of the pest.

II.2.4 Determination of infestation level in olive fruits

Samples of olive fruits (200 fruits) were collected randomly, on a weekly basis, when the sticky traps were replaced, from different places in the olive orchard. Each fruit sample was placed into labelled plastic bags and transported to the laboratory where they were stored at 22 ± 2°C, 36 RH. Collected fruits were examined under the stereomicroscope; 10X mag, RS Pro Deluxe Stereo Microscope, to detect and record the number of immature stages (eggs, larvae 1-2-3 instar, puparium or pupae, and galleries) in order to estimate the natural infestation level of *B. oleae* (Figure II.4). The fruit collection process was started in mid-July and continued periodically every week until the mid of October, approximately 3000 fruits were examined during the tested period, to evaluate natural infestation levels based on the number of larvae and pupae detected per single olive fruit (Allmuça et al., 2013).

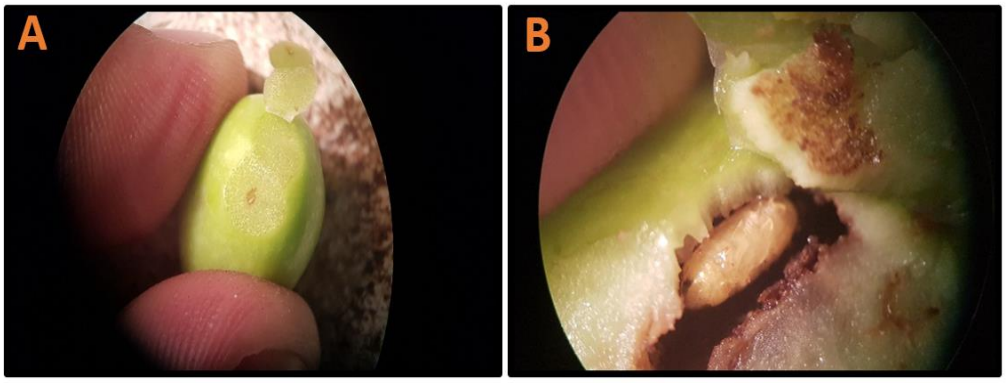

Figure II.4. Olive fruits under microscopic screening. (A) eggs of fruit fly and **(**B) the pupa of the fruit fly.

II.2.5 Treatments of olive trees in open field

The Calcium carbonate ($CaCo_3$) nanoparticles (NPs) suspension (nominal diameter 58 nm (20-100 nm)) was supplied by BIOSCINTIFICA S.R.L., (Via Flaminio- ITALY), and colloidal calcium was purchased from the Serbios CO., S.R.L. (Badia polesine, Italy). Field treatments were started on the 26th of September when the infestation of olive fruits reached

significant level; ~ 76% of the sampled fruits were infested and the number of captured females was at a high peak. The central part of the olive orchard was divided into three blocks (A1), (A2) and (A3) each block with dimension (20m X 50m). Blocks A1 and A2 were treated with Calcium carbonate formulas, respectively, Calcium carbonate NPs (A1) and colloidal calcium CaCol (A2), and the third block (A3) was treated with tap water as a control. Moreover, an equal number of trees (10 trees) were selected in each block, planted with the same cultivars, and almost has the same amount of olive fruits. The Calcium carbonate nanoparticles (NPs) and colloidal calcium (CaCol) solutions were prepared in the laboratory, diluted in distilled water to obtain Calcium carbonate (NPS) with final concentration 3gr/L, and for colloidal calcium 16ml/L as recommended by the producers. Each tree was spread with 6L of the corresponding solution to cover all the vegetation part, using handled sprayer pump; (GDM "Rosy" lt-16; R: ME2395017).

Due to the bad weather conditions (heavy rainfall and wind), and in order to compensate the leaching of suspensions caused by those conditions, the treatment was repeated on October 3rd. One week after treatments, samples of olive fruits (200 fruits) were collected from each treated block (A1, A2, and A3). The samples were placed in plastic bags, labeled with the collecting information (the block's name and the date), and transferred to the laboratory to evaluate the effect of both Calcium carbonate (NPs) and colloidal calcium on insect development in treated olive fruits, and compare them with non-treated fruits collected from control block.

II.2.6 Treatments of olive branches covered with muffs

Regarding the covered branches, the same previous processes were carried out, starting by dividing a part of the field to three groups (1), (2) and (3), and each group contained three covered branches belonging to the same cultivar Frantoio ; (A), (B) and (C) loaded with olive fruits. As it was mentioned previously, the branches were covered late of July, before the presence of the fly and they were healthy (free of infestation). Later, on October 3rd, the branches were uncovered to undergo treatments. In fact, the main reason to perform the treatments immediately after uncovering the branches was to guarantee that olive fruits are

definitely healthy thus, the effectiveness of the treatment through the infestation level will be precisely assessed, based on the number of new emerged adults from treated fruits (200 fruits from each group per week).

The branches (A, B and C) in each group were treated with the following suspensions, respectively (Calcium carbonate NPs 3g/L, colloidal calcium 16ml/L, and tap water as a control). The treatment was repeated twice, on 9th and 23rd October, to guarantee sufficient coverage of the protected olives and to ensure the treatment fidelity. After treatment, 200 olive fruits from each treated branch were picked and carried to the laboratory. Then, olive fruits were conditioned into small rearing boxes (Figure II.5) to evaluate the effectiveness of treatments on insect development.

Figure II.5. Treated fruits maintained in rearing boxes in the laboratory.

II.2.7 Evaluation of treatments effectiveness

The fruits collected on both trees and covered branches were placed in special rearing boxes (L40 cm, W20cm, and H15cm), and the upper side of each rearing box consisted of mesh tulle (0.5 mm). Olive fruits in boxes have been incubated under laboratory conditions (at 22°C ± 2, RH **63 ±2**), and remained under observation for ≈ 21 days (Pucci, 1992). After incubation period, the boxes were examined to evaluate the effect of each treatment on insect development. To this purpose, reared insects in boxes were killed using fast killing aerosol insecticide. After 10 minutes, boxes were opened separately, neatly screened and the number of emerged adults and immature stages were counted manually for each rearing box.

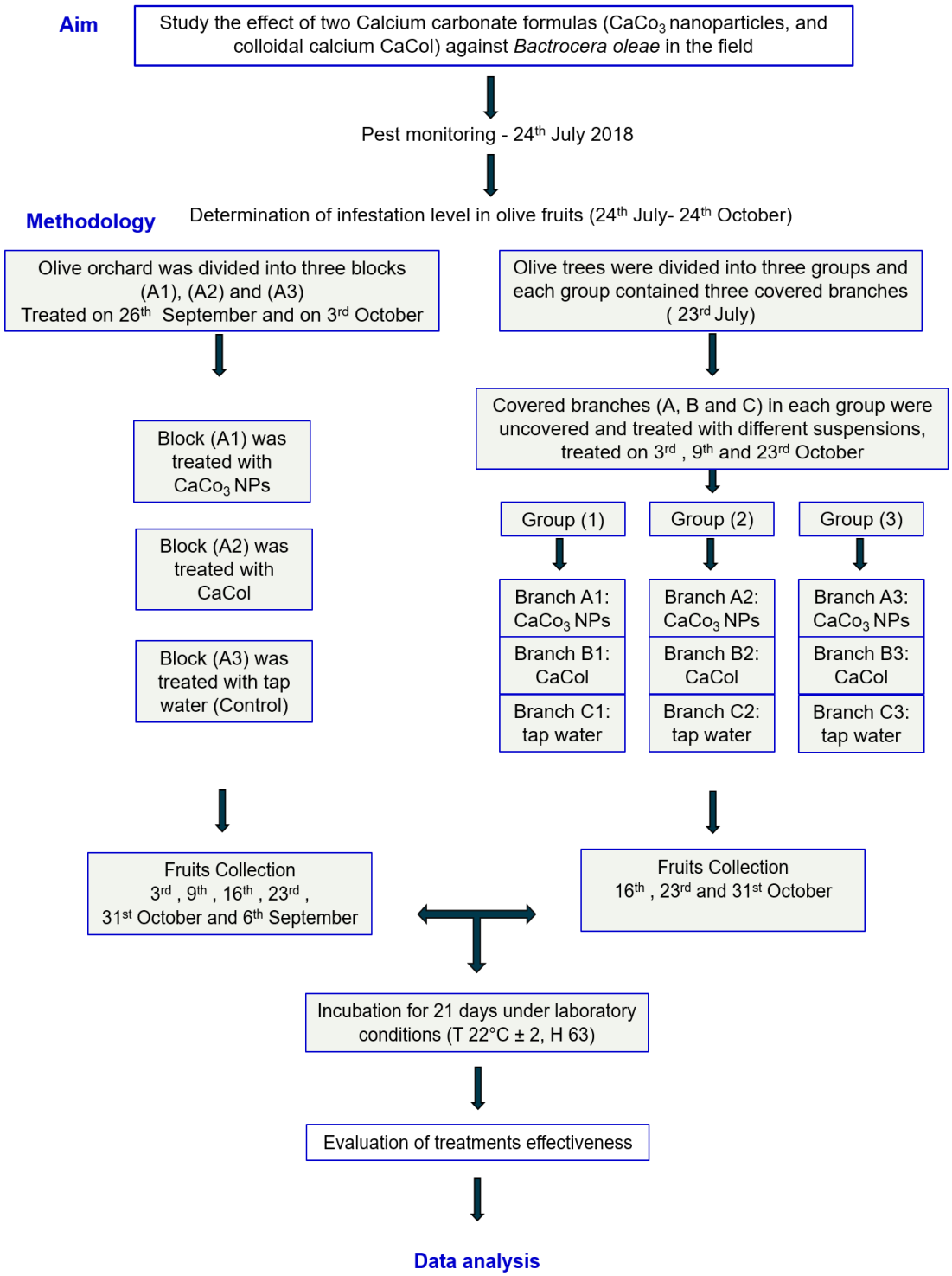

Figure II.6. Research methodology flow chart. The flow chart shows in steps the activities carried out in the study.

II.2.8 Data analysis

Data were recorded in a Microsoft Excel spreadsheet and used for statistical analysis to assess the effectiveness of suspensions ($CaCO_3$ NPs and CaCol) plus control, with six replicates per each treatment applied on different stages of insect development; based on the number of emerged adults for each treatment. Statistica software (version 8.0) was used for checking normality assumptions by using the Shapiro-Wilk test, and to analyze the data by one-way analysis of variance (ANOVA) ($P < 0.05$), followed by post-hoc Tukey HSD (Honestly Significant Difference) testing.

II.3 Results

II.3.1 Monitoring of adults in the field

The meteorological data show average temperature during the present assay of 21 ± 5°C (Figure II.7).

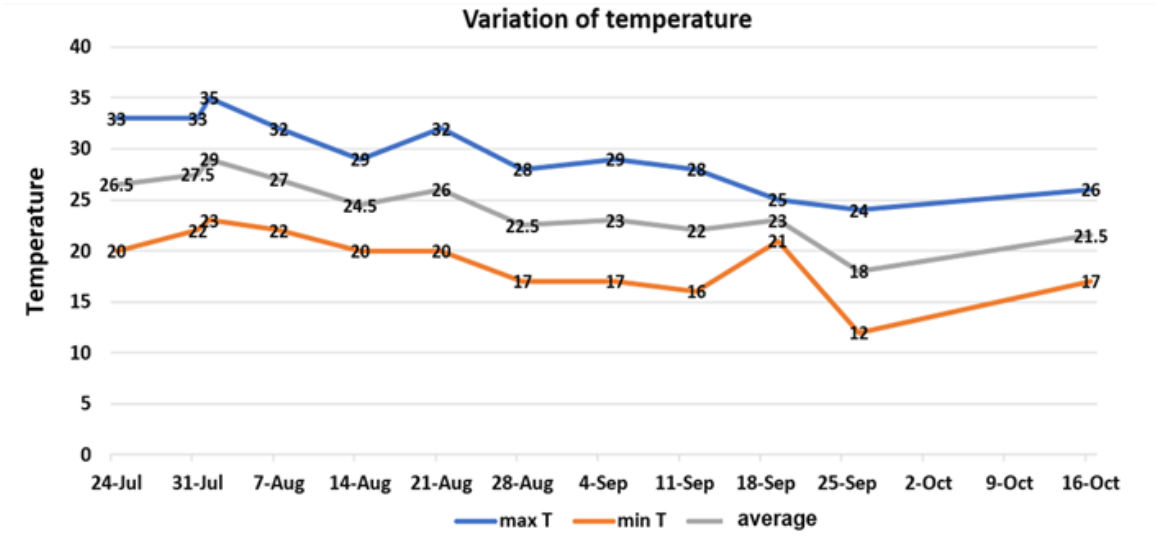

Figure II.7. Temperature variations during the field assay.

The data shown in (Figure II.8A) refer to the average number of captured adults per trap, divided by nine which is the number of traps. The monitoring processes have shown that *B. oleae* adults arrive at the olive orchard in mid-August (0.7 adult flies per trap) and rises

gradually up to reach their maximum in mid-October (88.4 adult flies per trap) to decrease slowly until the end of the present study (43.2 adult flies per trap). Temperature and captured adults illustrated in Figure II.8B indicate that there is correlation between *B. oleae* abundance and the effects of temperature.

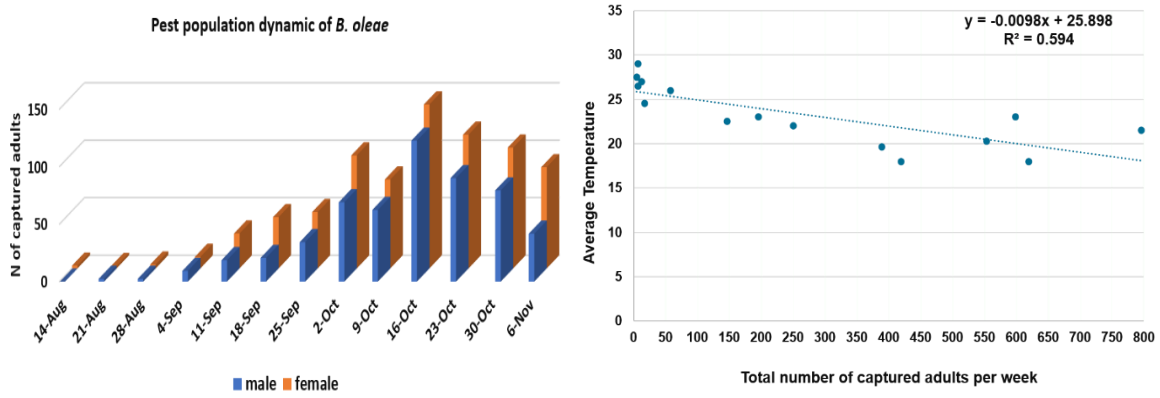

Figure II.8. (A) Average number of *Bactrocera oleae* adult captures (male and female) in yellow sticky traps during the monitoring period. (B) Relationship between the total number of captured adults and average temperature.

II.3.2 Infestation level in olive fruits

The insects detected in the fruits collected from the field were divided into three groups based on their stage as follows: egg+ 1st larval instar **L1**, 2nd larval instar **L2**+ 3rd larval instar **L3**, and pupae + galleries. The infestation level of *B. oleae* was evaluated, and the data showed that the percentage of the total (eggs+**L1**) was 0.02 individuals per fruit on 24th of July, while the percentage of the total 2nd and 3rd larval instars (**L2**+**L3**) was 0.00, the same goes for the percentage of the total (pupae + galleries) on the same mentioned date. However, the number of individuals gradually increased to reach the higher number of individuals detected (0.155 per fruit) for (eggs+L1) on 26th of September, whereas the higher density of (L2+L3) was 0.13 individuals per fruit, and for the (pupae + galleries) = 0.74 individuals per fruit, recorded on 16th of October (Figure II.9).

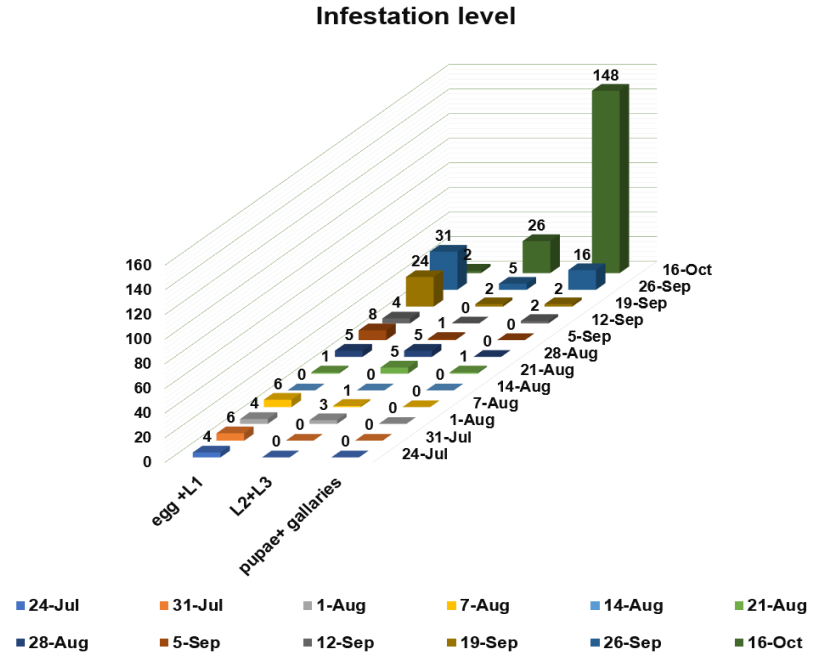

Figure II.9. Infestation level based on the detected instars in olive fruits starting from 24th July to 16th October.

II.3.3 Effect of applied treatments on insect development

The data presented in Table II.1 show the number of emerged adults by treatment and by date of collection.

Table II.1. Number of emerged adults from treated olive fruits.

A. The numbers of emerged adults from olives collected on trees				
N fruit/box	**Collection date**	**NPs**	**CaCol**	**Control**
200	03/10/2018	118	134	156
200	09/10/2018	112	144	166
200	16/10/2018	96	164	144
200	23/10/2018	140	138	141
200	31/10/2018	139	157	130
200	06/11/2018	108	124	132

B. The numbers of emerged adults from olives collected on covered branches				
N fruit/box	**Collection date**	**NPs**	**CaCol**	**Control**
200	16/10/2018	142	148	151
200	23/10/2018	136	144	150
200	31/10/2018	140	140	148

II.3.3.1 *Bactrocera oleae* adults emergence in olives collected on trees

Analysis of variance (ANOVA) revealed that the Calcium carbonate (NPs) had a significant effect on *B. oleae* development ($P < 0.05$). The $CaCO_3$ NPs treatment significantly affect the olive fruit fly development and reduced the number of newly emerged adults compared to colloidal calcium (CaCol) and control (Table II.2 and Figure II.10). Additionally, the analysis revealed that there was no statistically significant difference between the numbers of emerged adults from olives treated with colloidal calcium and those treated with water as a control (Figure II.10). Post hoc test revealed significantly effect of $CaCO_3$ NPs treatment compared to colloidal calcium CaCol and control (**$CaCO_3$ NPs**: Mean= 118.83, SEM= 7.16, Mean Diff (NPs vs. CaCol)= -24.67; **CaCol:** Mean= 143.5, SEM= 6.06, Mean Diff (CaCol vs. Control)= -1.33 and **Control:** Mean= 144.83, SEM= 5.69, Mean Diff (NPs vs. Control)= -26).

Table II.2. The One-way ANOVA for adults emergence of *Bactrocera oleae* from treated olives collected on trees.

Source of Variation	df	Sum Sq	Mean Sq	F-Value	P-Value
Treatment	2	2572.44	1286.22	5.334	**0.0178**
Residual	15	3617.16	241.14		
Total	17	6189.61			

*df. Degree of freedom; Sum Sq. sum of squares; Mean Sq. mean of squares.

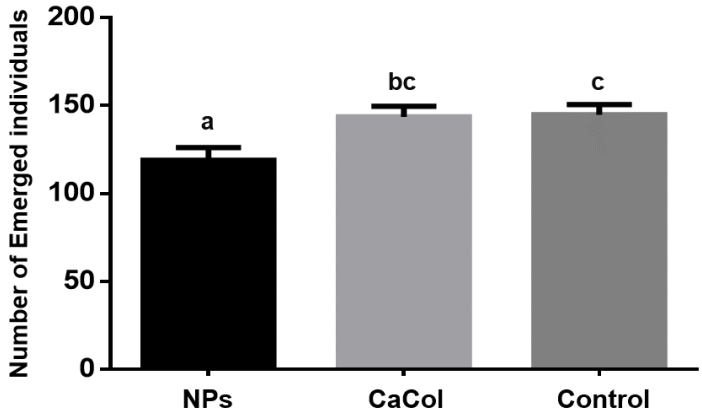

Figure II.10. Adults emergence of *Bactrocera oleae* (mean ± SEM) from olives collected on tree, treated with Calcium carbonate nanoparticles $CaCO_3$ (NPs) and colloidal calcium CaCol in comparison with control, Tukey HSD test P<0.05.

II.3.3.2 *Bactrocera oleae* adults emergence in olives collected on covered branches

ANOVA revealed that the application of $CaCO_3$ NPs significantly decreased adults emergence of *B. oleae* with respect to control ($P < 0.05$); the number of insects from fruits treated with $CaCO_3$ NPs was lower than that of insects emerged from untreated fruits (Table II.3 and Figure II.11). However, the number of new individuals that emerged from fruits treated with $CaCO_3$ NPs, and that of fruits treatd with CaCol was similar (Figure II.11). Post hoc test revealed the existence of a significant difference between $CaCO_3$ NPs and control, however there were no significant differences among other treatments (**$CaCO_3$ NPs**: Mean= 139.33, SEM= 1.76, Mean Diff (NPs vs. CaCol)= -4.66; **CaCol:** Mean= 144, SEM= 2.30, Mean Diff (CaCol vs. Control)= 5.66 and **Control:** Mean= 149.66, SEM= 0.88, Mean Diff (NPs vs. Control)= 10.33).

Table II.3. The One-way ANOVA for adults emergence of *Bactrocera oleae* from treated olives collected on covered branches.

Source of Variation	df	Sum Sq	Mean Sq	F-Value	P-Value
Treatment	2	160.66	80.33	8.71	**0.0168**
Residual	6	55.33	9.22		
Total	8	216			

*df. Degree of freedom; Sum Sq. sum of squares; Mean Sq. mean of squares.

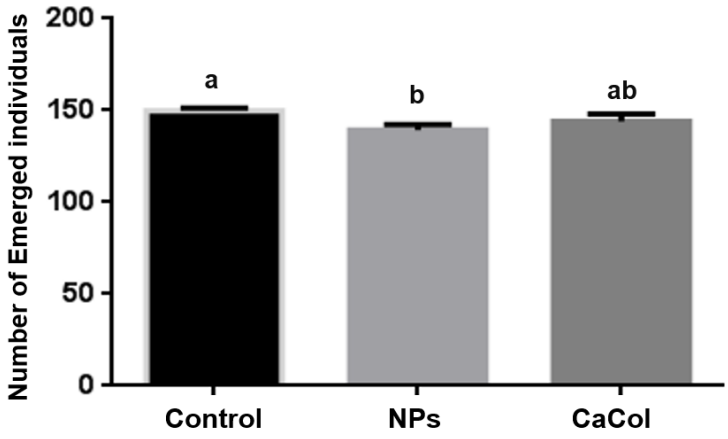

Figure II.11. Adults emergence of *Bactrocera oleae* (mean ± SEM) from olives collected on covered branches, treated with Calcium carbonate nanoparticles $CaCO_3$ (NPs) and colloidal calcium CaCol in comparison with control, and exposed to wild adult flies, Tukey HSD test P<0.05.

II.4 Discussion

The results of this research showed that Calcium carbonate (NPs) significantly reduce *Bactrocera oleae* development in their early life stages such as eggs and L1 instar. We suppose that uptake of NPs significantly decreased development of the immature stages, this finding is consistent with those obtained from studying the effect of Calcium carbonate (NPs) against *Bactrocera dorsalis* (Hua et al., 2015), that belongs to the same genus of our target insect ; the aforementioned authors reported that field tests of Nano-Calcium particles reduced the oriental fruit fly populations in their early stages, and performed well in terms of pest control and plant protection.

During field monitoring, climatic conditions played an important role in *B. oleae* populations dynamic, growing and dispersal as well. Temperature seems to determine *B. oleae* density level in the olive orchard because the number of adults captured was lower both at high (> 26 °C), and at lower temperatures (<18 °C), and the optimal temperature range for insect development ranged from 18 to 22 °C, with related humidity 64% ± 2. This result is similar to those obtained in an early study by Genç and Nation (2008), who reported the effect of temperature and RH on olive fruit fly *B. oleae* lifetime, oviposition and mating behavior,

development period and eggs hatching. Furthermore, the newly applied technique by covering the olive branches with muffs early in the season, made it possible to keep the olive fruits fresh longer and free of *B. oleae* infestations.

It is renowned that calcium carbonate involves 4% of the earth's crust. generally this component is primarily used in fertilizers for agriculture and forestry and numerous studies indicated that Ca sprays can reduce the severity of some post-harvest pathogens such as gray mold (Botrytis blight) in table grape, strawberries and rose flowers (Naradisorn et al., 2004; Nigro et al., 2006; Samarakoon and Faust, 2019; Volpin and Elad, 1991). Moreover, mineral particles in general attach to the insect body surfaces and produce cuticle abrasions leading to biological and behavioral changes, including the reduction of moving, feeding, and oviposition (Glenn and Puterka, 2005). In the present study, direct field application of Calcium carbonate NPs on the olive trees and also on the covered olive branches reduced the fly infestation; by decreasing the survival of immature stages (larvae and pupae), as well as for the application of colloidal calcium compared to the control (tap water). The obtained results are in line with Chen et al. (2015) and Araj et al. (2016), who reported that the uptake of different types of NPs affect the dipteran *Drosophila melanogaster*, causing a reduction in the survival of immature stages and female fecundity, as well as the developmental delay of their offspring. Our findings confirmed the hypothesis of efficacy of NPs to decrease fruit flies development.

Insect's cuticle or waxy layer, composed of a mixture of lipids, represents the first barrier to environmental threats. When this cuticle is covered with external smaller particles, like nanoparticles, they are absorbed into the cuticular lipids of the insects and subsequently cause damages to the protective wax layer leading to death by dehydration. Nair et al. (2010) have documented that nanoparticles (nano-silica) can cause damages to stored-product beetles of maize and wheat by absorbing the waxy cuticular layer of the beetles which leads to their death.

What is also worth noticing in our results, is the significant effect of $CaCO_3$ NPs on pest reduction. Our finding could be due to the fact that nanoparticles have a greater affinity

towards the insect cuticle, having at least one dimension less than 100 nm. This provides better coverage and adhesion of smaller particles, as well as higher solubility and mobility.

Moreover, the present study reported that the control by using NPs sprays on both covered branches (extensive treatment 3-times) and olive trees (2-times treatment) showed a similar reduction in the development of the early stages of olive fruit fly, although the variation in treatment repetitions. This indicate that the $CaCO_3$ NPs could provide phytosanitary efficacy with less spraying.

The current results observed the same trend as other studies that elucidated the effects of NPs in pest control compared to classical formulations. Mesbah et al. (2017) in studies on Nano-silica particles (NSPs), evaluated the efficacy of Nano-silica compared to the normal silica, against the bruchid beetle *Callosobruchus chinensis* (Linnaeus); Nano-silica NSPs were found to be more effective against *C. chinensis*, and showed more pick-up of silica particles. Also, Boehm et al. (2003) estimated the efficacy of nanosphere (NS) formulations to control aphids in cotton fields, compared to a classical suspension used as a reference. They found that NS formulations were better for reinforcing the systemicity of the active ingredient and improved the penetration through plant tissues due to their small size.

Calcium carbonate is an important ingredient used as plant protection product in fertilizers, in organic farming, but also for pre-harvest application for controlling some diseases like *Botrytis*. In fact, Calcium formulas used in this study have the advantage of lower resistance compared to the commonly used insecticides against the olive fruit fly, including organophosphates and pyrethroids. Another benefit is that they are relatively safe for human and environmentally friendly compared to conventional pesticides, though, it can be harmful to human health only in concentrated solid form or in very concentrated solutions.

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III. LABORATORY BIOASSAYS TO STUDY THE EFFECT OF CALCIUM CARBONATE FORMULAS ON *BACTROCERA OLEAE*

Although field studies have been made, the effectiveness of used materials (Calcium carbonate NPs and colloidal calcium) need further investigation. Therefore, the aim of the present study was to investigate the effects of Calcium carbonate NPs and colloidal calcium CaCol on adult's longevity and immature developmental stages of *Bactrocera oleae*, under laboratory conditions.

III.1 Materials and methods

III.1.1 Laboratory rearing and procedures used for colony maintenance

The *B. oleae* colony was established in a separate room at the laboratories of Tuscia University- Dipartimento di Scienze Agrarie e Forestali (DAFNE). Ripe and unripe infested olive fruits bearing the eggs of *B. oleae* were collected from an orchard located in Lazio region (Viterbo, 01100 VT 42.410092, 12.078059). Infested fruits were placed in transparent plastic bags and transferred to special rearing cages (Ø = 30 cm, h = 40 cm) to maintain them in a controlled environment: 26 ± 1°C under a 18:6 (L: D) photoperiod and 64% RH ± 2 (Figure III.1).

Figure III.1. Infested olive fruits collected from orchards.

The *B. oleae* adult flies were reared on fresh olive fruits after conditioning them in ventilated cylindrical cages made of Plexiglas (Figure III.2). The cage dimensions were Ø = 30 cm, h = 40 cm with a small side-window, removable bottom and the top is closed by 0.5 mm-mesh tulle (Sime et al., 2006). The adults were provided with a special diet *ad libitum*; the feeders used in rearing are made up of a glass cylinder container (Ø=3 cm and h=10 cm) and the top is closed by plastic screw. The diet has been used and developed by Professor Claudio Pucci in 2007 (Baratella, 2011). It consists of 1 mL of honey mixed with 9 mL of sterilized distilled water, and is considered to be healthy providing important compounds for the insect productivity (Genç, 2014).

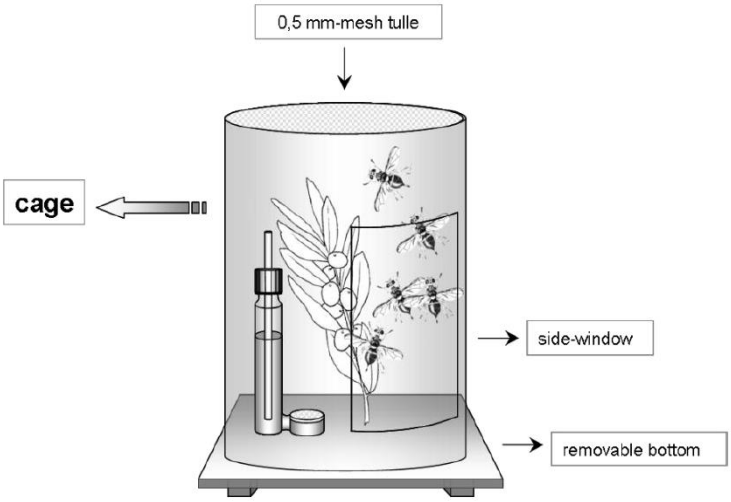

Figure. III.2. Cylindrical cages made of Plexiglas Ø = 30 cm, h = 40 cm and covered with mesh tulle, contain the diet feeder, used for adults rearing processes.

Some olive twigs were selected weekly from covered branches free of infestation; described in Chapter II. The branches were deprived of leaves to prevent a quick dehydration of olive fruits, then were disposed into a plastic vase containing water, and were placed in the cage as an oviposition substrate (Sivinski, 1992). The nutrition solution was added into the feeder and as soon as the feeder is filled up, it starts pouring into a little tray (Ø=4 cm, h=1 cm) connected through the bottom of the container. The little tray was plugged up with a porous material (as shown in Figure III.3) to have a slowly surfacing on the nutrition solution, allowing the adults to feed comfortably (Baratella, 2011). In order to avoid solution overflow, a glass

pipe must pass through the container's cap. This pipe has a free end outside of the container cap (Figure III.4). The feeders were cleaned up weekly, first with sodium hypochlorite, then soaked into water for 3 hours and once again washed in abundance of water. Moreover, common detergents were used to clean the rearing cages when needed.

Figure III.3. The feeder made up of a glass cylinder container (Ø=3 cm and h=10 cm) and a tray within the base plugged up with a porous material to let the adults feeding comfortably on a diet solution (water : honey, 1:10).

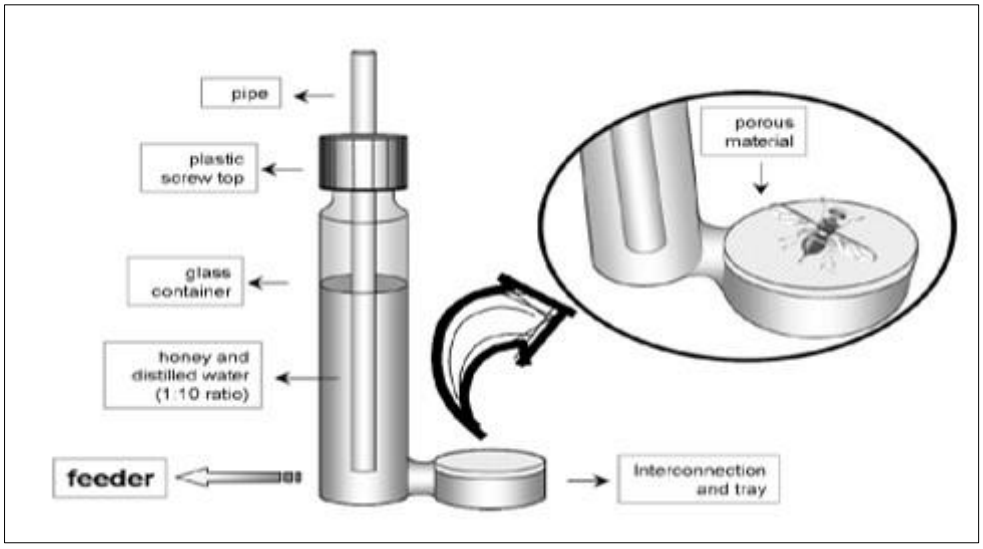

Figure III.4. Scheme of the feeder container used to feed olive fruit fly.

After incubation period, eggs were hatched in 2 - 3 days and the larvae completed their development and pupated in 10 - 20 days, then these pupae were allowed to emerge. Later, the emerged adults were displaced separately in a proper way to three cages, in order to maintain the new individuals in each generation. Fresh olives (200) were collected at weekly intervals from the covered branches and were exposed to female *B. oleae* (100 individuals) for 30 days. The adult flies were provided regularly with abundant nutrients, to avoid the effect of food competition that may influence the longevity and fecundity of the insects (Baratella, 2011).

III.1.2 Samples preparation

The experiment was designed to investigate the effect of the colloidal calcium CaCol and Calcium carbonate $CaCO_3$ nanoparticles (NPs) on egg oviposition of *B. oleae* and their development. The experiment was performed in laboratory conditions at temperature of 22 ± 2°C and relative humidity (RH) of 40 ± 10%. Three experimental cages (L= 35cm, W= 20cm and H= 13cm) were established simultaneously as follows: cage A contained olive fruit flies to be treated with nano-material Calcium carbonate $CaCO_3$ NPs, cage B contained *B. oleae* to be treated with colloidal calcium, and cage C contained insects to be treated with water as a control (Figure III.5).

Adult flies inside each cage (A, B, and C) were supplied with deprived twigs (the leaves were removed), containing 50 uninfested fruits per cage as a substrate for flies oviposition and larval development. Olive fruits were previously examined to ensure that none of them were infested. Then, ten adult flies (10-14 days old) were added to each experimental cage consisting of 6 females and 4 males per cage. The density is an essential factor for survival and insect fertility (Ben-yosef et al., 2015; Hua et al., 2015).

Figure III. 5. Left; is the experimental cage containing *B. oleae* to be used in the assay. Right; the experiment design with 3 replicates per each treatment; all the cages have similar dimensions; L= 35cm, W= 20cm and H= 13cm, each cage contains 50 fruits to be treated with a specific product, exposed to the flies and incubated until the emergence of new flies.

III.1.3 Application of colloidal calcium and Calcium carbonate ($CaCO_3$ NPs)

III.1.3.1 Suspensions preparation

The suspension of Calcium carbonate ($CaCO_3$) nanoparticles (NPs) with nominal diameter 58 nm (20-100 nm), was purchased from BIOSCINTIFICA S.R.L., (Via Flaminio-ITALY). All the products were diluted according to the mean doses as suggested by the producers for field applications. The treatment suspension is mainly composed of water and Nano-Calcium and was prepared by dissolving the small particles of Nano-Calcium $CaCO_3$ (NPs) in distilled water, in order to obtain the final volume of $CaCO_3$ (NPs) with a concentration of 3g/L (Bigiotti et al., 2019; Hua et al., 2015).

Colloidal calcium CaCOl suspension was prepared by suspending the concentrated solution of colloidal Ca in distilled water (16 ml/L). This concentration of colloidal Ca is registered and recommended by the manufacturer company (Serbios S.R.L.), as a sufficient fertilizer to manage a lot of problems occurring in several vegetable and tree crops such as (Olive, Melon, Maize, Rise, Potato, Pepper, Tomato, Eggplant, Citrus and many other plants in Italy).

III.1.3.2 Treatments application

After preparing the suspensions, the experimental cages were treated with the suspended materials. Each cage that contained the same number of insects and olive fruits (10 flies and 50 olive fruits), was treated with a specific suspension; cage A was treated with $CaCO_3$ (NPs), cage B was treated with colloidal calcium CaCOl, and cage C with tap water as a control. Fruits in each cage were treated, using commercial hand-held sprayers,with the same volume of products 500 mL (Bittelli et al., 2001). Indeed, this extensive treatment gave a good opportunity to inspect the indirect lethal effect i.e. (mortality) of the components used against *B. oleae* adults.

The experiment was replicated three times, within three weeks of treatment date, the treated olives were removed every week from all the experimental cages, and replaced by new 50 uninfested olive fruits. Simultaneously, collected fruits (treated) were transferred to new small separated boxes to maintain them until the eggs laid in the treated fruits, hatch. The new boxes were labelled with date and type of treatment and conserved during 21-25 days under controlled conditions, at 26 ± 1°C, 18:6 (L: D) photoperiod and (64% RH).

Despite of the conservation process of infested fruits, some pupae were found on the floor of the boxes. All these pupae were moved to new boxes containing sterilized soil covered with moistened paper towels, which were soaked with distilled water when required (Figure III.6), in order to provide a suitable media for pupae development and emergence of adults (Genç and Nation, 2008).

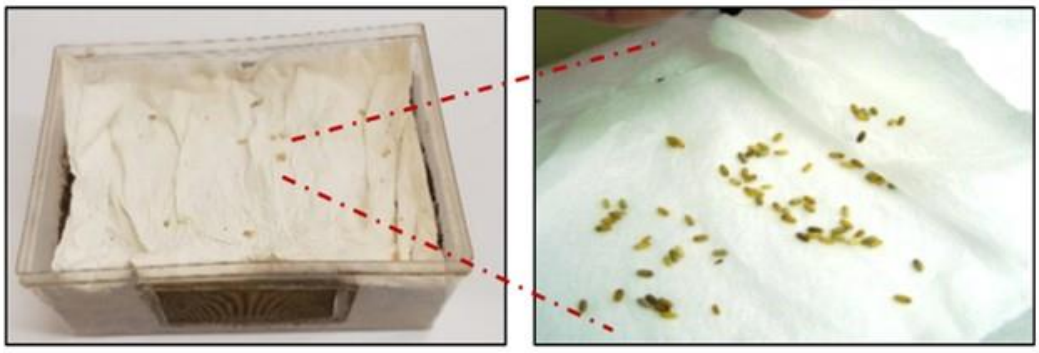

Figure III.6. Pupae collected from experimental cages and placed in new boxes supplied with sterilized soil and moistened paper.

Additionally, several fruits treated with $CaCO_3$ NPs, colloidal calcium, and water respectively (20, 18 and 12 olives) subsampled of the 50 olive fruits exposed to the attack of *B. oleae*; were placed in transparent plastic bags separately and conserved in a freezer at -20. This technique was performed to investigate the eggs and immature stages of olive fruit fly inside of the infested fruits (Yokoyama and Miller, 2004). This technique gave a satisfactory way for the observation and to see the immature stages, especially the eggs and 1st-instar larvae of *B.* oleae within the infested olives.

III.1.4 Evaluation of treatments effect on insect development

After 21-25 days of fruit exposition to *B. oleae* flies, the adults have emerged from the treated fruits and conserved in small boxes in the climatic chamber. Emerged flies were killed using aerosol insecticide. The impact of $CaCO_3$ NPs and CaCOl on insect development was studied by counting the number of developed and newly emerged individuals for each experimental cage operated with a given suspension. Also, treated fruits in each experimental cage were examined under an optical microscope (Motic Oculare- W10X/20) (Figure III.7). Data were recorded in a Microsoft Excel sheet and analyzed using statistical software NCSS 2020 (NCSS, LLC) by performing Repeated Measures ANOVA ($P < 0.05$), followed by Tukey-Kramer test for means comparisons.

Figure III.7. Detection of *B. oleae* eggs in treated fruits under an optical microscope.

III.2 Results and Discussion

The insects were checked weekly after fruit treatments with Calcium carbonate $CaCO_3$ NPs, colloidal calcium and water (control); and the results revealed that there was no effect on the lifespan of adult flies and all the tested flies remained alive during the tested period. Based on this finding, we suppose that the success of applied components on insect mortality is extremely based on the concentration of the used components. This fact was reported in a previous study by Alif Alisha and Thangapandian (2019), who conducted a comparative bioassay, testing different concentrations of silver nanoparticles and malathion, to control red flour beetle *Tribolium castaneum* (Herbst). Moreover, Rouhani et al. (2012), in their studies on silver and zinc nanoparticles against the oleander aphid, *Aphis nerii* (Fonscolombe), compared to Imidacloprid used as a conventional insecticide. They reported that these two nanoparticles could exert an effective pest control approach for *A. nerii*, although insect mortality using nanoparticles was slightly less than imidacloprid, one key fact is that mortality increased significantly with increase in NPs concentrations. Our results showed that there were no differences between the treatments; Calcium nanoparticles (NPs), colloidal Ca and tap water (control) with respect to insect mortality during the experimental period.

In fact, this study was carried out based on several researches that have studied the effects of other nanoparticle materials, and have shown that nanomaterials negatively affect the target insects by decreasing body weight, also interrupting the activity of detoxifying enzymes and inducing oxidative stress in larval guts, as put forward by Yasur and Pathipati (2015).

As a result, the data revealed that the number of new individuals emerged from fruits treated with Calcium carbonate nanoparticles was lower than the number of adults emerged from fruits treated with other materials (colloidal calcium and tap water). Consequently, the averages of emerged individuals from fruits treated with $CaCO_3$ NPs and CaCol were 23 and 28, respectively, whereas for untreated fruits (control) the average was 32 (Table III.1).

Table III.1. Number of new adults emerged from olive fruits treated with different materials (Calcium carbonate NPs, colloidal calcium and control).

Number of treated 50 fruits/ Cage	Emerged individuals (NPs)	Emerged individuals colloidal calcium	Emerged individuals (control)	Repetitions
	31	39	40	**Rep1**
	30	33	36	**Rep2**
	8	12	20	**Rep3**
	23	**28**	**32**	**Average**

However, a one-way repeated measures ANOVA showed that there is no significant effect on insect development in treated olive fruits when comparing Calcium carbonate (NPs) and colloidal Ca treatments. Moreover, our results demonstrated that there was no significant difference between the number of individuals emerged from olives treated with colloidal calcium and the number of individuals emerged from untreated fruits (control). Although the application of Calcium carbonate (NPs) showed a reduction in the number of emerged adults, ANOVA showed no significant effect when comparing Calcium carbonate (NPs) treatment and control (Table III.2 and Figure III.8). Tukey-Kramer test revealed a non-significantly effect of $CaCO_3$ NPs treatment compared to CaCol and control **: $CaCO_3$ NPs**: Mean= 23, SEM= 7.5, Mean Diff (NPs vs. CaCol)= -5; **CaCol:** Mean= 28, SEM= 8.18, Mean Diff (CaCol vs. Control)= 4 and **Control:** Mean= 32, SEM= 6.11, Mean Diff (NPs vs. Control)= 9.

Table III.2. The One-way repeated measures ANOVA for adults emergence of *Bactrocera oleae* from olives treated under laboratory conditions.

Source of Variation	df	Sum Sq	Mean Sq	F-Value	P-Value
Treatment	2	122	61	0.379	0.699
Residual	6	964	160.66		
Total	8	1086			

*df. Degree of freedom; Sum Sq. sum of squares; Mean Sq. mean of squares.

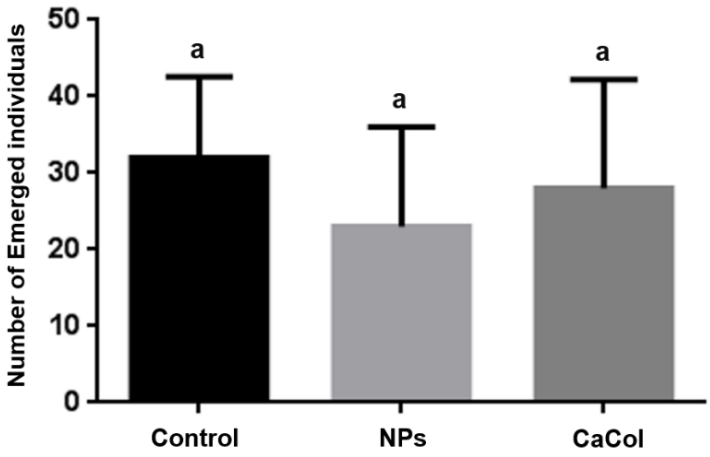

Figure III.8. Number of developed and newly emerged individuals from 50 olive fruits treated with different materials; control = number of adults emerged from olive fruits treated with tap water. NPs = number of adults emerged from fruits treated with Calcium carbonate nanoparticles. CaCol = number of adults emerged from fruits treated with colloidal calcium.

Laboratory experiments were carried out to verify the field application (discussed in Chapter. II), and to further study the impact of applied materials (Calcium carbonate NPs and colloidal calcium) on pest control. These compounds have been used in previous studies as foliar fertilizers for several plants (Hart, 1998). Our laboratory findings revealed that Calcium carbonate NPs reduced *B. oleae* adult's emergence, but had a lower effect compared to the field results. Moreover, there was no direct effect of applied products on insect mortality. The resulting effect could have occurred due to the influence of some factors such as the suspensions concentration, the artificial diet, the relationship olive fruits/adult flies and the fly physiology, beside the fact that treatment application was performed two times in the field assay; which resulted in higher efficacy against *B. oleae*. Lacey and Kaya (2007) quoted that "Some important factors may differ greatly between the field and laboratory".

III.3 References

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IV. STUDY OF THE EFFECT OF APPLIED MATERIALS ON THE BACTERIAL ENDOSYMBIONT "*CANDIDATUS* ERWINIA DACICOLA" AND ON EGG PRODUCTION OF *BACTROCERA OLEAE*

IV.1 Introduction

The importance of interaction between insect pests and their symbionts has been increasing in plant protection, to find out new and developed practices for sustainable integrated pest management (Nobre, 2019). Study of the relationship between the olive fruit fly, *Bactrocera oleae,* (Rossi, 1790) and its bacterial endosymbiont "*Candidatus* Erwinia dacicola" is an essential step toward performing efficient control of the insect pest population in the field. This bacterium is present in all life stages of *B. oleae,* and plays a crucial role in insect biology, allowing the larvae to survive inside unripe olive fruits, this by overcoming the "Oleuropein", a defensive polyphenolic compound produced by the olive tree (Ben-Yosef et al., 2015). Also, several studies have assumed that this bacterium has a great contribution to the fecundity of *B. oleae,* mating behavior and its fitness (Ben-yosef et al.,2010; Sinno et al., 2017). Consequently, interrupting this symbiosis relation between *B. oleae* and its symbiotic bacterium *Erwinia dacicola* might affect and reduce the population of olive fruit fly in the field.

Calcium carbonate ($CaCO_3$) and colloidal calcium are well known as fertilizers in horticultural crops all over the world. Calcium amendments has been used in agriculture to reduce the severity of gray mold (*Botrytis* blight) in rose flowers (Volpin and Elad, 1991), strawberries (Naradisorn et al., 2004) and table grape (Nigro et al., 2006); and to control bacterial wilt caused by *Ralstonia solanacearum* in tomato seedlings (Yamazaki, 2001). In the present study, we first investigated the effectiveness of Calcium carbonate (NPs) and colloidal calcium formulas against the targeted endosymbiont "*Candidatus* Erwinia dacicola", by evaluating the bacterial abundance in wild olive fruit fly population under laboratory conditions. Second, we evaluated the effect of these formulas on *B. oleae* female's fertility based on eggs production.

IV.2 Materials and methods

IV.2.1 Samples collection and rearing procedures

The wild olive flies were obtained by collecting the fruits suspected to be infested and contained the immature stages of the insect. Samples collection began in September 2019, and the fruits were brought from two olive orchards located in Viterbo-Lazio (the experimental orchard of Tuscia University), and in Perugia-Umbria. The fruits were examined visually and chosen olives contained unopened galleries created by the larvae, and most of the collected fruits contained the pupal stage (Figure IV.1). The collected fruits were placed in transparent plastic bags. Bags were kept open in order to avoid fungal growth, as well as to maintain the freshness of olives (Bigiotti et al., 2019). Harvested fruits were transferred to the laboratory (University of Tuscia, DAFNE Department) in order to start the rearing of the olive fruit fly under laboratory conditions.

Figure IV.1. Collecting the infested olive fruits to be used for insects rearing and propagation.

The infested fruits were sited into a special cubic rearing cage (L 55cm×W 55cm × H 75cm). The cage consisted of wooden base and the roof was covered with a plastic screen containing tiny holes to provide the adequate ventilation for the reared insects and avert the insect from escaping (Baratella, 2011; Domínguez, 1996; Hooper and Robinson, 1989). Moreover, the collected fruits were positioned in small plastic boxes (20cm×15cm×10cm) with sterilized soil in their bottom and covered by a soft film of moistened paper that serves as a proper surface for pupation (Genç and Nation, 2008) (Figure IV.2).

Figure IV.2. The small boxes containing a moistened soil where olive fruits were placed above the paper until the emergence of new adults.

In addition, collected fruits in the small boxes were moistened periodically every two days using a handled water pump spray, to prevent fruits hardening which might affect the development of larval stages of the fly (Baratella, 2011; Bigiotti et al., 2019; Domínguez, 1996). Also, the cage was supplied with nutrients feeder designed to be suitable for conducting the rearing process (Baratella, 2011; Cáceres et al., 2016). The feeder was provided with a specific diet for the reared insects composed of: honey mixed with distilled water (1/9 v/v), the diet is described as suitable for the insect's fitness and productivity as well for insect's development and maturation (Domínguez, 1996; Ras et al. 2017).

Inside the rearing cage, small plastic containers (250 mL) containing fresh olives attached on small twigs (deprived of leaves) was supplied (Baratella, 2011). The fruits in twigs were replaced periodically with new fresh ones collected from olive trees at the University of Tuscia (Viterbo), and the old fruits were discarded after the flies complete their development within these fruits. The collection of infested fruits was continued until the end of October 2019, and most of the adults have emerged between 7-9 days after fruit collection, obtaining enough number of adults to start the experiment.

IV.2.2 Experiment design

The experiment was performed at the laboratory of Dipartimento di scienze Agrarie e Forestali (DAFNE); University of Tuscia, Viterbo-Italy. The experiment involved three replicates for each treatment and control, in order to investigate the effects of Calcium carbonate nanoparticles and colloidal calcium (via oral route), on the symbiotic bacterium *E. dacicola*, which is present in the digestive system of all stages of wild olive fruit fly *B. oleae*.

The experiment consisted of three cylindrical (tube-shape) cages of 30 cm in diameter and 40 cm high (Baratella, 2011). Twelve reared adult flies, six males and six females, were collected from the main rearing cage and transferred to each experimental cage. Adult flies were carefully selected to be around the same age (10-15 days) as well as for fitness status. The flies were kept in the laboratory under controlled conditions at room temperature of 16 ± 2°C, under a 14h /10h (L:D) photoperiod (Bigiotti et al., 2019; Kounatidis et al., 2009). Each cage was supplied weekly with 30 olives (infestation-free) attached on small twigs, that were placed in a plastic vase containing distilled water, and used as an oviposition substrate for adult flies (Figure IV.3).

Figure IV.3. Fresh olive fruits serve as an oviposition substrate for the adult flies.

The $CaCO_3$ was prepared by dissolving 1gr of concentrated Calcium carbonate nanoparticles (NPs) in 99 mL of distilled water to yield a suspension of 0.01 M of $CaCO_3$ (NPs) following the manufacturers' instructions. Regarding the colloidal Ca, the treatment was prepared by diluting 1 mL of colloidal Ca in 99 mL of distilled water (the same volume of water) to give a suspension concentration of 0.01 M of colloidal calcium.

Diluted products were mixed with insect nutrients; 1mL of honey was added to 9 mL of each diluted product, which serves as a food source for the flies (Bigiotti et al., 2019; Sacchetti et al., 2008). Later, the final solutions (nutrients mixed with products) were transferred into three feeders. The first feeder (treatment 1) contains honey / $CaCO_3$ NPs (1:9 v/v), the second feeder (treatment 2) contains honey / colloidal calcium (1:9 v/v) and the third feeder containing honey mixed with tap water (1:9 v/v) as a control (Figure IV.4). Diet was replaced frequently (every 4 days) with a new mixture and the feeders were washed using sodium hypochlorite to prevent mold growth.

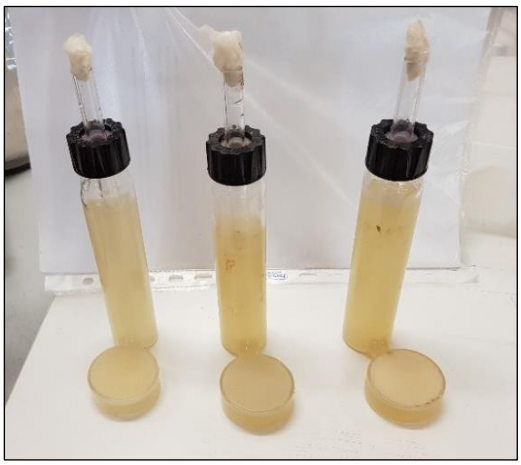

Figure IV. 4. Insect treatment by mixing the products with diet.

Adult flies have been tested during 27 days. During the experiment period, the treated flies were observed very frequently "three times a day" and day by day, to check and record adult mortalities. All replicates were placed randomly on a table and exposed to the same photoperiod (14h L and 10h D) using the neon lamp for each cage (Raspi et al., 2005).

IV.2.3 Insect and egg collection

Insect collection was performed at different intervals; treated flies were collected after the first, second and third week of exposure date, respectively, for each cage. Therefore, an equal number of treated flies; 4 individuals (2 males and 2 females) were randomly sampled with three replicates. Collected adults were transferred into Eppendorf tubes 2mL, and killed by exposing them to a low temperature degree (-18) for 5 minutes (Figure IV.5).

The experimental cages were checked daily and the new eggs were collected for each cage. The total number of eggs was counted to estimate the egg production and compare the effectiveness of different products on female's fertility.

Figure IV. 5. Adult flies collected from the cages were exposed to a low temperature and prepared for DNA extraction process.

IV.2.4 Insect dissections

Adult flies were extracted from the Eppendorf tubes, washed with a 2% sodium hypochlorite, rinsed with distilled water and then placed on tissue paper for 5 min to dry them. Subsequently, fly heads were carefully dissected under a microscope, the eyes were removed from the heads to avoid factor inhibitors that could affect negatively the DNA amplification process (PCR). This procedure was performed using convenient materials and techniques under Kenton JHP-2 laminar flow hood (Sacchetti et al., 2008).

IV.2.5 Bacterial DNA extraction

Different preliminary tests were run in order to estimate the sufficient amount of bacterial DNA for better extraction. Wild adult flies used in these tests were obtained from infested untreated olive fruits (not included in the experiment design). Six Eppendorf tubes were used and each tube contained a different number of wild *B. oleae* heads (tube A=1 head, B=2, C=4, D=8, E= 10 and F= 20) respectively. The heads were gently squeezed with a sterilized pipette tip and then relocated into Eppendorf tubes containing 100 µL NucleoSpin® DNA Insect (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. The

number of *B. oleae* heads to be used for bacterial DNA extraction will be determined based on a PCR analysis as described below.

IV.2.6 PCR analysis

The success of DNA extraction was confirmed by performing a preliminary polymerase chain reaction (PCR) targeting the 16S region in the rDNA of "*Ca.* Erwinia dacicola", using the DNA products from *B. oleae* head samples. PCR was performed using two different pairs of universal primers; forward primers 10F (5′-AGTTTGATCATGGCTCAGATTG-3′) and EdF1 (5'- CTAATACCGCATAACGTCTTCG-3) pairing with a reverse primer 1507R (5′-TACCTTGTTACGACTTCACCCCAG-3′) to generate nearly (~1300 bp) (Estes et al., 2012; Sandström et al., 2001). The reactions were run to detect the bacterial DNA in different numbers of head samples (1, 2, 4, 8, 10 and 20 heads respectively), positive and negative controls were also included in the PCR.

The PCR reactions were carried out using a C-1000 Touch™ Thermal Cycler (Bio Rad, USA). The PCR was standardized to amplify the 16S region, in 25 µL final volume containing: 2 µl template DNA (at a concentration of 5 ng), 12.5 µl of 1×IQ SYBRGreen Master Mix, 1 µl each primer and 4.6 µl sterile RNase-free water. The reaction involved an initial denaturation at 95°C for 2 min, followed by 35 cycles in series of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 90 sec, with a final step of one cycle at 72°C for 90 sec to final extension.

The PCR amplification products were separated by electrophoresis on a 1.5% agarose gel in 1.0× Tris-acetate-EDTA (TAE) buffer, at 80 V for 45 min, and then visualized after staining with Midori Green Advance DNA Stain, using a Biostep GmbH UV Transilluminator UST -20M- 8K. The GeneRuler™ 1 kb DNA Ladder, ready-to-use (Thermo Scientific™ Fermentas) was used as a molecular size marker.

IV.2.7 Real-time PCR analysis

Quantitative real-time PCR was performed in order to quantify the bacterial DNA of *Erwinia dacicola* in insect samples (heads), previously treated with the target products and

collected at different intervals. qPCR was used to determine variation in the abundance of *Ca*. Erwinia dacicola detected in treated olive fruit flies by making a proportion between the number of cycles (Ct) for each sample; the higher Ct values mean lower amounts of target DNA.

Amplifications were carried out using a Rotor-Gene Q - QIAGEN® Thermal Cycler with 10 µl reaction volumes consisting of 5 µl Xpert Fast SYBR Mastermix (Uni) –1mL (GriSP), mixed with 400 nmol/L (0.4 µl) of each primer and 2 µl of DNA sample. Two specific primers were used in the reaction, Forward primer EdF1 (5'-CTAATACCGCATAACGTCTTCG-'3), and Reverse primer EdEn (5'-CCACCTACTAGCTAATCCC-'3), targeting the 16S region in the bacterial rDNA (Estes et al., 2009; Munson et al., 1991). The amplifications were performed with the following conditions: denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec (Bigiotti et al., 2019). All reactions were run in triplicate for each sample (NPs, CaCol and control), negative controls were also included in each plot.

The fluorescence data were recorded at the end of hybridization stage and data were analyzed using the Rotor-Gene Q software version 2.02. Final report illustrated the cycle threshold (Ct) for each replicate in a sample. These Ct values were recorded on an Excel sheet to be analyzed statistically.

IV.2.8 Data analysis

Data analysis was performed to evaluate the efficacy of Calcium carbonate formulas; $CaCO_3$ NPs, CaCol plus control, based on females' egg production, as well as on the abundance of the endosymbiont *Ca*. Erwinia dacicola. Treatments effect was assessed at different intervals after treatment (1 ,2 and 3 weeks), with three replicates per each treatment. XLStat 2020 software (Addinsoft SARL) was used to check data for normality assumptions by using the Shapiro-Wilk test and data were analyzed using the two-way (factorial) analysis of variance (ANOVA), followed by the Tukey's multiple comparison test to determine significant differences between the mean values ($p < 0.05$).

IV.3 Results

IV.3.1 Adult mortality

All tested flies were not affected by applied products $CaCO_3$ NPs and CaCol, and no mortality was observed in *B. oleae* flies; they showed a survival similar to untreated flies (control). In this chapter, we investigated whether the longevity of *B. oleae* adults vary depending on the treatment modes (spraying or host feeding).

IV.3.2 Egg production

Data analysis (ANOVA) revealed a significant effect of the Calcium carbonate NPs and colloidal calcium CaCol on females' egg production ($P < 0.05$) (Table IV.1 and Figure IV.6A). The $CaCO_3$ NPs and CaCol treatments significantly reduced egg production compared to control treatment at different intervals: after 1 week, 2 and 3 weeks (Figure IV.6A). Furthermore, Calcium carbonate (NPs) treatment significantly decrease egg production of the female flies with respect to colloidal calcium treatment; NPs showed a higher reduction in eggs after 2 and 3 weeks of exposure date. In contrast, colloidal calcium affected the egg production after the 1st and 2nd week, while after the 3rd week; the number of eggs was higher but similar to the control (Figure IV.6B).

Table IV.1. The Two-way ANOVA for egg production of *Bactrocera oleae* females, treated with different materials (Calcium carbonate NPs, colloidal calcium and control) through feeding.

Source of Variation	df	Sum Sq	Mean Sq	F-Value	P-Value
Treatment type	2	272.889	136.444	21.051	**0.000019**
Collecting interval [week]	2	28.667	14.333	2.211	0.138
Treatment type*Collecting interval [week]	4	79.778	19.944	3.077	**0.043**

*df. Degree of freedom; Sum Sq. sum of squares; Mean Sq. mean of squares.

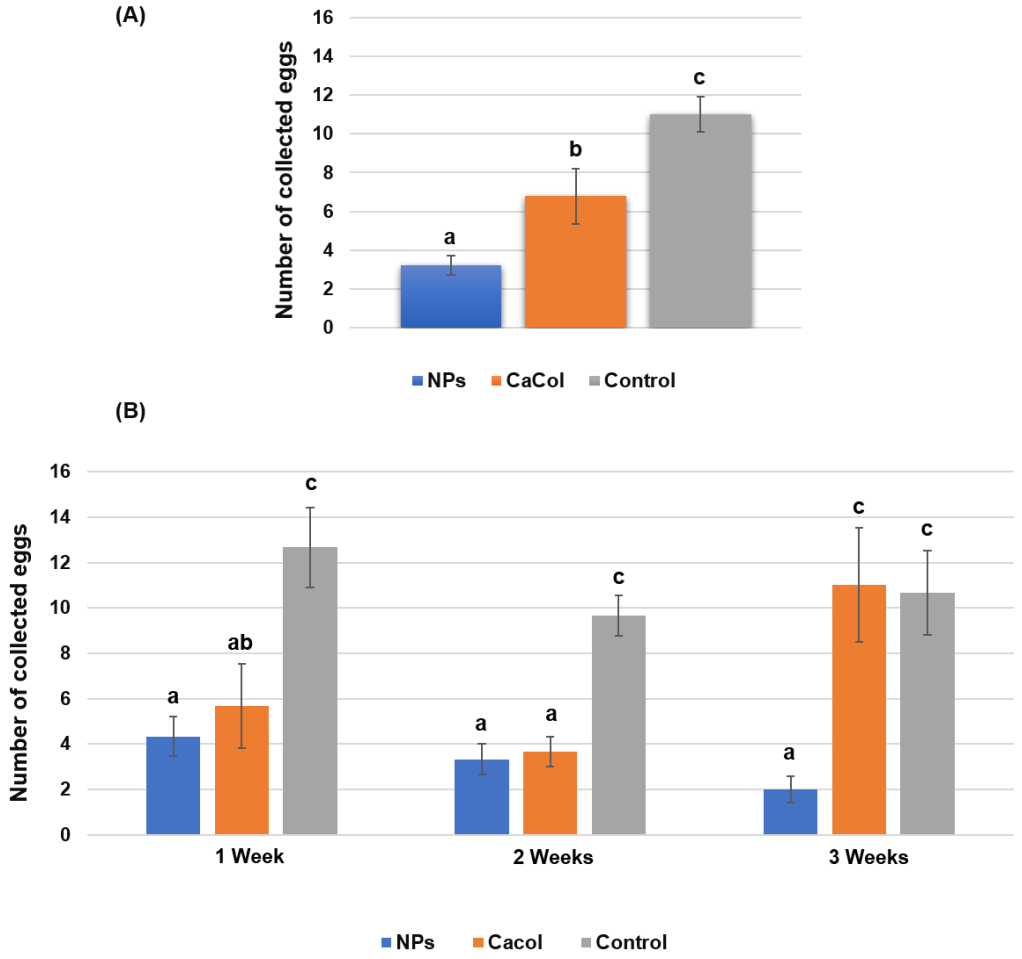

Figure IV.6. Number of eggs laid by female flies exposed to different products $CaCO_3$ NPs, CaCol and Control at different intervals after treatment (1 ,2 and 3 weeks). Data were analyzed by two-way analysis of variance (ANOVA): main effect (A) and interaction (B). Bars indicate standard error and letters above bars designate significant differences at $p < 0.05$ (Tukey's Honestly Significant Difference, HSD).

IV.3.3 Molecular detection and quantification of *Candidatus* E. dacicola

Visual examination of the gel images indicated the presence of *Ca.* E. dacicola. PCR products of all samples tested, consisting respectively of a different number of insect heads (1, 2, 4, 8, 10 and 20 heads), showed single bands of the expected size ≃ 1300 bp, obtained with EdF1 and 1507R primers, whereas no band was observed for the negative control (Figure IV.7).

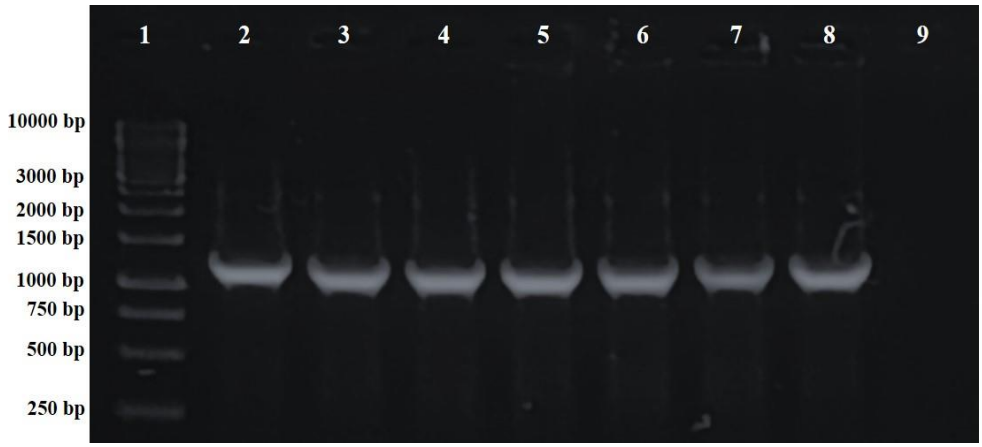

Figure IV.7. Agarose Gel Electrophoresis of 16S rDNA gene fragments of "*Candidatus* Erwinia dacicola", obtained by amplification with the EdF1/1507R primer set. Lane 1: 1 kb DNA Ladder; Lane 2: 1 head; Lane 3: 2 heads; Lane 4: 4 heads; Lane 5: 8 heads; Lane 6: 10 heads; Lane 7: 20 heads; Lane 8: positive control; Lane 9: negative control.

Real-time PCR illustrated the curves pattern for each sample that was exposed to different treatments and collected at different intervals (Figure IV.8).

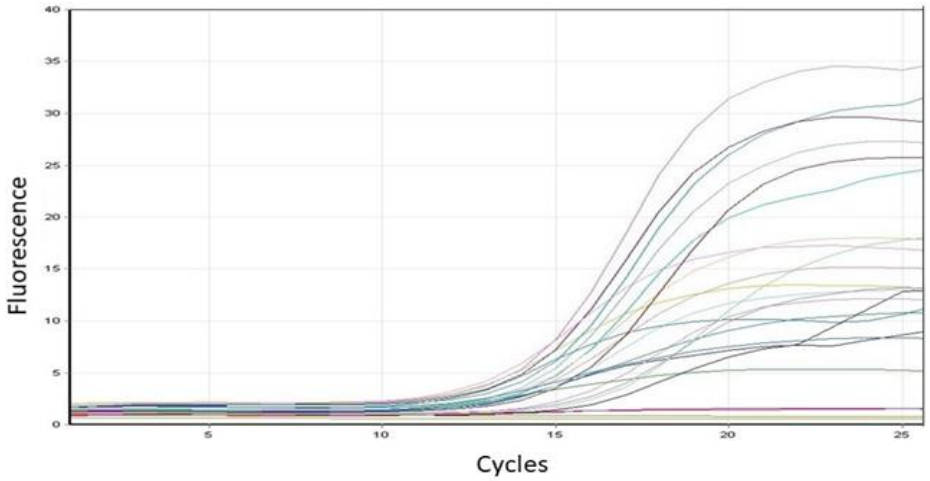

Figure IV.8. Real-time PCR amplification curves of the bacterial endosymbiont *Candidatus* Erwinia dacicola, extracted from different *Bactrocera oleae* adult samples at different intervals.

The Ct value was calculated for each replicate in a sample. As a result, the qPCR on the head samples revealed a negligible reduction in the bacterial abundance in *B. oleae* flies exposed to NPs and colloidal calcium treatments; they had approximately an equal abundance

of *Ca*. E. dacicola compared to the control, throughout the experimental period (Figure IV.9A). It is to note that adult flies treated with colloidal calcium showed a decrease in bacterial abundance after 2 weeks (Figure IV.9B). ANOVA analysis showed that there was no significant effect on the bacterial abundance of *Ca.* E. dacicola in adult flies treated with NPs and CaCol in comparison with non-treated adults (Control). In addition, no significant difference was noted between the adult flies exposed to NPs and those exposed to CaCol throughout the experiment (Table IV.2 and Figure IV.9B).

Table IV.2. The Two-way ANOVA for the abundance of *Candidatus* Erwinia dacicola, detected in *Bactrocera oleae* flies treated with different materials (Calcium carbonate NPs, colloidal calcium and control) through feeding.

Source of Variation	df	Sum Sq	Mean Sq	F-Value	P-Value
Treatment type	2	3.173	1.586	1.695	0.212
Collecting interval [week]	2	4.451	2.226	2.378	0.121
Treatment type*Collecting interval [week]	4	1.993	0.498	0.532	0.714

*df. Degree of freedom; Sum Sq. sum of squares; Mean Sq. mean of squares.

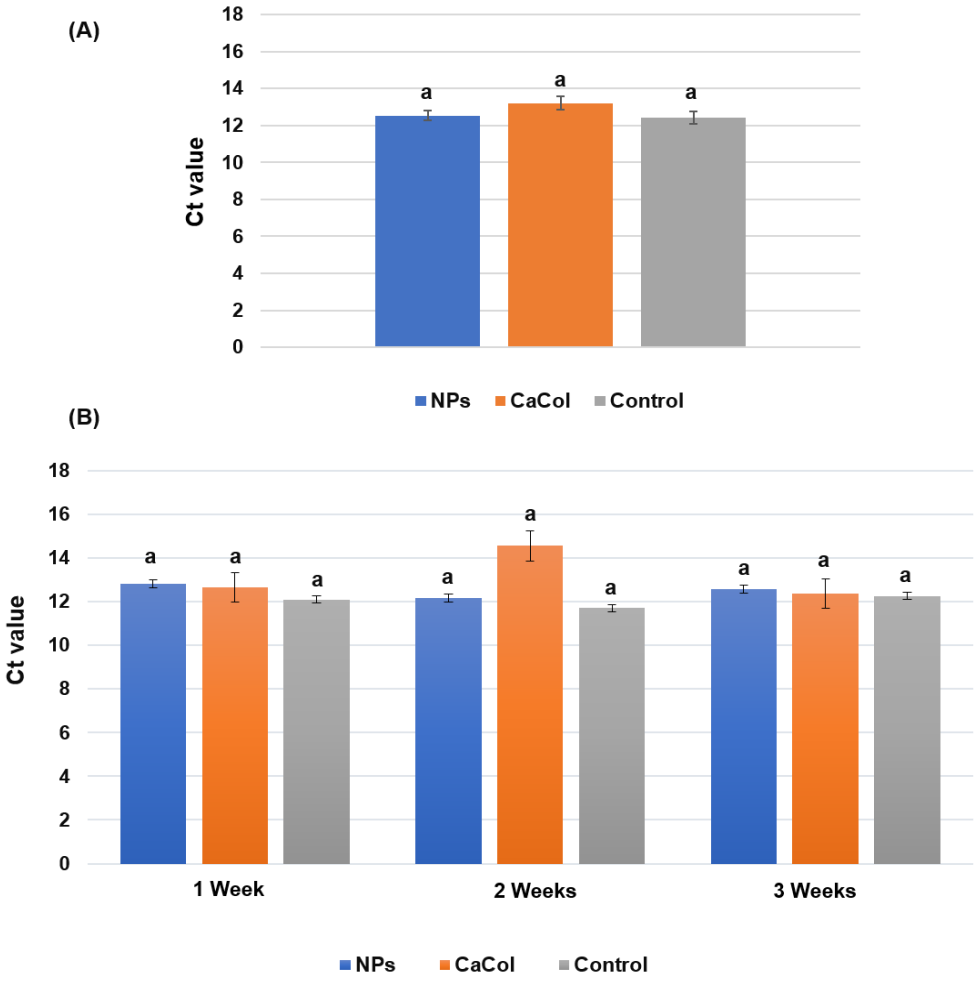

Figure IV.9. Abundance of *Candidatus* Erwinia dacicola in the heads of *Bactrocera oleae* fruit flies treated with Calcium carbonate nanoparticles NPs, colloidal calcium CaCol compared to control after 1, 2 and 3 weeks. Data were analyzed by two-way analysis of variance (ANOVA): main effect (A) and interaction (B). Bars indicate standard error and different letters above bars denote significant differences at $p < 0.05$ (Tukey's Honestly Significant Difference, HSD).

IV.4 Discussion

Given that one of the essential parts of this study was to determine whether the tested products (Calcium carbonate NPs and colloidal calcium) could negatively affect both the olive fruit fly *B. oleae* and the abundance of its endosymbiotic bacterium *Ca*. E. dacicola. Our research has hypothesized that these products may affect the oviposition behavior in the olive fruit fly *B. oleae*, as well as the abundance of *Ca*. E. dacicola present in the cephalic part of the adult fly.

As a fact, the two different formulas of calcium carbonate, NPs and colloidal calcium, affected the adult flies' fertility and NPs significantly decreased egg production in the female flies examined during the trial period. This result is in line with a study done by Bigiotti et al. (2019), where they found that two Copper suspensions (5% and 20%) reduced egg production in *B. oleae* females. Our findings suggest that NPs could have a negative effect on the physiology of adult flies, including female productivity.

Afterward, our results showed that both products tested through host feeding (Calcium carbonate NPs and colloidal calcium) did not affect the insect's lifespan and no mortality was recorded during the tested periods. This finding lead us to believe that insect mortality is influenced by the concentration of applied products, similar observation was reported in previous studies by Alif Alisha and Thangapandian (2019), who conducted a comparative bioassay, testing different concentrations of silver nanoparticles and malathion, to control red flour beetle *Tribolium castaneum.* Therefore, we propose that exposing adult females to higher concentrations of the tested products, as well as for longer intervals, might negatively affect the insect fitness and cause mortality.

The endosymbiotic bacterium *Ca*. E. dacicola is widespread and its presence in all sampled *B. oleae* adult flies was confirmed by conducting a PCR amplification on our tested samples. Our original hypothesis suggested that Calcium carbonate formulas could have antimicrobial activity by interrupting the symbiosis interaction between this bacterium and its insect host. As a matter of fact, our finding revealed that both products tested have no antibacterial activity. More specifically, the products showed a slight reduction in the abundance of *Ca*. E. dacicola. This is in agreement with results obtained by Bigiotti et al. (2019) which stated that the propolis has no action on the abundance of *Ca*. E. dacicola in the oesophageal bulbs of the olive fruit fly. The reduction in egg production in adult flies exposed to Calcium carbonate NPs, could be as a result of inhibition of bacterial metabolism. This is in line with Jayaseelan et al. (2012), that report a strong inhibitory activity of nanoparticles against the bacterial species *Pseudomonas aeruginosa* which has been shown with zinc NPs.

Chapter IV. Study of the effect of applied materials on the bacterial endosymbiont "*Candidatus* Erwinia dacicola" and on egg production of *Bactrocera oleae*

The experimental period and product concentrations are critical factors for successful treatment. In this context, Chen et al. (2019) tested different concentrations ranging from 50-250 mL^{-1} of copper oxide NPs against the soilborne bacterium *Ralstonia solanacearum*, and their results reported a more significant effect using higher concentrations. Therefore, we could hypothesize that the tested products in the present study could show an antimicrobial action against the endosymbiotic bacterium *Ca.* Erwinia dacicola, if used with higher concentrations and for a longer experimental time.

Hence, this study led to a better understanding and contributed to the study of the possibility of applying the Calcium carbonate NPs to control olive fruit fly populations, which could increase the perspectives for plant protection using low-environmental-impact approaches, opening up new prospects for sustainable integrated pest management programs.

IV. 5 References

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V. GENERAL CONCLUSIONS

In conclusion, the current research represents the first study on the effectiveness of Calcium carbonate $CaCO_3$ nanoparticles, in comparison with the conventional colloidal calcium CaCol, as a new alternative component to control the olive fruit fly *B. oleae*, in the field. Furthermore, this research investigated the antimicrobial activity of both $CaCO_3$ NPs and colloidal calcium CaCol on the bacterial endosymbiont *Ca*. Erwinia dacicola, associated with *B. oleae*, under laboratory conditions.

The field assays have evaluated the pest infestation level in olive fruits and highlighted the relationship between temperature changes and population dynamics of target pest. Moreover, the field trial revealed that the $CaCO_3$ NPs sprays demonstrated activity, remarkably reducing the population of olive fruit fly *B. oleae* in the field, by reducing insect development during early life stages (Chapter II).

The laboratory bioassays showed that calcium carbonate NPs decreased *B. oleae* adult's emergence; although the effect was lower compared to the field. Moreover, there was no direct effect of calcium carbonate formulas on insect longevity. These facts could be due to the concentrations of applied products and the experimental period, or to the laboratory conditions which impact the fly's physiology (Chapter III). Eventually, the two Ca carbonate forms could show better efficacy on treated *B. oleae*, if higher appropriate concentrations are used.

Afterwards, the productivity of *B. oleae* females decreased significantly with the two different treatments ($CaCO_3$ NPs and CaCol) incorporated into insect diets. Moreover, treated females showed a greater reduction in egg production, when exposed to Calcium carbonate NPs than to colloidal calcium during the laboratory trial (Chapter IV). These results were supported by those obtained in the field trials and confirmed the effectiveness of NPs on insect development and female's fertility.

In contrast, molecular findings based on quantitative real-time PCR, revealed that the $CaCO_3$ NPs and CaCol treatments had no significant impact on the abundance of *Ca*. Erwinia dacicola compared to the control treatment. The insects exposed to both Ca carbonate formulas

contained approximately equal amounts of *E*. dacicola bacterial DNA throughout the different exposure intervals (Chapter IV). This could be due to the same reasons that we mentioned previously (product concentrations and experiment period).

To our knowledge, this research is the first study on the effectiveness of Calcium carbonate NPs and colloidal calcium against the olive fruit fly and its endosymbiotic bacterium *Ca*. E. dacicola. Thus, further trials should be performed testing different concentrations of Calcium carbonate NPs and colloidal Ca. Moreover, laboratory trials should be established for a longer period, which may lead to better evaluate the effectiveness of these products on the viability of the insect pest and its endosymbiotic bacterium. The use of new concentrations should be established while evaluating the effect of these concentrations on agriculture and environmental systems, and their interaction with the human health. In addition, Future studies should investigate their selectivity by evaluating their negative impact on the population density of beneficial organisms in olive groves, as well as on other useful insects.

The present study indicates an overall trend that the Nano-calcium carbonate could reduce the olive fruit fly populations in the field and has performed better in terms of pest control than colloidal calcium, thus forming the basis for further investigations for its possible implementation in crop protection and integrated pest management strategies.