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TITOLO TESI

Natural and anthropogenic factors affecting the life cycle of exotic and native insect species

(Fattori naturali e antropici che influenzano il ciclo biologico di specie di insetti esotici e nativi)

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TITLE:

NATURAL AND ANTHROPOGENIC FACTORS AFFECTING THE LIFE CYCLE OF EXOTIC AND NATIVE INSECT SPECIES

GENERAL INTRODUCTION

The approach of the modern agricultural method foresees the use of pesticides not as the only one system for the defense of crops, but includes the action of natural enemies of the pest agents. As in these last years there is a better sensitivity and awareness towards the importance of health of the planet, this system permits to operate in agriculture reducing the environmental impact.

My Phd thesis is the result of three years of work mostly focused on the study of strategies for a sustainable control towards exotic insect species introduced and established in Italy. In particular, the first two chapters are focused on the relationships between alien species and indigenous parasitoids, in order to evaluate the action of these entomophagous insects as potential natural enemies of the target alien pests.

In the first chapter, I compared, in laboratory conditions, the longevity and the reproduction capacity of two coccinellid species, the exotic *Harmonia axyridis* (Pallas) and the native *Adalia bipunctata* (L.) after the exposure to the indigenous parasitoid *Dinocampus coccinellae* (Schrank) (Hymenoptera Braconidae). The aim was the evaluation of the effects induced by the parasitoid on the fitness of coccinellid females, with a particular stress on the Asian *H. axyridis*. This exotic species was introduced some years ago in Europe and reared in biofactories for augmentative biological control purpose, but now, for its voracity and prolificacy, it is even considered by some

entomologists as an invasive pest so that it is no longer commercialized in Europe (Burgio et al., 2008; Kenis et al., 2008; Lombaert et al., 2014).

The second chapter is focused on the assessment of the parasitization effect of the native dipteran tachinid *Exorista larvarum* (L.) on the exotic box tree moth *Cydalima perspectalis* (Walker).

This lepidopteran is one of the most recent exotic pests introduced in the European habitats (Kruger, 2008). In the experiment this alien species was compared with the factitious laboratory host *Galleria mellonella* (L.). This latter species is not a natural host of *E. larvarum* , but in laboratory conditions, it can support its maintenance and during the experiment acted as control. The aim was to check the possibility for *Cydalima* larvae to be accepted and/or successfully parasitized by the tachinid, with a complete development of the parasitoid through formation of puparia and emergence of adults.

In the third chapter I studied the lethal and sublethal effects of two different insecticides - widely used in agriculture in orchards and vegetables - on the two coccinellid species *H. axyridis* and *A. bipunctata*. The products selected were Imidacloprid, a neonicotinoid, and Spinetoram, a compound derived by toxins produced by the bacterium *Saccharopolyspora spinosa* (Mertz and Yao, 1990). The aim was to evaluate the acute and long-term effects of the two different insecticides on these insect predators with standardized methods and their fitness in laboratory (Stark and Banks, 2003). The final purpose was to investigate the laboratory response of the two coccinellid species to the two insecticides and mostly, to observe the effects of the treatments on the exotic ladybird, considered invasive. The results obtained in this laboratory research were also supposed to be a starting point in the perspective of a future study performed under field conditions.

The studies were performed in the framework of the GEISCA Project (Globalization Exotic Insects Sustainable Control Agro-forestry ecosystems) targeted at the sustainable control of exotic species, especially (but not exclusively) with respect to the action of native entomophagous insects (Maini et al., 2010).

The last part and fourth chapter of my thesis is related to my abroad experience. I carried out two laboratory experiments at the Department of Plant Sciences at Wageningen University (The Netherlands). The study was inserted in the AMIGA Project (Assessing and Monitoring The Impacts of Genetically Modified plants in Agro-Ecosystems) that is focused on the evaluation of the risk due to genetically modified organisms in the environment, following protocols set by the European Food Safety Autority (AMIGA Collaborative project proposal, 2011). In this study it was evaluated the impact of GM potato plants, resistant to the fungus *Phytophthora infestans* de Bary,

towards the tritrophic system plant-herbivorous-entomophagous insects. The aim of the first experiment was to evaluate the impact of these GM plants on the development time of the hymenopteran braconid *Aphidius colemani* Viereck. The parasitoid was, maintained on the green peach aphid *Myzus persicae* Sulzer reared on trans-genic and cis-genic potato plants infected by *Phytophthora* compared with the development time tested on plants non infected by the fungus.

In the second experiment it was evaluated the development time of *A. bipunctata* (from the third instar until the adult stage) fed on *Myzus,* that was reared on non- GM potato plants. The aim was to compare the development time occurred on plants infested and non-infested by *P. infestans* and to observe some effects on the third trophic level, represented by this coccinellid predator.

Chapter 1

1. LONGEVITY AND REPRODUCTION CAPACITY OF TWO COCCINELLID SPECIES PARASITIZED BY *DINOCAMPUS COCCINELLAE* (SCHRANK) (HYMENOPTERA: BRACONIDAE)

INTRODUCTION

The application of biological control often refers to the use of entomophagous insects (predators and parasitoids). These beneficial organisms may also be introduced from another Country for the biocontrol of phytophagous insects come from the same exotic Country (classical biological control) or when they play a role of high containment towards indigenous pests (Lockwood,1993) (neoclassical biological control). When an alien insect succeeds to survive and reproduce in the new habitat, it may spread and very often its populations may constitute a threat, since in the new eco-system natural enemies lack. Sometimes, also the exotic insects introduced as natural enemies against pest agents may represent this kind of problem.

In this study the exotic species that was considered as beneficial and subsequently a potential competitor towards indigenous natural enemies is the coccinellid *Harmonia axyridis* (Pallas). This aphid predator was tested in comparison with the native species *Adalia bipunctata* (L.). Both of them are Coleoptera Coccinellidae.

Historically, ladybirds have had importance as biocontrol agents. Some of them were also introduced from other countries to solve huge pest infestations in the field (e.g. the Australian coccinellid *Rodolia cardinalis* Mulsant was introduced in California in 1888by C.V. Riley in citrus orchards against the cushiony scale insect *Icerya purchasi* Maskell; the first big action of biological control recorded (Roy and Wajnberg, 2007).

Harmonia axyridis, the multicolored Asian lady beetle is most likely native of a region that extends from Altai Mountains to the Pacific Coast and from southern Siberia to southern China (Koch, 2003). It is also called Halloween beetle for massal groups of this coccinellid that is possible to observe in late October (Halloween time) in North America (Koch, 2003).

This polyphagous species feeds on aphids, Tetranychidae, Psillidae, Coccoidea, Curculionidae, Lepidoptera and first stages of Chrysomelidae, but also nectar and pollen (Koch, 2003). This coccinellid is so voracious that in the larval stages can consume from 100 to 400 aphids and the consumption of preys increases with the amount ofpreys and their degree of aggregation (Koch, 2003). For this reasons and for its adaptability to several environments and crop systems, *H. axyridis* has been considered an optimal biocontrol agent. The case of *Harmonia* is the example of how an entomophagous insect was introduced and used for pest control in a situation in which the native species could not contain enough the degree of infestation, not for the specific control of alien species in which it was requested the action of an exotic natural enemy.

The first introduction as pest control agent was in the USA in 1916 using *H. axyridis* of Japanese origin (Brown et al., 2011). Subsequently in 1964-65, in Europe the first releases occurred in the 1980s and the 1990s with a rapid spread, until the ascertainment of the establishment after about ten years (Roy and Wajnberg, 2007).

In Italy the “harlequin ladybird beetle” was reared in bio factories and then released in the field for the biocontrol in the 1990s, but the massal production was interrupted in 2000 for a hypothetical invasiveness towards other beneficial arthropods (Burgio et al., 2008). The coccinellid was detected for the first time in October 2006 in an urban area around Turin and again observed in the same area in 2007. Then the species was recorded in other Northern Italy regions (Burgio et al., 2008).

Brown (2011) in his study, comparing the exotic species with native coccinellids, reports that in East-England *H. axyridis* was the most abundant species. In particular the decline of native species was likely caused by competition for prey and intraguild predation of eggs, larvae and pupae by the exotic ladybird.

Kovach (2004) and Ejbich (2003) explained how the case of *Harmonia* is emblematic. In fact, usually ladybirds are considered beneficial insects for excellence, but this exotic species is now very unpopular. This not only for the interspecific competition towards native coccinellids, but also for damages caused to humans. In fact, in autumn, it is common to find adults of this coleopteran feeding and overwintering on grapes, and then being pressed and altering the quality of wine. Furthermore, they overwinter in buildings in massive groups representing a nuisance.

About the interspecific competition, in this study it was analyzed, in laboratory conditions, the comparison in several qualitative parameters between *H. axyridis* and the native "two-spots ladybird" *A. bipunctata* that shares with the alien species the same ecological niche and in time could be substituted by *Harmonia* for a natural process of competitive exclusion.

The effect of a cosmopolitan parasitoid of coccinellids, *Dinocampus coccinellae* (Schrank) (Hymenoptera Braconidae) [first recorded in Italy on *H. axyridis* in 2010 (Francati, 2013)] was tested in order to ascertain if the parasitoid may represent a good natural enemy of this alien predator.

1.1 EXOTIC INSECTS AND INDIGENOUS PARASITOIDS

The introduction and establishment of exotic species in new areas are consequent of the continuous process of globalization. Furthermore, the climate change (the global warming) favours a big spread of these species until to more temperate zones. The invasion of new alien species is particularly relevant in Italy for its geographical position and for its optimal climate (Jucker and Lupi, 2011), especially in the South, also allowing survival and reproduction of subtropical species.

The Program GEISCA (Globalization Exotic Insects Sustainable Control Agro-forestry ecosystems) was developed in order to join seven research groups in Italy and an abroad collaboration for studying the phenomenon of the invasion by new hosts, developing methods for a sustainable control, mainly through the use of native natural enemies.This because the adaptation process of native natural enemies to exotic insects is only partially known. The plan was mainly focused on the role of native natural enemies in a sustainable control context towards the new intentionally or unintentionally introduced species. It has to be stressed that the success of alien species in new

environments is also due to the scarcity of coevolved indigenous enemies (predators and parasitoids) that can balance their fitness (enemy release hypothesis) (Keane and Crawley, 2002) (Maini et al., 2010. PRIN Project 2010-2011 prot.2010CXXHJE).

In the list of insects cited in this research project it is also inserted *H. axyridis*. As already said, the Asian ladybird beetle is an active aphid predator, introduced in USA and Europe in the last century for the biological control in the field and greenhouses.Then it was considered invasive because threating for the native biodiversity, in particular towards the smaller *A. bipunctata,* annoying for humans and being pest for grapes and wine (Kenis et al., 2008). **In this framework, the aim of this study was the assessment in laboratory of some effects induced by *D. coccinellae* on longevity and reproduction capacity (fitness) of *Harmonia* and *Adalia* females in order to predict the possible role of this parasitoid in a field context.**

Recent studies in Italy (Dindo et al., 2014; Di Vitantonio et al., 2014) and researches in other European Countries (Berkvens et al., 2010; Kojama and Majerus, 2008) have reported a new association between *Harmonia* and the indigenous parasitoid in consolidation (Maini et al., 2010. PRIN Project 2010-2011 prot. 2010CXXHJE).

1.2 *Harmonia axyridis* (Pallas)

Harmonia axyridis belongs to the Coleoptera order, family Coccinellidae and subfamily Coccinellinae (Hodek et al., 2012). This coccinellid of Asian origin is common in China, South of Siberia (Koch, 2003), but also in Japan, Korea, Taiwan, Bonin and Ryukyu islands (Dobzhansky, 1933; Chapin, 1956; Iablokhoff-Khnozorian, 1982).

Initially, the description fell on the taxonomic definition *Coccinella axyridis* Pallas, but Jacobson and Timberlake ascribed the species under the *Harmonia* genus (Koch, 2003).

In Asia the "harlequin ladybird" completes only two generations per year (Sakurai et al.,1992), also in North America (LaMana and Miller,1996) and Europe (Ongagna et al., 1993). But it can arrive to have five generations per year as described by Wang (1986) and Katsoyannos et al. (1997).

The body of adults is convex shaped. The head has a facing down prognathism, well developed eyes, clubbed antennae with 11 articles and chewing mouth parts (Masutti and Zangheri, 2001).

The adults measure 4.9-8.2 mm in length and 4-6.6 mm in width. The livery is variable and the head can be black, yellow or yellow with black markings. The yellowish pronotum is provided of black markings, that can be simply black spots, a black M or W-shaped, a black trapezoid (Koch, 2003).

The high polymorphism in colors and pattern of elytra in adults is on genetic basis, controlled by a multi-allelic gene (Koch, 2003). Coloration and maculation can be influenced by larval diet and temperature to which pupae are exposed (Koch, 2003). The polymorphism can vary also seasonally and spatially (Koch, 2003).

Photo 1. *Harmonia axyridis*

(from http://www.insects.at/view.php?img=Coleoptera/Cucujoidea/Coccinellidae/Coccinellinae/Harmonia_axyridis_A_0408C.jpg)

Depending on the coloration of elytra it is possible to separate melanic forms from non-melanic forms. The melanic forms have a basic black color, and among these the typologies are:

- *spectabilis* Fald, with black elytra and four red spots (two per elytra);

- *conspicua* Fald, with black elytra and two red spots (one per elytra);

- *aulica* Fald, with black elytra and two big yellow spots.

The non-melanic forms are extremely various in number of black spots on the red, yellow, or orange elytra:

- *novemdecimsignata* Fald, with 19 black spots on yellowish back;

- *succinea* Hope, with yellow elytra and without spots.

Nalepa et al. (1996) report a correspondence between the geographic distribution and the livery coloration. They underline a higher frequency of melanic forms in altitudes increasing.

Three practical systems are useful to detect the sex in the adult individuals:

- different coloration of labrum: in male the color is white or anyway lighter than the female;

- different morphology of the distal margin of the fifth abdominal sternite: in males the distal margin is concave, in females it is convex;

- chromatic difference of thoracic sternites: in male the color is lighter than female.

Furthermore, usually, females are bigger than males, but it is not an absolute condition because of the high intraspecific variety.

This is a polyphagous species, feeding on aphids, coccoides, psyllas, mites, etc. However, *Harmonia* is mainly an aphidophagous coccinellid. The adult is very cold-resistant, it can survive until -30°C (Iablokoff-Khnozorian, 1982).

In spring, with a milder temperature, at least 10°C (Ongagna et al., 1993), and the increasing of the photoperiod, the wintering individuals leave their protected site (small clefts in the soil or in the trees) and mate (LaMana and Miller, 1996).

Each female can lay 20-30 eggs per day, but in laboratory condition they can arrive to 3000 eggs with a mean of about 25 eggs per day, depending on quantity and quality of food (Hukushima and Kamei, 1970).

Generally yellow batches eggs are laid close to growing aphid colonies, in order to guarantee the sustainment to the progeny. This behaviour can be explained through the capacity of the females of using volatiles for an evaluation of development stage of the colony or if it is not suitable, because already parasitized by other insects (Osawa, 2000).The larvae prey from 90 to 370 aphids a day, depending on the aphid species and the instar of the larvae (Koch, 2003).

Harmonia axyridis is a holometabolous insect with four larval stages, pupa and adult (Koch, 2003).

LaMana and Miller (1998) showed that in laboratory condition, at 26°C and with a diet of *Acyrthosiphon pisum* Harris the day for each phase are: egg 2.8 days, first instar larva 2.5 days, second instar 1.5 days, third instar 1.8, the fourth instar 4.4 days, the pupa 4.5 days, 20 days totally.

The eggs are yellow when freshly laid and become grey-dark before the hatching. Usually, they are laid glued to a leave, or another substrate. Larvae are very different from adults. The elongated body is covered with tubercles "scoli" and in late instars also orange bands appear. They have a chewing mouth part and start to prey immediately after the egg hatching (Koch, 2003).

At the first stages, they suck the internal fluids of preys, but with the dimension increasing they also eat solid components. Larvae of the second instar are the most voracious, but in the subsequent phases the aphids request decreases (Koch., 2003).

A frequent phenomenon in *Harmonia*, but also in other coccinellids, is the cannibalism. This is a survival system in condition of lack of prey. The sibling cannibalism occurs when the larvae feed on eggs not yet hatched from the same batch; the non-sibling cannibalism occurs in case larvae feed on eggs of different batches. The cannibalism is inversely related to aphid density (Burgio et al., 2002). The sibling cannibalism is more common because often the close larvae are in contact to each other. *Harmonia* larvae, however, prefer the non-sibling cannibalism, for the capacity to recognize relative larvae. This evolutionary strategy allow the survival of the species (Joseph et al.,1999; Michaud, 2003).

After the fourth instar, the transformation in pupa occurs. The pupa is fragile and vulnerable because the body is fixed at the substrate and exposed to external attacks. The stage of pupa is not completely immobile. In fact, if annoyed, the pupa makes shot of defense (Majerus and Kearns, 1989; Eisner and Eisner,1992). The newly emerged adult breaks the involucre and its coloration is very clear and susceptible to the external adversity because not yet sclerified.

In nature, *Harmonia* is a predator, but it has several natural enemies, both in the original area of distribution and also in the introduction areas. Among these, the braconid *Dinocampus coccinellae*

(Schrank), the fungi *Beauveriabassiana* (Bals.Criv.) and *Hesperomyces virescens* (Thaxter). Two kinds of defence systems may be adopted by coccinellids, including *Harmonia*: 1) Reflected Autohemorrhage, exudation of hemolymph of acrid smell and 2) Thanatosis, immobility that mimics death (Hodek et al., 2012).

1.3 *Adalia bipunctata* (L.)

Also known with the appellative of "Two spot Ladybird," this species, belonging to the subfamily Coccinellinae, is widespread in Europe and Central Asia, imported later in North America (Hodek and Honek, 2013). In our habitats it actively preys on aphids present on tree species, shrubs and herbaceous plants both cultivated and wild (Pollini , 1998). Its size is about 3.5-5.5 mm with a quite variable color. The most typical chromaticity is red-orange with two black points on the elytra. Also melanic forms with reddish-orange spots are frequent. The pronotum is black with white spots on the sides, but also cream-colored with black stain shaped central M. The body is dorsally convex and ventrally flattened, head buried in the prothorax. The antennae are filiform with the last three items slightly dilated. The legs have tarsi composed of 4 short articles, of which the second and the third bilobed is very small.

Photo 2. *Adalia bipunctata*, adult with aphids

(from. http://bioplanet.it/it/controllo-biologicoadalia-bipunctata)

Adalia bipunctata is a multivoltine coccinellid, it performs many generations per year. In many areas, however, it completes only one generation per year, as wintering adult undergo diapause in sheltered areas (Hodek and Honek, 2013; Hodek et al., 2012). After mating, each female lays her eggs in bright yellow clusters (which show a palisade structure). The eggs are laid on the undersides of leaves (500-800 per year, depending on the prey species) (Hodek and Honek, 2013). The larval stages are 4 and they are followed by the pupal phase, which later emerges as adult. The larvae are black with white and yellow spots and are very voracious

preying about one hundred aphids per day, while for adults the daily ration is around 50 individuals (Hodek and Honek, 2013).

1.4 *Myzus persicae* (Sulzer)

Myzus persicae (Sulzer, 1776), the green peach aphid, is a tinygreen aphid (1.5-2 mm length) belonging to the order Hemiptera and family Aphididae. Other possible primary hosts are *Prunus davidiana* (Carrière) Franch. and *Prunus serotina* Ehrh. The origin is Palearctic, currently is widespread worldwide (Pollini, 2013). The overwintering egg is laid on the peach tree *Prunus persica* (L.) Batsch (the main primary host), where the aphid also completes some spring generations. From June migrant forms appear and move onto herbaceous species (secondary hosts), both cultivated and wild (about 400 species belonging to different families including Cruciferae, Umbelliferae, Compositae, Solanaceae, Chenopodiaceae). In late summer, winged males and fall migrant females are produced and fly back to peach trees. On these, the fall migrant females produce wingless egg-laying females which mate with the males and lay the overwintering egg (Pollini, 2013).

Photo 3: *Myzus persicae*

(from http://www.nbair.res.in/Aphids/images/Myzuspersicae/Myzus%20persicae%20(10).jpg)

Gatehouse et al. (1996) reports that the importance of this pest is due to its worldwide distribution, its polyphagous nature and to the fact that it is a vector of over a hundred kinds of plant viruses. *M. persicae* causes several damages on potato, sugar beet and brassicas, and in glasshouses where it can compromise the marketability of ornamental crops (Gatehouse et al.; Pollini, 2013).

1.5 *Dinocampus coccinellae* (Schrank)

Dinocampus coccinellae (Schrank) is a wasp belonging to the Order of Hymenoptera, Family Braconidae, Subfamily Euphorinae. The species is cosmopolitan, except for the Antarctic Continent. The origin of distribution is uncertain (Balduf, 1926), maybe Palearctic or maybe Ethiopian and accidentally introduced in new Countries with ladybirds released for the biological control. For Timberlake (1918) the wasp probably reached Hawaii islands with the host *Olla v-nigrum* (Mulsant); for Gourlay (1930) it was introduced in New Zeland with the other control agent *Coccinella undecimpunctata* (L.).

Dinocampus coccinellae is a solitary endoparasitoid and exclusively attacks the subfamily Coccinellinae (Balduf, 1926). But the study of Ceryngier et al. (2012) showed that occasionally the braconid can parasitize other coccinellids subfamilies in a laboratory context. The way of reproduction is parthenogenesis, thelytokous type, that is, from non-fertilized eggs only female individuals are originated. In extremely rare cases, males were obtained (Berkvens et al., 2010). The species is multivoltine over much its distribution area (Berkvens et al.,2010), for instance 2 generations in Central Europe, 3 generations in France and until 5 generations in Italy. In general about 40 species can represent its hosts, among these, *H.axyridis*. In Europe one of the most favourite hosts is *Coccinella septempunctata* (L.) (Iperti, 1964). Francati (2013) reports that *D. coccinellae* does not successfully parasitize *A. bipunctata*, most likely because of its small size that does not allow the development of the larva of the parasitoid (Hodek, 1973). From the field collection, the successful attack was detected for *H. axyridis*, *C. septempunctata*, *Hippodamia variegata* (Goeze). Berkvenset al. (2010) recorded for the first time the new field association between the exotic coccinellid *H. axyridis* and the indigenous *D. coccinellae* in Europe. In Italy the new association was detected for the first time in 2010 in Emilia Romagna (Francati, 2013). Mostly, *D. coccinellae* oviposits into adult ladybirds, but in case of their scarcity, also in larvae and pupae (Filatova, 1974; Obrycki, 1989). The indigenous wasp is also able to choose between the female and male of *H. axyridis*. In fact the females are parasitized much more than males (Maeta., 1969).

Coccinellids are attacked and parasitized when are mobile; if not, the braconid stimulates the coccinellid to walk with the antennae and the ovipositor (Balduf, 1926; Walker, 1961), so that the abdomen of the host is more exposed to the action of the parasitoid.

According to Richerson and DeLoach, (1972), the action of attack can be synthesized in three moments:

- Pursuit and search of the host without extending the ovipositor;
- Positioning of the ovipositor in the ventral part of the host between the legs
- Attack with introduction of the ovipositor.

Photo 4. *Dinocampus coccinellae* detected an individual of *H. axyridis*.

(from http://bugguide.net/node/view/468476

The egg of *Dinocampus* is elongate and, when introduced into the coccinellid body, pedunculated. Growing, it becomes oval, increasing also in lenght and stretching four times in three days. The incubation time is about five to seven days (Balduf, 1926; Sluss, 1968).

For some authors as Oglobin (1924), three are the larval stages of *D. coccinellae*, but for others as Balduf (1926) the stages are four. The second molt of the insect parasitoid occurs just before the exit from the host; the mature larvae leave the coccinellid body through the membrane between the fifth and sixth or sixth and seventh urite. Then it weaves the cocoon among the legs of the host and transforms into pupa.

The coccinellid, before the leakage of the larvae from the abdominal region, becomes almost immobile and this condition can extend until death, after few days. The pupa of *Dinocampus* in the cocoon is well protected and can exploit the advantage furnished by the structural body and the aposematic coloration of the coccinellid as defense from natural enemies.

Photo 5. *H. axyridis* on pea shrub infested by aphids and with cocoon of *D. coccinellae* emerged from its abdomen. Entomology archive.

The wasp, when adult, leaves the cocoon chewing the apex, and each female is already prepared to attack a possible host. Each adult female emerged, in fact, is ready to oviposit.

Obrycki and Tauber (1978) detected as a temperature increasing, from 15.6°C to 26.7°C can induce a decrease of the mean time of the larval development from 47.9 to 16.3 days; a decrease of the pupal stage from 20.8 to 7.1 days and the total development time from egg to adult, from 65.8 to 23.3 days. They also observed that the development time is influenced by the host species and by the parasitized stage.

In laboratory, *D. coccinellae* can die for starvation in 3.6 days, if in condition of 27°C , but its longevity can increase until 20-25 days if maintained at 19°C and fed on a solution with honey or sugar (Filatova, 1974).

2. MATERIALS AND METHODS

2.1 INSECTS USED FOR THE EXPERIMENT

The laboratory experimental trial took place at the Entomology area in the Department of Agricultural Science, University of Bologna. It started in March 2013 and ended in early July 2013. The insects used in the experiments were reared in climate rooms located in the basement of the Entomology area.

2.1.1 *H. axyridis, A. bipunctata* and *M. persicae* rearing.

Both the coccinellid species used for tests came from the rearing maintained in the climate cells at T: 25±1°C, 70±10% H.R., 16L: 8D photoperiod (Lanzoni et al., 2004); the colonies were started from individuals collected in a biological orchard and in the educational garden of the Department. Adult males and females were kept in plexiglass cages (40x30x30 cm).

Photo 6. Cage for coccinellids rearing. Entomology archive.

The cages were provided with metal net for ventilation and a small door for internal cleaning, renewal of food and collection of eggs. For the oviposition, sheets of bubble wrap sticked to the walls with scotch tape were regularly changed. Namely,two times a week or every day, if necessary, the eggs were collected cutting out fragments of bubble wrap on which they were deposited. To avoid cannibalism events and to facilitate rearing operations, all the development cycle, from egg to adult emergence, took place in plastic boxes (30x20x10 cm) with pierced lid and covered with wire mesh fine mesh for ventilation. After the emergence that requires 20 days, the adults were

transferred in the cage for mating and oviposition. Both adults and larval stages were fed with aphids of the *Myzus persicae* species, reared on pea buds. For the rearing of *M. persicae*, twice a week from 12 to 18 plastic boxes, each containing 3 bowls filled with expanded perlite on which pea seeds were laid, were used. Pea seeds were then covered with expanded perlite and watered with 500 mL of water put at the bottom of the box.

Photo 7. Box for sowing of pea. Entomology archive.

Boxes with bowls were kept in the climate rooms at condition of T: 20±1°C, 75% H.R., 16:L 8:D photoperiod. Tuesdays and Fridays were generally the days of pea seed sowing and transfer of aphids from old infested seedlings to new shoots. These last were used as food for the two coccinellids species when necessary. Boxes and bowls were washed and sterilized with hypochlorite at the end of their use.

2.1.2 *D. coccinellae* rearing.

The parasitoid was reared in a different climate room (but with the same climate conditions for the coccinellids, see above), located in the opposite area of the Department in order to avoid the risk of a possible escape and contamination of the coccinellids rearing.

The rearing of *D. coccinellae* started in 2010 from specimens emerged from adult individuals of *H. axyridis* collected in the field and maintained following techniques described by Francati (2013). The maintenance was achieved exposing newly emerged adults of *H. axyridis* to the braconid wasp (in relation 1 adult parasitoid: 10 adults of *Harmonia*) and keeping them in the same plexiglass cage for a time of 2 hours. The operation was weekly repeated for steadily obtaining new parasitoids ready to use in the experiments. After the exposition to the *Dinocampus*, the coccinellids were collected and put in plastic boxes; they were fed for about 20 days (the mean time for the parasitoid cocoon emergence) with an artificial diet containing pork liver (Sighinolfi et al., 2008), using the Francati (2013) technique. The successful parasitization occurs in case of emergence of the cocoon under the abdomen of the coccinellid. All found cocoons were taken and kept in the same cage of the adult wasps, for a continuous substitution of the old individuals. The adults were fed with honey drops taken with a small and plastic fork and put on an oil-paper strip, then glued on the internal wall of the cage.

2.2 EXPERIMENTAL TRIALS

2.2.1 Parasitoid performance test.

For testing the parasitoid performance, coccinellids obtained from the mass rearing were used. Firstly, in order to obtain adults for the exposition, pupae from the rearing were taken and individually put into small see-through plastic cylinders (5 cm height, 4 cm diameter). This operation was important for checking the emergence date, the sex of each individual, as well as the impossibility of a randomly mating before the exposition or the pair composition. Females of *H. axyridis* and *A. bipunctata*, emerged a maximum of 4 days before, were separately exposed to *D. coccinellae* into microperforated and see-through plastic cylinders (20 cm height, 9 cm diameter) in relation 1 parasitoid: 1 coccinellid female for 30 minutes, as described by Francati (2013). The individuals of *D. coccinellae* used for the test came from the rearing and were at maximum 72 hours old to avoid shortage of performance for old age.

Then, these coccinellid females were mated with coetaneous non-exposed males and kept into small cylinders (8 cm height, 6 cm diameter) containing strips of bubble wrap for collecting eggs. Pea shoots infested by aphids (*M. persicae*) were daily furnished as food to the couples.

For the experiment 80 couples were totally used; they were subdivided as follows: 40 of *H. axyridis* (20 couples with exposed females and 20 with non-exposed females, as control) and 40 of *A.*

bipunctata (20 couples with exposed females and 20 with non-exposed females, as control). Each couple was marked with a code. The eggs laid by the females were daily collected, counted and kept in petri-dishes on which the date of oviposition and the parent couple code were reported. *Ephestia kueniella* eggs (Zeller) furnished by Biotop (Cape d'Antibes, France) were introduced to feed the emerged larvae and to avoid to lose data for the cannibalism that they might have exerted on each other.

2.2.2 Parameters considered in the study

The parameters considered in this study were:

- The longevity of adult females from emergence (in days);
- The pre-oviposition time (in days);
- The oviposition time (in days);
- The eggs laid in the first 10 days (E10)(Ferran et al., 1998 Sighinolfi et al., 2008);
- The eggs laid for the total time considered (=24 days, see explanation below) (E tot);
- The fertility (=% of hatched eggs) in the first 10 days;
- The total fertility.

2.3 DATA ANALYSIS

The data analysis was performed with a factorial ANOVA (2x2), in which the first factor was the coccinellid species (*Harmonia/Adalia*) and the second factor was the exposition of the coccinellid to the *Dinocampus* (exposed/non-exposed). When the data were not homogeneous, they were transformed for the analysis using the radq or the log10 transformation. When heteroscedasticity occurred despite transformation (oviposition time) the data were analyzed using the Kruskal-Wallis non parametric test, considering the factors "species" and "exposition" separately. The % values were ASN transformed for the analysis (Zar, 2010). The % parasitization (n. of formed cocoons/ n. of exposed females x100) was also considered.

Longevity was also analyzed by 2x2 contingency tables.

3. RESULTS AND DISCUSSION

An immediate result of the experiment carried out was that the larvae of the braconid parasitoid completed the development until the pupal cocoon formation, into 4 females on 20 *H. axyridis* exposed. In the exotic species it was thus attained the 20% successful parasitization.

In no female of the exposed *A. bipunctata* the development of *D. coccinellae* was completed.

The exposition and the likely partial development of the parasitoid in the hosts of both the species, however, produced some effects as shown in the tables below.

Table 1a. Biological parameters referred to the coccinellids *H. axyridis* (*H.a*) and *A. bipunctata* (*A.b.*) exposed or not-exposed to the parasitoid *D. coccinellae* as related to the combination of the factors "coccinellid species" and " exposition to the parasitoid"(means±SE). The number of couples (=replicates) are given ( ) above the means. Original number of couples = 20 per thesis

Parameters	Coccinellid species	Exposition to the parasitoid		ANOVA Results		
		Yes	No	Effectof the host species	Effect of the exposition	Interaction
	H.a.	(20) 20±0.7	(20) 22.2±0.9			
				F= 0.03	F=3.44	F=0.3

				gl= 1,76	gl= 1,76	gl= 1,76
				P= 0.87	*P*=0.07	*P*=0.58
Female Longevity within 24 days from emergence (days)	*A.b.*	(20) 20.7±1.2	(20) 21.9±0.8			
Pre-oviposition time	*H.a.*	(18) 6.28±0.8	(19) 5.11±0.4			
				F= 13.78	F=1,96	0.59
				gl=1,71	gl=1,71	gl=1,71
				P=0.0004**	0.17	0.45
	A.b.	(19) 4.21±0.3	(19) 3.95±0.3			
Oviposition time (days) (non parametric test of Kruskal-Wallis, for non homogeneous data, also with transformation)	*H.a.*	(18) 9.1±1.43	(19) 16.6±0.72			
				H=3.65	H=14.75	
				N=75	N=75	
				P=0.06	*P*=0.0001**	
	A.b.	(19) 12.9±1.5	(19) 17.1±0.9			
Number of eggs laid in 10 gg (E10) (data transformed for the analysis in log10)	*H.a.*	(20) 149.1±38.1	(20) 308.9±29.2			
				F=0.06	F=6.66	F=2.54
				gl= 1,76	gl= 1,76	gl= 1,76
				P=0.8	*P*=0.01*	*P*=0.11
	A.b.	(20) 143.8±24.2	(20) 173.5±20.6			
	H.a.	(20) 211.7±62.1	(20) 484.6±55.81			
				F=0.07	F=8.6	F=2.66
				gl= 1,76	gl= 1,76	gl= 1,76
				P=0.8	*P*=0.004**	*P*=0.11

Number of total eggs (=laid within the 24 days) (data transformed for the analysis in log10)	*A.b.*	(20) 230.5±42.6	(20) 289.7±31.5			
Fertility referred to E10 (= n.larvae/n.laid eggs in 10 days x100) (data transformed for the analysis in ASN)	*H.a.*	(18) 19.3±4.5	(19) 35.4±4.1			
				F=5.87	F=9.25	F=1.02
				gl=1,71	gl=1,71	gl=1,71
				P=0.02*	*P*=0.003**	*P*=0.32
	A.b.	(19) 13.7±3.3	(19) 20.6±3.7			
Fertility referred to total eggs (n.larvae/n.total eggs x100) (data transformed for the analysis in ASN)	*H.a.*	(18) 19.7±4.8	(19) 34.9±3.8			
				F=6.08	F=8.62	F=1.34
				gl= 1,71	gl= 1,71	gl= 1,71
				P=0.02*	*P*=0.004**	*P*=0.25
	A.b.	(19) 13.8±2.9	(19) 19.6±3.5			

Table 1a. The parameters considered in the table refer to all the total 80 couples of the two coccinellid species in which the females were exposed or non-exposed to the parasitoid. The longevity was the elapsed time (in days) between the emergence of the female and its death. The observation was kept until the 24th day following the emergence, because within this time the successful parasitization (= cocoon emergence) could occur (Francati, 2013). In this case, as usual, the cocoon appeared after about 17 days.

Table 1b. Biological parameters referred to the coccinellids *H. axyridis* (*H.a*) and *A. bipunctata* (*A.b.*) exposed or non-exposed to the parasitoid *D. coccinellae* as related to the combination of the factors "coccinellid species" and " exposition to the parasitoid"(means±SE). The number of couples (=replicates) are given ( ) above the means. For the exposed couples, only those which did not produce parasitoid cocoons (=unsuccessfully exposed) were originally considered.

Parameters	Coccinellid species	Exposition to the parasitoid (coccinellids from which the cocoonhasnotemerged)		ANOVAResults		
		Yes	No	Effect of the hostspecies	Effect of theparasitization	Interaction
Longevity (days)	*H.a.*	(16) 20.1±0.9	(20) 22.2±0.9			
				F= 0.02	F=2.97	F=0.23
				gl= 1,72	gl= 1,72	gl= 1,72
				P= 0.9	*P*=0.09	*P*=0.63
	A.b.	(20) 20.7±1.2	(20) 21.9±0.8			
Pre-oviposition time (days)	*H.a.*	(14) 5.93±0.97	(19) 5.11±0.4			
				F= 8.99	F=1.3	0.34
				gl=1,67	gl=1,67	gl=1,67
				P=0.004**	0.26	0.56
	A.b.	(19) 4.21±0.3	(19) 3.95±0.3			

Oviposition time (non parametric test of Kruskal-Wallis, for non homogeneous data, also with transformation)	*H.a.*	(14) 10.1±1.7	(19) 16.6±0.72			
				H=1.91	H=13.01	
				N=71	N=71	
				P=0.17	*P*=0.0003**	
	A.b.	(19) 12.9±1.5	(19) 17.1±0.9			
Number of laid eggs in 10 days (E10) (data transformed for the analysis in log10)	*H.a.*	(16) 171.1±45.9	(20) 308.9±29.2			
				F=0.07	F=5.83	F=2.2
				gl= 1,72	gl= 1,72	gl= 1,72
				P=0.79	*P*= 0.02*	*P*=0.14
	A.b.	(20) 143.8±24.2	(20) 173.5±20.6			
Number of total eggs (=laid for all the life) (data transformed for the analysis in log10)	*H.a.*	(16) 249.4±74.9	(20) 484.6±55.81			
				F=0.03	F=7.3	F=2.18
				gl= 1,72	gl= 1,72	gl= 1,72
				P=0.86	*P*=0.009**	*P*=0.14
	A.b.	(20) 230.5±42.6	(20) 289.7±31.5			
Fertility referred to E10 (=	*H.a.*	(14) 22±5.4	(19) 35.4±4.1			
				F=7.36	F= 6.75	F=0.39
				gl= 1,67	gl= 1,67	gl= 1,67
				P=0.008**	*P*=0.012*	*P*=0.54
	A.b.	(19) 13.7±3.3	(19) 20.6±3.7			

n.larvae/n.laid eggs in 10 days x100) (transformation in ASN)						
Fertility referred to total eggs (n.larvae/n. total eggs x100) (data transformed for the analysis in ASN)	*H.a.*	(14) 22.6±5.8	(19) 34.9±3.8			
				F=7.8	F= 6.15	F=0.56
				gl= 1,67	gl= 1,67	gl= 1,67
				P=0.007**	*P*=0.02*	*P*=0.46
	A.b.	(19) 13.8±2.9	(19) 19.6±3.5			

Table 1b. The parameters considered in the table refer to the couples of the two coccinellid species in which the females were exposed or non-exposed to the parasitoidand in which the cocoon did not emerge. The longevity was the elapsed time (in days) between the emergence of the female and its death. The observation was kept until the 24th day following the emergence, because within this time the successful parasitization (= cocoon emergence) occurs (Francati, 2013). In this case, as usual, the cocoon appears after about 17 days.

As reported in tables 1a and 1b, the longevity indicates the lifetime of the females ladybirds. In this experiment the lifetime considered was set at 24 days; in this period the emergence of the parasitoid cocoon could be expected (Francati, 2013). In this parameter, the species, the exposure to the parasitoid and the interaction between the two factors had no significant effects.

The pre-oviposition time, that is the time between the mating and the oviposition, was influenced by the species. In fact, in *Harmonia* the pre-oviposition time recorded is longer then in *Adalia*, both for exposed and non- exposed females.

The oviposition time is the period starting from the first oviposition until the end of the observation (24 days), or the death of the female, if occurred within 24 days. In this case the exposure to the

parasitoid had a negative effect on the parameter, reducing the days of oviposition for both the species (Kruskal-Wallis test: a) H=14.75; N=75; *P*=0.0001; b) H=13.01; N=71; *P*=0.0003). The species instead, did not influence the parameter statistically (Kruskal-Wallis test: a) H= 3.65; N=71; *P*=0.0003; b) H=1.91; N=71; *P*=0.17).

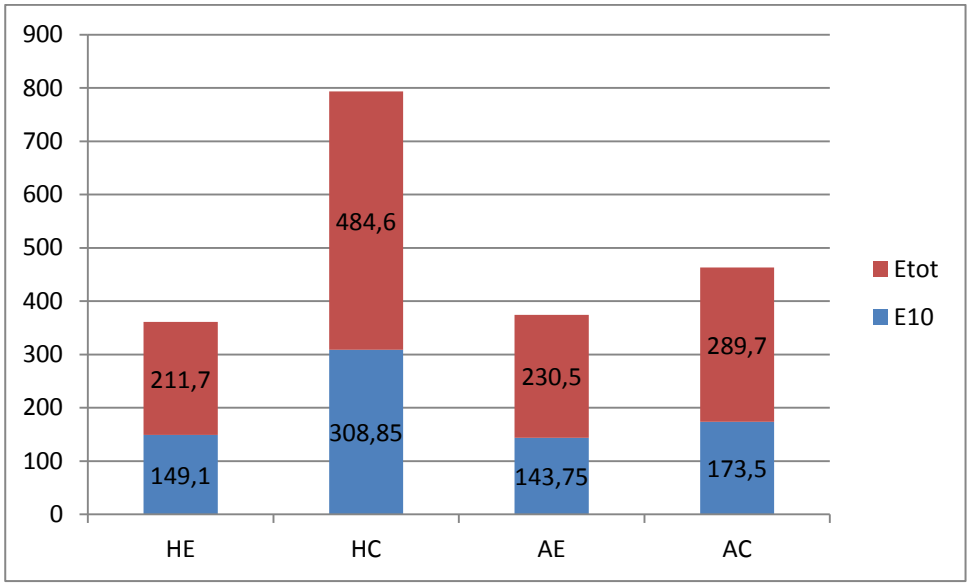
The parameter E10 refers to the number of the eggs laid in the first 10 days from the oviposition starting. Also in this case the exposure to *D. coccinellae* negatively influenced the fecundity in both the species (even independently of successful parasitization). In *H. axyridis* the number of eggs laid by the exposed ladybirds was much lower than that of eggs laid by the controls, especially when also the successfully parasitized ladybirds were considered (table 1a and table 1b).

The Etot is the number of eggs laid for all the oviposition time considered in the study, in this case 24 days (period in which it is possible to obtain the emergence of the parasitoid cocoons). The results resemble that for E10. The exposure negatively affected the fecundity of both the species, even halving the number of eggs laid by *H. axyridis* (table 1a). In *A. bipunctata* was instead recorded a less dramatic, but significant decrease of eggs laid by the exposed females.

The fertility for E10 and Etot are percentage values obtained from the number of hatched larvae related to the laid eggs respectively in the first 10 days of oviposition and in total (until the end of observation or until the death of the female). Both the parameters showed significant effects assignable to both the species and the exposure to the parasitoid. The interaction was not significant.

It has to be stressed that similar results were obtained when the successfully parasitized (= from which the cocoon emerged) *H. axyridis* were either included (table 1a) or excluded (table 1b) from the analysis. The incomplete parasitoid development has thus produced some effects at least on fecundity and fertility. The number of successfully parasitized *Harmonia* was, however, very low and no cocoons were obtained from *Adalia*.

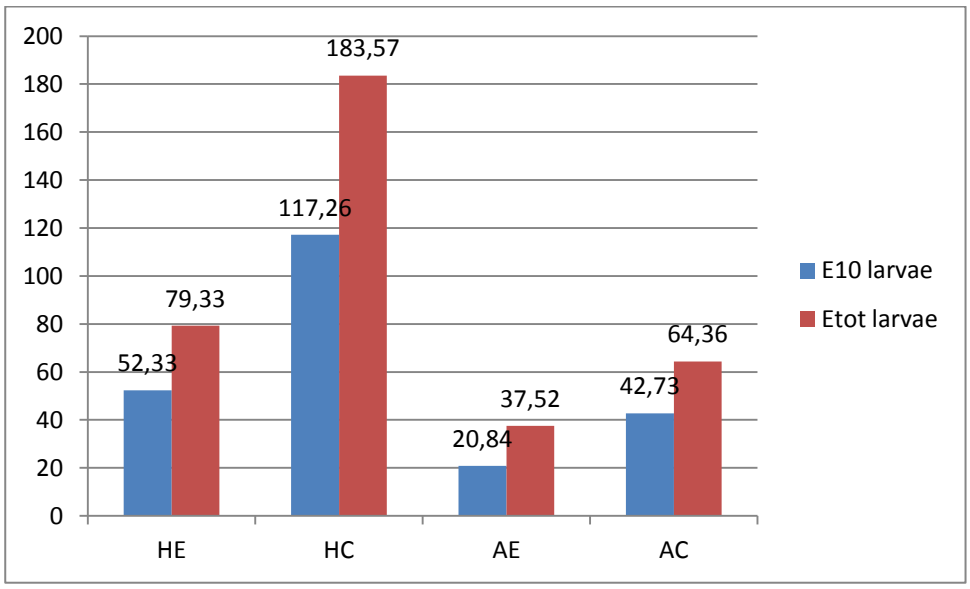
Graph 1. The mean values of fecundity (=number of eggs laid) of *H. axyridis* and *A. bipunctata* exposed (E) and not- exposed (C = Control) to *D. coccinellae* are reported in the graph below.



Graph 1. Significant effect of the factor "exposure to the parasitoid" for the parameters E10 and Etot (*P*=0.01 and *P*=0.004).

The results of the fecundity in *Harmonia* and *Adalia* exposed and notexposed to *Dinocampus*, reported in the graph, show the impact of the exposure. In *Harmonia* a considerable difference between the exposed and not-exposed females occurred, more than in *Adalia*.

Graph 2. The mean values of fertility (=number of hatched larvae/laid eggs) of *H. axyridis* and *A.bipunctata* exposed (E) and non- exposed (C= Control) to *D. coccinellae* are reported in the graph below.



Graph 2. Significant effect of the factor "species" and the factor "exposure to the parasitoid" for both the parameters related to E10 and Etot (*P*=0.02 and *P*=0.003/0.004)

The fertility of *Harmonia* and *Adalia* was affected by both the factors and the results are visible in the graph. The values of fertility in the exposed females were always lower compared to the values in the not-exposed females. In this case also the species had a significant statistical effect.

Table 2. In the 2x2 contingency tables *Harmonia* and *Adalia* exposed or not exposed to the parasitoid were compared in order to measure separately the effect of the exposure and the species on coccinellid longevity.. Any possible combination was tested.

	Alive (at 23th day)		Dead (before the 23th day)		χ^2 (gl=1) (Yates)	*P*
	n.	% (±SE) (1)	n.	%		
H. axyridis Control	14	70% ± 10.5	6	30%± 10.5		
H. axyridis parasitized	5	25%± 9.9	15	75%± 9.9	6.42	0.011*
H. axyridis Control	14	70%± 10.5	6	30%± 10.5		
A.bipunctata Control	14	70%± 10.5	6	30%± 10.5	0.12	0.73
A.bipunctata Control	14	70%± 10.5	6	30%± 10.5		
A.bipunctata	12	60%± 11.2	8	40%± 11.2		

parasitized					0.11	0.74
H. axyridis parasitized	5	25%± 9.9	15	75%± 9.9	3.68	0.055
A.bipunctata parasitized	12	60%± 11.2	8	40%± 11.2		
H. axyridis Control	14	70%± 10.5	6	30%± 10.5		
H. axyridis exposedbut non successfullyparasitized	5	31.3%± 11.9	11	68.7%± 11.9	3.91	0.047*
A.bipunctata parasitized	12	60%± 11.2	8	40%± 11.2	1.91	0.17
H. axyridis exposedbut non successfullyparasitized	5	31.3%± 11.9	11	68.7%± 11.9		
A.bipunctata Control	14	70%± 10.5	6	30%± 10.5	3.91	0.048*
H. axyridis exposedbut non successfullyparasitized	5	31.3%± 11.9	11	68.7%± 11.9		
H. axyridis Control	14	70% ± 10.5	6	30%± 10.5	0.11	0.74
A.bipunctata parasitized	12	60%± 11.2	8	40%± 11.2		
H. axyridis parasitized	5	25%± 9.9	15	75%± 9.9	0.001	0.97
H. axyridis exposed but non successfully parasitized	5	31.3%± 11.9	11	68.7%± 11.9		

Table 2. In the 2x2 contingency tables the comparisons 2x2 between the longevity of *Harmonia* and *Adalia* exposed and not-exposed are shown. The comparison involved the alive coccinellids until the end of the observation (=24th day from the exposure) and those dead before the 24th day. The χ^2 values are reported for each combine. The significant *P* values are marked with a star (*)

The 2x2 contingency tables showed that in all the possible combinations, significant results were recorded in case of exposed vs not exposed *Harmonia*. The parasitoid affected the exposed females, reducing the lifetime. The same significant result was also observed between the control and the exposed, but non successfully parasitized, females. In fact, also these females died before the 24th

day in more cases compared to the control. The same difference is also evident in the comparison between *Adalia* control and *Harmonia* exposed but non successfully parasitized. In all the other cases the results did not show significant differences.

It is important to underline that all the *Harmonia* successfully parasitized (from which the cocoon emerged) died before the 24th day.

4. CONCLUSIONS

The study was performed within a research project (GEISCA) aimed at testing and demonstrating the potential of control action of *Dinocampus coccinellae* (Schranck) as natural enemy of the exotic coccinellid *Harmonia axyridis* (Pallas) compared to the native *Adalia bipunctata* (L.).

In the test it was evaluated which of the two coccinellid species was more successfully parasitized and if the parasitization mainly influenced longevity, pre-oviposition time, oviposition time, fecundity and fertility of the females of one or the other species.

As regards longevity, the factorial analysis of variance did not show any influence of either species or exposure. However, the 2x2 contingency tables showed that in exposed *Harmonia* more individuals died before the 24th day. This result suggests that the effect of *D. coccinellae* on this parameter deserves more research.

The exposure to the *D. coccinellae* did not affect the pre-oviposition time of the two species. However the exposure affected the oviposition time, reducing it in both *Adalia* and *Harmonia*. This phenomenon was probably related to the development of the parasitoid larvae in the ladybird body.

It is also likely that the parasitoid development (if any) stopped in the exposed *Adalia*, because the females of this species continued to oviposit over the 10th day of the experiment. Instead, the exposed *Harmonia* stopped ovipositing at the 9th day. This parameter was mainly negatively affected in the exotic coccinellid.

Within the time of observation of 24 days, about the control females, *Harmonia* laid a bigger number of eggs than *Adalia*, as expected, but the oviposition time was not significant different between the species.

Some exotic ladybird were successfully parasitized, but the number of cocoons emerged from the hosts was only 4/20 with a percentage of parasitization of 20%.

The action of the parasitoid wasp negatively affected the number of laid eggs (fecundity), as well as the number of hatched larvae from the eggs (fertility) in both the coccinellid species. In this last parameter also the species influenced the hatching of the eggs. These significant differences occurred both in E10 (first 10 days from the oviposition beginning) and in E tot (total time of oviposition until the end of observation).

The results obtained in this experiment showed that the exposure to the *Dinocampus* negatively affected the fitness of both the coccinellid species, but in lesser way in *A. bipunctata*. Probably these effects were the sum of physical and/ or physiological damage caused by the parasitoid larvae during the development into the body of the coccinellid, as observed in many host-parasitod systems (Vinson and Iwantsch, 1980; Dindo, 1987; Koyama and Majerus, 2008). This phenomenon was maybe less impactful in *Adalia*, which, due to its smaller size and other unknown factors, did not allow a complete development (if any) to the parasitoid. Other laboratory experimental trials demonstrated the poor fitness of native *Adalia* as host of *D. coccinellae* (Honek, 1993; Francati 2013). Due to its preference for the exotic species, the indigenous parasitoid can contribute to the containment of *H. axyridis* (also with a reduction of its fitness) without representing a big threat for the small *A. bipunctata*.

Dinocampus coccinellae is already known as a potential natural enemy of *Harmonia* in different Countries, for instance Belgium (Berkvens et al., 2010), Canada (Firlej et al., 2005) and in Italy too (Francati 2013). This study confirms its possible contribution to the control of *Harmonia* populations, although at little extent.

It is important to observe that the study was carried out in laboratory conditions and in the field the situation is mitigated by many variables: the effect of *Dinocampus* is thus likely to be reduced. It would be desirable to extend the study in a field context.

Chapter 2

1. PARASITIZATION EFFECT OF THE TACHINID *EXORISTA LARVARUM* (L.) ON THE BOX TREE MOTH *CYDALIMA PERSPECTALIS* (WALKER) (LEPIDOPTERA: CRAMBIDAE)

INTRODUCTION

The alien species increasingly represent a great ecological and economical problem, negatively influencing the biodiversity of our habitats and also compromising our traditional landscapes (Bella, 2013). Hulme and Roy (2010) report the high threat level due to the alien insects, as one of the most dangerous groups towards the European economy. About the order Lepidoptera, more than the 70% of introductions in Europe occurred during the last century. It was also calculated that 2 new arrivals established per year between 2000 and 2007 (Lopez-Vaamonde et al., 2010). Examining 78 exotic lepidopteran species, it was shown that a bigger number came from Asia compared to the other geographic zones (Lopez-Vaamonde et al., 2010).

This progressive introduction was unintentionally increased through an increasing requirement of the commercialization of plants (Bella, 2013).

The box tree moth *Cydalima perspectalis* (Walker) was one of the most recent exotic species arrived in Europe. It was recorded for the first time in 2007 in south-western Germany and the Netherlands, probably introduced with *Buxus* seedlings from East Asia (Kruger, 2008). In fact China, Japan, Korea and India are its original Countries (Wan et al., 2014). In this last years the species continued to spread in the rest of Europe (Nacambo, 2013). According to Nacambo et al. (2013) the insect will likely invade all the European areas, except the Northern Scotland, Northern Fenno-Scandinavia and the high mountain regions. In Italy the first record of the species was in 2010 in Lombardia region, in Como province. It probably came from Switzerland and now it is spreading across the Northern part of our Country (Bella, 2013). In Emilia Romagna the first record was in 2012, and, subsequently Tuscany and Marche regions were also colonised. The most recent Italian finding sites of *Cydalima* were in Catania province (Sicily), on plants of *Buxus sempervirens* L. "Rotundifolia" imported from Tuscany (Bella, 2013) and Perugia province (Umbria) in several gardens and nurseries during the summer 2014 (Salerno et al., 2014).

This moth is a pest of plants belonging to the genus *Buxus* (family Buxaceae) that counts about 90 species of primitive angiosperms, common in most tropical regions and in the Mediterranean basin (Leuthardt et al., 2013). In its original area, the insect mainly feeds on *Buxus microphylla* Siebold e Zucc. in Japan and in *B. microphylla* spp. *sinica* (Rehd. et Wils.) in China. It was probably introduced in the Russian Far East, since in that area *Buxus* spp. are not native plants (Kirpichnikova, 2005). Other plants confirmed in China and Japan as hosts of this lepidopteran are *Euonymus alatus* (Thunberg) Siebold, *Ilex purpurea* Hasskarl (Aquifoliaceae) and *Euonymus japonicus* (Thunberg) (Celastraceae) (Uezumi, 1975; Shi and Hu, 2007). In the introduced habitats, the larvae extend their diet on new varieties (Leuthardt et al., 2013). In Europe, the damages caused by *C. perspectalis* are particularly serious in natural areas where the native *Buxus sempervirens* is an essential component of a unique forest eco-system, such as the Southern part of the Massif Central in France and the Pyrenees (Di Domenico et al., 2012; Kenis et al., 2013). The box tree is a typical ornamental plant of public and private gardens (e.g. the Italian style geometric gardens), nurseries, cimiteries and parks (Bella, 2013). In Italy, the already fragmented distribution of *B. sempervirens* could be seriously compromised by repeated attacks of *Cydalima*.

This Asian lepidopteran was inserted in EPPO Alert list in 2007, but, until 2010, none of the member Countries suggested an international action to contain this new alien. In 2011, therefore, the deletion from the Alert list followed, after a consideration of sufficient alert towards the pest (EPPO, website). Factors that can control its spread are attributable to temperature, photoperiod and humidity (Nacambo et al., 2013). In the new environments *C. perspectalis* seems not having natural

enemies, except for the tachinid *Pseudoperichaeta nigrolineata* (Walker), present in Europe but not widely spread (Salerno et al., 2014). *Cydalima perspectalis* may display some competition with other herbivores, but not at high level (Nacambo et al., 2013). Instead, in Asia besides this dipteran, other two tachinid parasitoids attack larvae of the pest, *Exorista* spp. and *Compsilura concinnata* (Meigen) (Shi and Hu, 2007). In the Xinyang region of China, percentages of more than 30% (Shi and Hu, 2007) and more than 47% (Wan et al., 2014) of larval and pupal mortality caused by *Exorista* spp. were recorded. The hymenopteran braconid *Chelonus tabonus* (Sonan) is considered the most common egg-larval parasitoid of *C. perspectalis* in China with a successful parasitism over the 50% in some areas (She and Feng, 2006). It is widespread across East Asia, including Japan, Korea, several Chinese Provinces , Yunnan and Taiwan, Indonesia (She and Feng, 2006). In China, another braconid wasp, *Dolichogenidea stantoni* (Ashmead) is ranked by Chen et al. (2005) as gregarious larval endoparasitoid of this moth. Instead, the same authors reported *Brachymeria lasus* (Walker) (Hymenoptera: Calcididae) as the only pupal parasitoid. It seems, from field observations, that birds are not good predators of *Cydalima* because, when the larvae were grabbed, they were left or regurgitated, likely for the presence of alkaloids in the larval body resulting from the digestion of the leaves of box trees (Leuthardt et al., 2013). The use of bioinsecticides in China (based on Neem oil and *Bacillus thuringiensis* (Bt) var. *kurstaki*) have shown satisfactory results as control systems against this pest (Li et al., 2004). Two soil nematode species collected in Korea, *Steinernema carpocapsae* (Rhabditida: Steinernematidae) and *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) were tested in laboratory and the results showed their potential effectiveness towards *Cydalima* (Choo et al., 1991).

In Italy, the tachinid *Exorista larvarum* (L.) is a polyphagous gregarius larval parasitoid of *Lymantria dispar* (L.) and other lepidopteran defoliators (Dindo et al., 2003; Dindo and Grenier, 2014). This indigenous fly, is currently reared in the Entomology laboaratory of the Department of Agricultural Sciences in Bologna, on the factitious host *Galleria mellonella* (L.) (Dindo et al., 2003; Dindo et al., 2016). In the past, *E. larvarum* has shown a potential of contributing to the control of another alien lepidopterous pest, the Geranium Bronze, *Cacyreus marshalli* Butler (Dindo et al., 2013).

The aim of this laboratory study was to test the effectiveness of *E. larvarum* on larvae of *Cydalima*, in order to evaluate a possible adaptation of this tachinid to this exotic pest and the exploitation of this native parasitoid as natural enemy of the alien lepidopterous defoliator.

1.1 *Cydalima perspectalis* (Walker)

The box tree moth *Cydalima perspectalis* (Lepidoptera: Crambidae), already known under the genera *Phakellura*, *Glyphodes*, *Diaphania*, *Neoglyphodes* (Mally and Nuss, 2010) is an indigenous pest of *Buxus sp.* trees in the Asian habitats (Wan et al., 2014).

The adult moths are generally white with wings slightly iridescent bordered by a large dark brown band, the melanic (completely dark brown) variants are less common (Bella, 2013). The wingspan is of about 4 cm, they are good flyers and live around two weeks. The mating occurs only once in their lifetime and lasts for about 2 hours (Cheng, 2005). The fecundity of females depends on the generation, decreasing from the overwintering generation to the third generation, from about 500 eggs to about 200 eggs respectively (Wan et al., 2014). The eggs are laid on the undersides of the leaves of the host plant, in clusters of about 20 (Bella, 2013). The fresh eggs are pale yellow, but before the hatching, they become dark for the visible black heads of the larvae (Bella, 2013). Studies carried out in Japan reported a different duration of the egg development of *Cydalima* at different temperatures and it was shown that with the increasing of temperature the development (in days) decreases from 15 to 3 days at temperatures values from 15° C to 30°C (Wan et al., 2014).

Photo 8. *Cydalima perspectalis*, larva. Photo Martini A.

The larvae are light green and have a black head and black stripes with white dots and hairs, an aposematic coloration that alludes to toxicity for defensive purpose (Leuthardt et al., 2013). The larvae of *C. perspectalis* are able to sequester toxic alkaloids contained in the *Buxus* leaves. The study of Leuthardt et al. (2013) showed that they store large amount of dibasic alkaloids (containing two basic amine groups) and they can metabolize or eliminate with excretions monobasic alkaloids (with only one amine group). The larvae emerged from a single egg stay on the

portion of the host plant in a range of 20-25 cm diameter until the pupal stage (Leuthardt et al., 2013). The larvae are visible, in fact they do not usually hide under the leaves or inside the shrub. Wan et al. (2014) reported that in laboratory at 25°C larvae of *C. perspectalis* developed through 6 larval stages. The stages 1-5 developed in 3 days and the 6th stage developed within 8 days. In the areas of origin, the complete development (of the 2nd and 3th generations of the same year) occurs in 24 days at 25°C (Japan) and 27°C (North-China) (Wan et al., 2013). The larvae feed on the leaves of different species of *Buxus* and can also attack the burk causing defoliation and in extreme cases the death of plants for withering (Bella, 2013). The pupae measure about 2 cm of lenght and are first green with dark strips and then, close to the emergence of the adult, they become brown with a dark pattern corresponding to the dark bands of the adult wings (Bella, 2013).

The species overwinters as larva, inside a silky cocoon between the box tree leaves. The number of generations of this lepidopteran change, depending on the climatic conditions of the region (Wan et al., 2014). In Asia 3-5 annual generations occurr (Wan et al., 2014) (in Japan 3 generations from May to September), instead in Central Europe Nacambo et al. (2013) reported only 2 generations per year. The diapause is induced by a photoperiod of 13.5 hrs of daylight (Salerno et al., 2014).

In Noth-East Italy Santi et al. (2015) found that *C. perspectalis* completes 3 generations per year. The three flight peaks occurred at the beginning of June, at the beginning of August and half September, respectively.

1.2 *Exorista larvarum* (L.)

This tachinid fly, belonging to the Diptera Order and Tachinidae Family, is a polyphagous and gregarious larval parasitoid widespread in a range that includes Europe, North-Africa, some Asian regions and North America, in which it was introduced in the early 1900 for the control of *Lymantria dispar* (L.) (Herting, 1960).

The life history of this species is divided in: egg, three larval stages, a pupal stage and adult (Hafez, 1953). The development time from egg to adult requires about two weeks depends on environmental conditions: Marchetti (2006) showed that in laboratory conditions, at 27°C, it took 16 days. The females of *E. larvarum* lay eggs directly on the host larvae, through their extensible ovipositor. The eggs are white when just laid and then become pale yellow (Hafez, 1953). Often some laid eggs can be lost for a detachment from the integument of the host larva, due to its movement or its molt (Hafez, 1953; Mellini & Campadelli, 1996a). The larva hatches after three

days at 26-27°C and through its special hook-like labrum perforates the integument of the host (Hafez, 1953). During its development, the larva inside the host breathes the atmospheric air, exploiting its respiratory funnels (Mellini, 1991; Valigurova et al., 2014). In the above mentioned condition, the mean time of development from the first instar larva to puparium was 6-7 days. Since *E. larvarum* is a gregarious parasitoid, more than one larva can develop in a single host (Michalkova et al., 2009). The pupa is protected by a hard structure called puparium, which is derived from the third instar larva. The puparia become dark red during the pupation process and they are often visible outside the host's body (Hafez, 1953). Marchetti (2006) showed that in laboratory at 27°C the time required for the emergence of the adult from the puparium is about 8 days. Usually, females emerge a few days later than males (Hafez, 1953). The two adult sexes are distinguished for two forks much more evident on the pretarsi of the males (Hafez, 1953). The mating occurs soon after the emergence (Marchetti, 2006) and generally, males and females mate more than once and they can also change partner (Hafez, 1953). The preoviposition time depends on temperature and season employing three days in summer and six days in winter (Hafez, 1953). According to Dindo et al. (2007) this time can be reduced to one day at laboratory condition of 31°C. In nature, for the host location, the parasitoids trust in olfactory cues (volatiles) released by plants when they are attacked by herbivores (Depalo et al., 2012). A female that intercepts a potential host (mostly an advanced larval instar) extends its ovipositor and lays an egg, gluing it with a sticky substance (Hafez, 1953). In this species, the superparasitoidism is a common condition (Hafez, 1953). The females oviposit for about 20-25 days (Hafez, 1953) and mostly in the first ten days from the starting of the oviposition (Dindo et al., 1999). The adult longevity depends on availability of food, temperature and sex. Generally females survive more than males (Hafez, 1953).

A particular characteristic of this tachinid is that its life cycle is not synchronized with the host and also for that reason it can be reared also *in vitro* (Dindo et al., 1999; Dindo, 2011). Most of the natural hosts of *E. larvarum* belong to Lepidoptera order, but the records are scarse and incomplete (Cerretti and Tschorsnig, 2010); Hafez (1953) and Harting (1960), however, mentioned over 45 natural hosts, including a few species belonging to Hymenoptera Symphitae.

Photo 9. Eggs of *Exorista larvarum* laid on larva of *C. perspectalis*. Photo Martini A.

Among these hosts, some are pest species such as *L. dispar*, *Hyphantria cunea* (Drury) (Cerretti and Tschorsnig, 2010), *Dendrolimus pini* (L.) (Csoka et al., 1989) and *Spodoptera littoralis* Boisduval (in Egypt) from a record of Hafez et al. (1976). In the laboratory of DipSA of Bologna University, a colony of *E. larvarum* is maintained on a factitious host, the lepidopteran *Galleria mellonella* (L.), an insect that in nature would not be attacked by the parasitoid for ecological differences, but that supports its development in artificial conditions. In fact it is convenient to use this factitious host because it is easy to rear in high quantity on artificial diet; the production process is less expensive compared to the rearing of the parasitoid on natural hosts (De Clercq, 2004). Since *E. larvarum* has polyphagous habits it is more adaptable to factitious hosts than monophagous or oligophagous species (Riddick, 2009).

Exorista larvarum is considered as the second most important natural enemy of *L. dispar* (Herting, 1960). Yet, its use as a biological control agent against this lepidopterous defoliator has been limited to a few inoculative and augmentative releases in North America, where it has become established (Sabrosky and Reardon, 1976; Kenis and Lopez-Vaamonde, 1998).

2. MATERIALS AND METHODS

2.1 INSECTS USED FOR THE EXPERIMENT

2.1.1 *C. perspectalis* rearing

The experiment took place in the laboratory of DipSA, University of Bologna. It lasted from May to the end of June 2015 and started from a collection of *C. perspectalis* larvae from a box tree located in the experimental garden of the University of Bologna Experimental Farm, located in Cadriano (Bologna) (44°32’57’’N 11°23’15’’E 28m a.l.s).

After the collection, the larvae were brought into the laboratory, closed in plexiglas cages (40 x 30 x 30 cm) with ventilation holes covered by fine mesh and kept in climatic rooms at 25°C, 65% R. H., 16:8 L:D of photoperiod. The larvae were fed on field- collected box leaves constantly renewed to avoid their withering.

2.1.2 *E. larvarum* rearing

Exorista larvarum was and is still maintained in a climatic room at the same conditions and in similar cages as those above described. The adults of the parasitoid were kept in number of about 50-70 individuals per cage, fed on lump sugar and cotton balls soaked in a solution prepared with 20% of honey and 80% of water (Depalo et al., 2012). Generally, the change of this cotton balls occurs three time a week in order to avoid drying and mold contamination. This tachinid species is reared on the factitious host *G. mellonella* [in turn reared on artificial diet prepared with honey, beeswax, glycerin, flours, and kept in a climatic cabinet at 30-32°C in the darkness (Depalo et al., 2010)]. Once a week mature lepidopteran larvae are exposed to the parasitoid. Namely, about 80 larvae of *Galleria* are introduced in the parasitoid cage for 30 minutes: the females need some time before laying eggs on the integument of the host. When it is possible to count at least 4-5 eggs per larva, then it is also possible to remove the host larvae and keep them in a separate box, in order to obtain puparia and new *Exorista* adults. After their emergence, the parasitoid adults are transferred into a new cage.

2.2 EXPERIMENTAL TRIALS

2.2.1 Description

For the experiment, a total number of 34 *Cydalima* and 34 *Galleria* mature larvae were separately exposed to the parasitoid in 4 replicates (corresponding to the field collections). At the same time 34 *Cydalima* and 34 *Galleria* mature larvae were not exposed and maintained as control. For each replicate, different numbers of larvae per species were used (11,4,4,15), upon the availability of *Cydalima* larvae. The exposure occurred in plexiglas cages (20 x 20 x 20 cm) for a time of three hours at a ratio per cage of 2 host larvae for 1 mated parasitoid female (obtained from the standard colony) not older than one week from the emergence. After the exposure, the parasitoid females were removed and put in the adult cage; the parasitoids eggs on larvae were counted; the larvae of

both species were individually placed in 9-cm diameter plastic petri-dishes, kept in the climate room at the same conditions as above, and daily monitored until visible effects of parasitization/non parasitization by the tachinid (larval death; puparium formation; lepidopterous adult emergence) could be detected. The larvae were considered as "accepted" when at least one parasitoid egg was found on their integument.

Photo 10. *C.perspectalis* exposed to *E. larvarum*. Photo Di Vitantonio C.

2.2.2 Parameters considered in the study

The results were evaluated in terms of the following parameters:

eggs/accepted larva;

puparial yields (% calculated on the parasitoid egg number)

adult emergence (% calculated on the puparium number)

number of exposed larvae with no eggs (non-accepted larvae)

C. perspectalis or *G. mellonella* adults emerged from exposed or not exposed larvae (% calculated on the larval number)

The following indices (per host species) summarizing host-parasitoid interactions were also calculated (modified from Chabert et al., 2012).

DI(=T-di/T x100): Degree of infestation, this measures the proportion of hosts that following exposure to *E. larvarum*, died due the parasitoid larval activity.

SP(=pi/(T-di) x100): Success rate of parasitism, this measures the probability that an infested host (= a host containing larvae of this gregarious parasitoid) would give rise to an adult fly at least.

T= number of lepidopterous adults obtained from control larvae (=not exposed to *E. larvarum*)

di= number of lepidopterous adults obtained from larvae exposed to *E. larvarum*

pi= number of lepidopterous larvae, exposed to the parasitoid, which produced adults

In some cases, pi was higher than (T-di); for these cases SP was set as 1(Chabert et al., 2012).

2.3 DATA ANALYSIS

The data were analysed by one way ANOVA or Kruskal-Wallis test when heteroscedasticity occurred. The percentages (%) were transformed for the analysis using ASN transformation (Zar, 1984).

3. RESULTS AND DISCUSSION

Table 1. Parasitization of *Cydalima perspectalis* and *Galleria mellonella* by *Exorista larvarum*: parasitoid eggs/accepted larva(no.) , puparial yields(%), adult emergence (%), exposed larvae with no eggs(no.). For the eggs /larva, means(±SE) in a column followed by the same letter are not significantly different (one- way ANOVA). Number of replicates =4. Number of lepidopterous larvae per species /replicate= 11(1), 4(2), 4(3),15(4).

Host Species	Eggs/larva (no.)	Puparial yields (%)[1] *	Adult emergence (%)[2] *	Exposed larvae with no eggs *
C. perspectalis	4.15±2.64a	0	----	12.27±9.49
G. mellonella	7.26±1.76a	15.53±5.43	100	0
F (df)	3.81 (1,6)			
P	0.98			

1 Percentages based on eggs 2 Percentages based on puparia * Statistical analysis not performed

Table 2. Lepidopterous adults emerged from control *C. perspectalis* or *G. mellonella* larvae (control = not exposed to *E. larvarum*). Means(±SE) in a column followed by the same letter are not significantly different (one- way ANOVA). Number of replicates =4. Number of lepidopterous larvae per species /replicate= 11(1), 4(2), 4(3),15(4).

Host species	Lepidopterous adults emerged/larvae (%)

C. perspectalis	77.95±1.75a
G. mellonella	96.06±2.33b
F (df)	35.18 (1,6)
P	0.001

Table 3. Lepidopterous adults emerged from *C. perspectalis* or *G. mellonella* larvae which had been exposed to *E. larvarum*). Means(±SE) in a column followed by the same letter are not significantly different (Kruskal- Wallis test). Number of replicates =4. Number of lepidopterous larvae per species /replicate= 11(1), 4(2), 4(3),15(4).

Host species	**Lepidopterous adults emerged/larvae (%)**
C. perspectalis	12.5±7.22a
G. mellonella	10.19±5.3a
H (N)	0.088 (1,6)
P	0.77

Table 4. Indices (%) summarizing host-parasitoid interactions per host species. DI: Degree of infestation, this measures the proportion of hosts that following exposure to *E. larvarum*, died due the parasitoid larval activity. SP: Success rate of parasitism, this measures the probability that an infested host (= a host containing larvae of this gregarious parasitoid) would give rise to an adult fly at least. Means(±SE) in a column followed by the same letter are not significantly different (Kruskal-Wallis test). Number of replicates =4. Number of lepidopterous larvae per species /replicate= 11(1), 4(2), 4(3),15(4).

Host species	**DI (%)**	**SP***
C. perspectalis	91.65±8.35a	0
G. mellonella	95.73±2.54a	100
H (N)	0.00 (8)	

P	1.0000	

*** Statistical analysis not performed**

This laboratory test, in which *C. perspectalis* was exposed as host to *E. larvarum*, that has never been reported as a natural enemy of the lepidopterous pest showed that this exotic species is accepted by the parasitoid females. In fact, the females of *Exorista* laid a number of eggs on *Cydalima* larvae that was not significant lower than the eggs laid on the factitious host *G. mellonella* (Table 1).

Some development of the parasitoid occurred in both hosts. No puparia, however, formed in any exposed and accepted larva of *Cydalima*. This phenomenon probably occurred because the parasitoid larvae were encapsulated by the host, which showed no suitability for this tachinid species. Conversely, the factitious host *Galleria* confirmed to be well accepted and greatly suitable for *Exorista* as shown by the % of puparia and the % of adult parasitoid emergence. Moreover, in *C. perspectalis* there were a few larvae on which the parasitoid did not lay any egg, instead in the factitious species, all exposed larvae showed eggs on their body (Table 1).

In the control (larvae of *Cydalima* and *Galleria* not exposed to *E. larvarum*) more than 75% of *C. perspectalis* larvae reached the adult stage. This value was significant lower compared with that of *G. mellonella*, in which more than 95% of larvae developed to adult. This was possibly due to the fact that the field- collected box moth larvae were not well adapted to be kept in captivity (Table 2).

About the larvae of both species that were exposed to *E. larvarum* and became adults, the results were not significantly different (Table 3). In fact, *Cydalima* and *Galleria* showed similar percentages of lepidopterous adults obtained. For *Galleria*, the mortality can be ascribed mainly to the parasitoid larval activity, but for *Cydalima* the factors that caused the larval mortality may also be different (as shown in the control, where a lower percentage of *Cydalima* larvae unexposed became adults).

The comparison of the Degree of infestation, that is the proportion of hosts that were successfully parasitized (Chabert et al., 2012), showed a not significant difference between the values obtained in the two lepidopterous species, despite of a lower percentage recorded in *Cydalima*. Instead, the Success rate of parasitism, that is the probability that an infested host will give rise to an adult parasitoid (Chabert et al., 2012), showed that in the two host species opposite results occurred: 0% of success rate on *Cydalima* and 100% of success on *Galleria*. This results confirm the unsuitability

of *Cydalima* for *E. larvarum* which may, however, kill the alien host for incomplete development (Table 4).

4. CONCLUSIONS

The aim of this experiment was to assess the parasitization effect of the tachinid *E. larvarum* towards the exotic box tree moth *C. persepctalis* (Walker). This Asian lepidopteran is one of the most recent alien species introduced in Europe, where it was first recorded in Germany and The Netherlands in 2007 (Kruger, 2008). In Italy, the first reporting occurred in Lombardia region, in 2010 (Bella, 2013). This exotic pest attacks different species of *Buxus*, generally present as ornamental plant in public and private gardens, cemeteries, nurseries. The larvae feed on leaves but also on bark (Wan et al., 2014). The species was inserted in EPPO Alert list in 2007, afterwards removed from the list (in 2011) as none of the member Countries suggested an international action to contain this new alien (http://www.eppo.int/).

The indigenous parasitoid *E. larvarum*, a tachinid fly that attacks many lepidopterous species, was chosen for this study in order to evaluate its effectiveness as potential natural enemy of *Cydalima*. It has to be stressed that unidentified species of *Exorista* have been reported as parasitoids of *C. perspectalis* in the Countries of origin (Shi and Hu, 2007). A number of 34 mature instar larvae of *C. perspectalis* were exposed to the parasitoid females in comparison with the same number of exposed larvae of *G. mellonella*. This factitious host is commonly used in the laboratory of DipSA for the *E. larvarum* rearing, although, in nature, the two insects do not share the same ecological niche, something very common in parasitoid rearing on factitious hosts (De Clercq, 2004).

The results showed a good acceptance of the exotic species by the parasitoid, but, although the eggs were laid in high number on the body of *Cydalima* larvae, the parasitoid could not complete the development. This was possibly due to the effect that the parasitoid I instar larvae were encapsulated inside the host's body without possibility to survive. As a consequence, puparia did not form and adults of *E. larvarum* did not emerge from *C. perspectalis*. However, a high number

of *Cydalima* larvae died when they were exposed to *E. larvarum* compared to those that were not exposed. Therefore, as also shown by the DI, *E. larvarum* has the potential to kill *C. perspectalis* in spite of lack of complete development (similarly to what observed by Dindo et al. [2013] for another alien lepidopterous species, *C. marshalli*).

Conversely, *G. mellonella* larvae were all accepted by the tachinid and almost all the exposed larvae produced puparia. The only *Galleria* larva which developed to adult could eliminate the host from its body.

About the control, *Cydalima* larvae non exposed to the parasitoid completed the development until the adult stage, but due to a mortality for other indipendent factors, they recorded a significantly lower percentage of adults (about 75%) compared to that of *Galleria* larvae in which the percentage of adults obtained was more than 95%.

However, it needs to be emphasized that the exotic insect is subject to an increase of mortality, following the tachinid parasitoid activity, although it is not suitable for its development. Since *E. larvarum* is a widespread parasitoid species in Italy, it may contribute to control this invasive pest in a context of conservative biological control.

Chapter 3

1. LETHAL AND SUBLETHAL EFFECTS OF TWO DIFFERENT INSECTICIDES TOWARDS A NATIVE AND AN EXOTIC COCCINELLID

INTRODUCTION

The modern society is alert to the quality of products as well as to the sustainability of production system. The main issue that the agriculture faces all over the world, is the maintenance and the increase of productive yields, reducing inputs, mainly pesticides. This is consistent with the fulfillment of laws that protect the agro-ecosystems landscape, maintaining it as intact as possible, minimizing soil exploitation and preserving natural habitats.

The integrated pest management (IPM) is a current solution that allows to maintain high yields, reducing chemical compounds. It is a defence practice of agriculture production which provides a decrease of pesticides, carrying out several measures. Among these, the use of selective pesticides to reduce the action of pests and minimize toxic or non-toxic effects on beneficial insects, humans, producers and consumers. (Desneux et al., 2007, Rimoldi et al., 2012, Gentz et al., 2010)

In 2011 The Agriculture Commission of the Italian Parliament established the "National Quality System of Integrated Production" (Repubblica Italiana, Legge n.4, 3 febbraio 2011). This regulation provides a rational use of pesticides and the obligation for all farms to adopt the principles of integrated production starting from January 1st 2014.

The technical standards provided by IOBC (International Organization of Biological Control) consider the importance of the reduction of treatments and the quantity of pesticides used, the

evaluation of necessary treatments and the identification of the most suitable acting time, and the choice of products at the lowest impact on the environment (Hassan, 1998). The IOBC involves "Pesticides and Beneficial Organisms" working group, whose aims are the development of standardized methods to test collateral effects of pesticides on natural enemies and the use of selective pesticides suitable for pest controlling (Sterk et al., 1999; Hassan, 1988).

The guidelines for these analyses were published by Hassan et al. (1985) and Hassan (1992) and consider a sequential screening of chemicals molecules through multilevel tests. The first step is represented by laboratory tests to evaluate the toxicity of products, classified according to mortality, parasitization capacity and oviposition caused to beneficial organisms. Then if the active ingredients show some toxicity, the investigation is extended to semi-field and field.

For a complete study of pesticides impact on beneficial organisms, would be necessary also to consider the sub-lethal effects (Stark and Banks, 2003). The organisms that survive to chemical exposition (common condition for insecticides of new generation) could still suffer significant damages (Stark and Banks, 2003). Sublethal effects as decrease of life expectation, decrement of development rate, reduction of fecundity and fertility, imbalance of sex-ratio, modification of the behavior, food searching and oviposition reduction can appear (Stark and Banks, 2003). It is always important to stress that the laboratory studies do not exactly show the real situation in the field, where the distribution of insect populations and the chemical components degradation are influenced by several environmental variables.

The selectivity of pesticides is evaluated on beneficial organisms, such as predators and parasitoids (Gentz et al., 2010). One of the fundamental criteria of the integrated pest management is therefore the protection and the enhancement of beneficial arthropods, valuable organisms for pollination and pest control.

Coccinellidae are appreciated coleoptera in agriculture for their role of natural predators of aphids and other pests (Hodek and Honek, 2013; Hodek et al., 2012; DeBach and Rosen, 1991).

The aim of this study was to evaluate the selectivity of two different insecticides of different composition, a neonicotinoid pesticide and a new generation compound of biological matrix, chemically modified, on two coccinellid species, the exotic ladybird *Harmonia Axyridis* Pallas and the native two-spots ladybeetle *Adalia bipunctata* (L.)

1.1 COCCINELLIDS AND PESTICIDES

Belonging to Coleoptera order, Coccinellidae are worldwide present with approximately 6000 species described (Hodek et al., 2012) and grouped in 7 subfamilies (Chilocorinae, Coccidulinae, Coccinellinae, Epilachninae, Ortallinae, Scymninae, Sticholotidinae).

Coccinellids mainly prey insects and mites, (Hodek et al., 2012), but sometimes they feed on vegetal substances. The relevance of many Coccinellid species is their function as predators of sucking pests in agro-ecosystems (Hodek et al., 2012).

Insect belonging to Homoptera Sternhorryncha as aphids and scales are the usual preys of ladybirds, but, also Coleoptera Crisomelidae, Lepidoptera, Orthoptera, Tisanoptera, and Acarina are included (Hodek et al., 2012).

The contribution of Coccinellids in pest management systems was already found more than one century ago (Obrycki and Kring., 1998), when *Rodolia cardinalis* Mulsant was introduced in California for the biological control in the citrus industry versus the cottony cushion scale *Icerya purchasi* Maskell (Hemiptera Monophlebidae) (DeBach, 1964; DeBach and Rosen, 1991).

The Coccinellids are endopterygota holometabolous insects with a life cycle composed of three preimaginal stages (egg, larva, pupa) and adult (Hodek and Honek, 2013; Hodek et al., 2012).

Fourth instar larvae and adults are the most active predators (Hodek et al., 2012). Moreover the fecundity of females is particularly linked to specific prey availability, such as aphids (Obrycki and Kring, 1998). Migration, reproduction, gregarious habits constitute a population dynamic dependent by environmental and climatic factors, first of all, food availability and temperature (Jalali et al., 2010; Mills, 1981).

When phytophagous infestations are considerable, Coccinellids can reach great densities and have effects similar to insecticides (Hodek et al., 2012). The density of coccinellid population is negatively affected when the natural development cycle of these predators is altered (Hodek et al., 2012). The IOBC/WPRS Working Group evaluated the selectivity and the appropriate timing to use chemicals, in relation to population dynamics to preserve predators and parasitoids and the healthiness of the ecosystems (Sterk et al., 1999).

1.1.1 COCCINELLIDS

1.1.1.0 *Adalia bipunctata* (L.) (see the description of the species in chapter 1)

1.1.1.1 *Harmonia axyridis* (Pallas) (see the description of the species in chapter 1)

1.2 SELECTIVITY OF PESTICIDES

In the last years the agriculture adopted a conservative approach towards agro-ecosystems, preferring low toxicity pesticides to preserve beneficial organisms and high environmental and economical sustainability (Gentz et al 2010). The combined use of selective products and natural enemies in IPM programs can provide very effective pest management (Gerwick and Sparks 2014; Koss et al. 2005). The rational use of these compounds in the integrated pest management (IPM) is favored by an increased interest and awareness of farmers and consumers towards human and environmental health, and from financial incentives provided by law (Repubblica Italiana, Legge 3 febbraio 2011 n.4).

The selectivity of pesticides is determined by the chemical characteristics of the molecule or by the mode of action of the active ingredient. These novel insecticides can act through:

a) Ingestion, causing the death of pests when they feed on treated parts of plants.

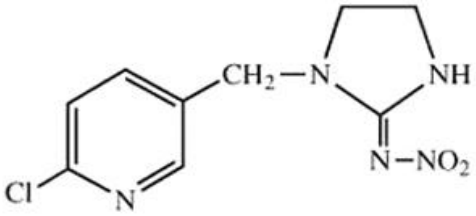
b) Contact, both through the direct contact on phytophagous during the spraying and through contact between the treated surface and the body of insect. In this case the products are relatively selective, because the life cycle of beneficial organisms and pests usually overlaps.

c) Asphyxia, inducing the death of pests that breathe the volatile substance in gaseous state. These products are not selective towards beneficial organisms. This mode of action is the least used in pesticides of new generation and formulation.

Therefore, it has to be stressed that Coccinellids can be exposed to insecticides, by direct contact, during spraying, at a later time on chemical residues, or indirectly, through the consumption of poisoned preys (Katsarou et al., 2009).

1.2.1 Imidacloprid

Imidacloprid (Confidor®) (1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine) is a insecticide belonging to the class of Chloronicotinyl compounds discovered in these last 20 years. This substance is currently commercialized under Confidor name. It was synthesized in February 1985 by Nihon Bayer Agrochem K.K.

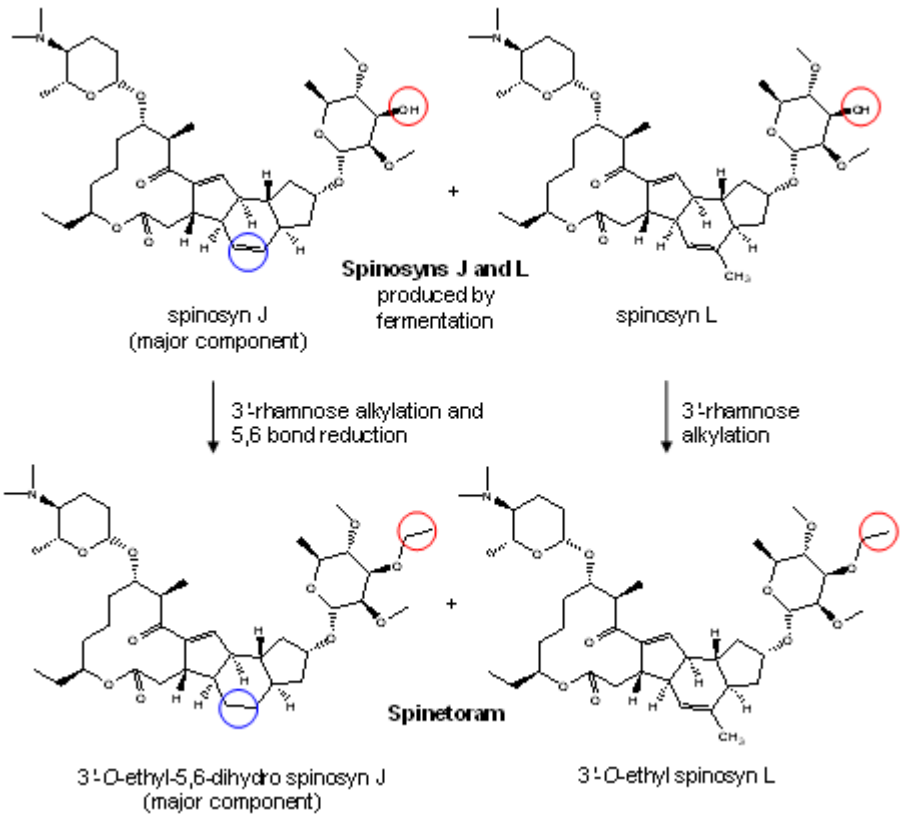


Imidacloprid (1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine)

It is a nitroguanidine systemic molecule which acts as the nicotinic acetylcholine receptor of insects competitor (Suchail et al., 2000). Imidacloprid is a pesticide effective against sucking and biting plant pests such as aphids, leafhoppers and planthoppers, thrips and whiteflies and some Heteroptera, Coleoptera and Lepidoptera species (Mullins, 1993),but it has no effect on nematodes or spider mites (Elbert et al., 1991). Imidacloprid is a Nicotinic acetylcholine receptor (nAChR) competitive modulator (www.irac-online.org, website). It has a mode of action acetylcholine-mimetic type, permanently binding to nicotinic receptors of the acetylcholine and causing an over excitement of the nervous cells that ends with the death of the insect. The molecule is active byingestion and direct contact, inactive as vapor phase (Elbert et al., 1991).

1.2.2 Spinetoram

Spinetoram (Delegate ™) is a new insecticide belonging to the biological origin products class, formulated by Dow AgroSciences (Spinetoram, Technical bulletin, 2006). It was registered with the commercial name Delegate. As the other spinosyns, Spinetoram is derived from *Saccharopolyspora spinosa* Mertz & Yao, an actinomycete bacterium, common in the soil (Spinetoram, Technical bulletin, 2006). This product formulated by Dow AgroSciences, is the result of fermentation of the biological matrix, followed by a chemical modification to create the active ingredient. The composition is constituted by two components. The major component (3'-ethoxy-5,6-dihydro spinosyn J) and the minor component (3'-ethoxy spinosyn L) (Spinetoram, Technical bulletin, 2006).



Spinetoram works as broad spectrum on many insects in different crops. From previous studies it results to have low impact on beneficial organisms. As the other spinosyns, this new formula acts trough a different site in the nicotinic receptor than neonicotinoids and other active ingredients (Dripps et al., 2008, website). Spinetoram is a Nicotinic acetylcholine receptor (nAChR) allosteric modulator (IRAC MoA Cassification, website), that affects nicotinic acetylcholine receptors and γ-aminobutyric acid (GABA) receptors on postsynaptic membranes, causing an overexcitation of nervous cells of the insect (Sumitomo-chem.co, website, 2012). An immediate effect of its ingestion is the feeding cessation and after 24 hours, paralysis and death (Mahmoud et al., 2009). Field experiment demonstrated efficacy on pests of a large variety of crops, such as, the codling moth *Cydia pomonella* (L), the armyworm *Spodoptera* spp., thrips, leafminers, etc (Dripps et al., 2008, website; Tescari et al., 2014).

2. MATERIALS AND METHODS

The laboratory experimental trials about the selectivity of insecticides towards *A. bipunctata* and *H. axyridis* started at the end of October 2013 and ended in May 2014.

The study was carried out following the advice and the guidelines of Dr. Edison Pasqualini of the Entomology area of the Department of Agricultural Sciences (DipSA) , University of Bologna.

These essays are included in the GEISCA project and in a project dealsing with the selectivity and collateral effects of pesticides on helpful arthropods of Emila-Romagna Region.

In laboratory, two different kinds of experiments were performed

1) Test on acute toxicity: mortality effects
2) Test on chronic toxicity: sublethal effects on fecundity and fertility

A residual type exposure on peach seedlings treated with mixed insecticides was chosen, according to the required dose for field treatments.

The experiments were carried out following the guidelines of the IOBC/WPRS (Pesticides and Beneficial Organisms working group) (Sterk et al., 1999).

2.1 INSECTS USED FOR THE EXPERIMENT

2.1.1 *A. bipunctata* rearing (see the description of its rearing in chapter 1)

2.1.2 *H. axyridis* rearing (see the description of its rearing in chapter 1)

The insects utilized in the experiments were reared in the Entomology area of the Agricultural Science Department, University of Bologna.

Both *H. axyridis* a dn *A. bipunctata* specimens used for tests were from these stock colonies, which were maintained at DipSA in climate cells at T: 25±1°C, 70±10% H.R., 16L: 8D photoperiod (Lanzoni et al., 2004). The colonies were started from individuals collected from a biological orchard and from the educational garden of the Department.

2.2 EXPERIMENT 1-ACUTE TOXICITY: mortality effects

The test was performed in climate cells at DipSA at the conditions described above. The toxicity of two different insecticides, Imidacloprid (Confidor) and Spinetoram (Delegate™) towards *H. axyridis* and *A. bipunctata*, was tested Experimental units consisted of micropropagated peach seedlings (30 cm height), decanted in 8-cm diameter pots.

For this test, third instar larvae and adults were used.

Three treatments were compared. The insecticide doses in the tested theses were selected according to the required dose for field treatments. The recommended doses for field treatments are established by the chemical companies of production, that report on label of each pesticide product the suitable concentration for kind of pest and for crop to be treated.

- Imidacloprid (Confidor ® 50 mL/hL Water)
- Spinetoram (Delegate™ 23.33 g/hL Water)
- Control: Tap water

Both for larvae and adults 5 replicates for each thesis were carried out. The seedlings were sprayed with a manual spray dispenser until leaf dripping according to a complete random experimental scheme. Once dry, on each plant 15 individuals of *H. axyridis* and 15 individuals of *A. bipunctata* were separately introduced. Each seedling was inserted in a plexiglass cylinder, 9 cm of diameter, 30 cm of eight, isolated at the bottom with a breathable tissue and provided with a perforated cap at the top and a further lateral hole, 9 cm of diameter, to insure the ventilation.

Photo 11. Seedlings of peach inserted in cylinders for the experiment. Entomology archive

During the experiment, the coccinellids were nourished with *Ephestia kueniella* eggs, glued on strips of wax paper with a solution of water and honey and inserted every two days into the cylinder. The mortality was detected after 2 and 7 days for adults and after 2 and 10 days for larvae (10 days are the time necessary to obtain the 80% of new emerged adults in the control, from the third instar larvae) , in this last case when in the control, the 80% of adults has emerged.

For the result comprehension, it has to be pointed out that, according to the evaluation scale of "IOBC"(International Organization for Biological Control) related to laboratory analyses and based on their mortality effect (found in several tests), the insecticides are classified in 4 categories of toxicity (Hassan, 1998; Hassan, 1992; Hassan, 1988):

1- Harmless (< 30%);
2- Slightly harmful (30-79%);
3- Moderately harmful (80-99%);
4- Harmful (> 99%).

2.3 EXPERIMENT 2-CHRONIC TOXICITY: sublethal effects on reproduction

The chronic toxicity of Imidacloprid (Confidor®) and Spinetoram (Delegate™) insecticides towards *H. axyridis* and *A. bipunctata* was also evaluated in the laboratories of (DipSA).

On the base of the mortality detected in the first test, also in this case it was decided to use the recommended doses for peach cultivation:

- Imidacloprid (Confidor ® 50 mL/ hL Water)
- Spinetoram (Delegate ™23.33 g/ hL Water)
- Control: Tap water

The materials and methods followed in this test were the same as those used in the first experiment, but the number of replicates per treatment was different, according to the availability of the emerged coccinellids.

The day after their emergence, adults of *H. axyridis* and *A. bipunctata* were were subjected to contact with treated seedlings for 48 hours. At the end of exposure, the alive individuals were sexed under a stereo microscope. Pairs composed by 15 treated-female/ 15 untreated-male, with males obtained from the standard rearing, were then formed. Each pair was kept in a small standard plastic cylindrical container, height 8 cm, diameter 6 cm. As oviposition support, a bubble wrap strip was applied to the internal container borders and daily replaced. The couples were fed with pea shoots infested by aphids (which were daily replaced) and kept in climate chambersat conditions of T 25°C, R.H.. 75%, 16:L 8:D photoperiod.

Photo 12. Cylinders for the maintaining of couples of coccinellids during the experiment. Entomology archive

The experiment took place according to a standard protocol (Lanzoni et al., 2004). For fertility evaluation, the collected eggs were transferred into identical plastic containers, where the hatched eggs were daily checked and the larval study followed. Into the plastic containers *E. kueniella* eggs were introduced on wax paper strips glued as food to avoid cannibalism events. The newly-hatched larvae, after hatching, were immediately counted and removed. Moreover the longevity of fertile females, the development time and survival of 60 larvae for each thesis with the sex- ratio of these last become adults, were evaluated.

2.4 Parameters considered in the study

The parameters considered in the study for the acute effects are:

-larval mortality at 2 and 10 days from the treatment

-adult mortality at 2 and 7 days from the treatment

The parameters considered in the study for the sublethal effects are:

-Mortality effects of the adults exposed to the treatments

-Survival time (Longevity) of adult females

-Fecundity of females

-Fertility of females

-Oviposition rate

-Development time of larvae

-Percentage of adults emerged

-Sex-ratio of adults emerged

2.5 DATA ANALYSIS

The data related to the mortality of larvae and adults were processed with one way ANOVA analysis, followed by the Tukey test ($p \leq 0.05$) for the means separation of the three treatments in each species. The mortality caused by the tested substances was corrected through the Abbott formula in order to compare the insecticide effects purified from the natural mortality of the control. The mortality of larvae and adults was calculated in pure numbers of deaths and percentages.

In order to investigate the sublethal effects, which affects the fitness of the two coccinellid species, were before evaluated the mortality effects of the treatments on the adults of the two species. The females survived from the treatments were then monitored for the evaluation of the other parameters.

The data related to mortality effects (for the study of sublethal effects) were analyzed with a factorial ANOVA (2*2) followed by Bonferroni test for mean separation ($p \leq 0,05$) (Soliani, 2008).

Kaplan- Meier estimators were used to calculate mean survival times and 95% confidence interval. Since all adult individuals were daily followed until death, no data were censured.

The Log-Rank test was used to identify significant difference ($p \leq 0.05$) between treatments and coccinellids species. In particular it is a test that verified/checked differences among survival curves. In case of statistical significance detected by this test, Holm- Sidak multiple comparison method was applied. The statistical programme used to the survival time evaluation was Sigma – Stat (Masetti et al., 2008).

The effects of the two insecticides on fecundity and fertility were analyzed only for data related to *A. bipunctata*. For *H. axyridis* it was not possible for the death of all females before starting the oviposition.

The fecundity and fertility of *A. bipunctata* were analyzed with one way ANOVA. Levene's test for Homogeneity of variances was performed.

The oviposition rate was calculated to know how many eggs were laid by each female of *Adalia* and *Harmonia* per day. The calculation was carried out dividing the total number of eggs produced by each female for the total number of days of her life. The calculation was repeated for each thesis.

In order to study the development time of larvae egg hatching until the adult stage, a number of 60 larvae from each thesis were tested.

The percentage of adults emerged were also calculated. This parameter was obtained from the number of hatched eggs that completed the development until the adult stage, on the starting 60s.

The 2x2 contingency table of emerged adults were performed for testing the independence among the theses; all the possible combination were calculated and compared.

The percentage of females and males were also calculated from the total of adults emerged in order to know the sex-ratio balance.

The 2x2 contingency table concerning the sex-ratio, were performed for testing the independence among the theses; all the possible combination were calculated and compared.

3. RESULTS AND DISCUSSIONS

3.1 EXPERIMENT 1-ACUTE TOXICITY: mortality effects

Table 1 reports the mean percentages of larval mortality of *Adalia bipunctata* for each thesis and after 2 days from the treatment.

LARVAE	% mean mortality	ST ERR fra %	ST DEV fra %
C-CONTROL	17,33	9,09	20,33
A-IMIDACLOPRID	57,33	8,06	18,01
B-SPINETORAM	20,00	2,98	6,67

Table 1. Mean percentages (%) of mortality in the third instar larvae of *A. bipunctata* after 2 days from the treatment. The percentages were calculated on the original number of larvae.

In figure 1 the results of the acute toxicity test carried out on third instar larvae of *A. bipunctata* are reported. The data were collected after 2 days from the treatment (speed of action). The results are expressed as larval mortality.

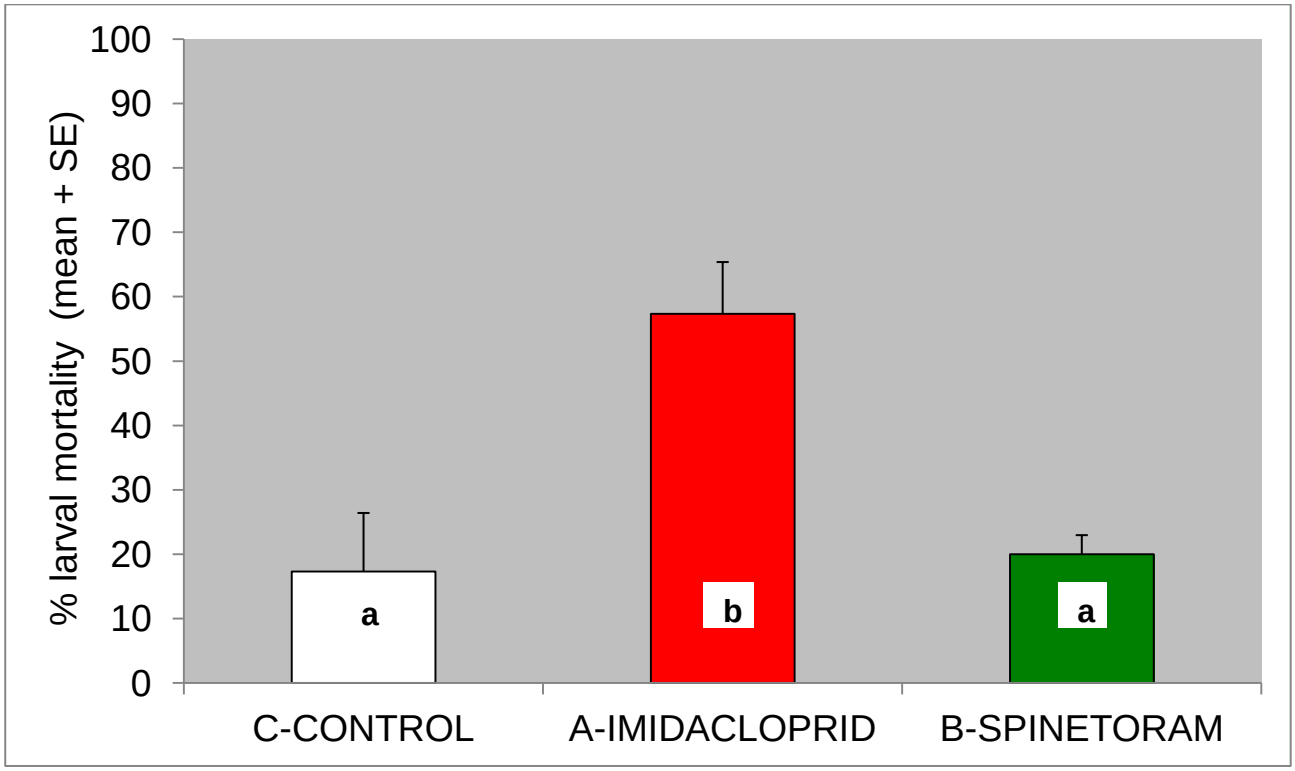


Fig.1. Acute toxicity trial on larvae: effect of the insecticides tested on larval mortality of *A. bipunctata* (expressed in %) at 2 days from the treatment. The data were analyzed by one way ANOVA followed by HSD Tukey test for mean separation ($p \leq 0.05$). The bars represent the standard errors.

The results show that there were not significant differences in % larval mortality between Spinetoram and the control. Instead, Imidacloprid was significantly different (higher larval mortality) both from Spinetoram and the control.

Table 2 reports the mean values related to the larval mortality, expressed as pure numbers, of *A. bipunctata* for each thesis and after 2 days from the treatment.

Product	MEAN deaths	ST DEV
C-CONTROL	2,60	3,05
A-IMIDACLOPRID	8,60	2,70
B-SPINETORAM	3,00	1,00

Table 2. Mean values and standard deviation of mortality (expressed as pure numbers) in third instar larvae of *A. bipunctata* after 2 days from the treatment.

Figure 2 reports the mean values of the larval mortality (pure numbers) of *A. bipunctata* for each thesis, after 2 days from the treatment.

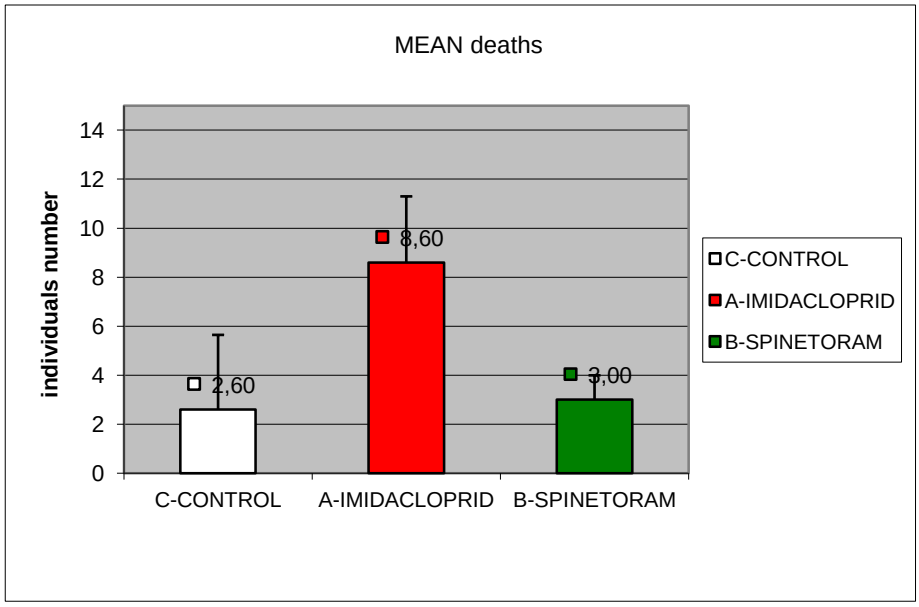


Fig. 2. Mean values (in columns) of mortality of the third instar larvae in *A. bipunctata* after 2 days from the treatment. Bars represent the standard deviations

The data concerning *A. bipunctata* mortality, expressed as pure number and reported in Table 2 and Figure 2, confirm that Imidacloprid was distinctly different compared to Spinetoram and the control. On the contrary Spinetoram and the control were similar.

Table 3 reports the mean percentages of larval mortality of *A. bipunctata* for each thesis and and after 10 days from the treatment.

LARVAE	% mean mortality	ST ERR fra %	ST DEV fra %
C-CONTROL	37,33	5,81	13,00
A-IMIDACLOPRID	64,00	7,77	17,38

B-SPINETORAM	34,67	3,89	8,69

Table 3. Mean percentages (%) of mortality in the third instar larvae of *A. bipunctata* after 10 days from the treatment. The percentages were calculated on the original number of larvae.

Figure 3 reports the results of the acute toxicity test carried out on third instar larvae of *A. bipunctata*. The data were collected after 10 days from the treatment (10 days= the necessary time for obtaining the 80% of adult emergence in the control).

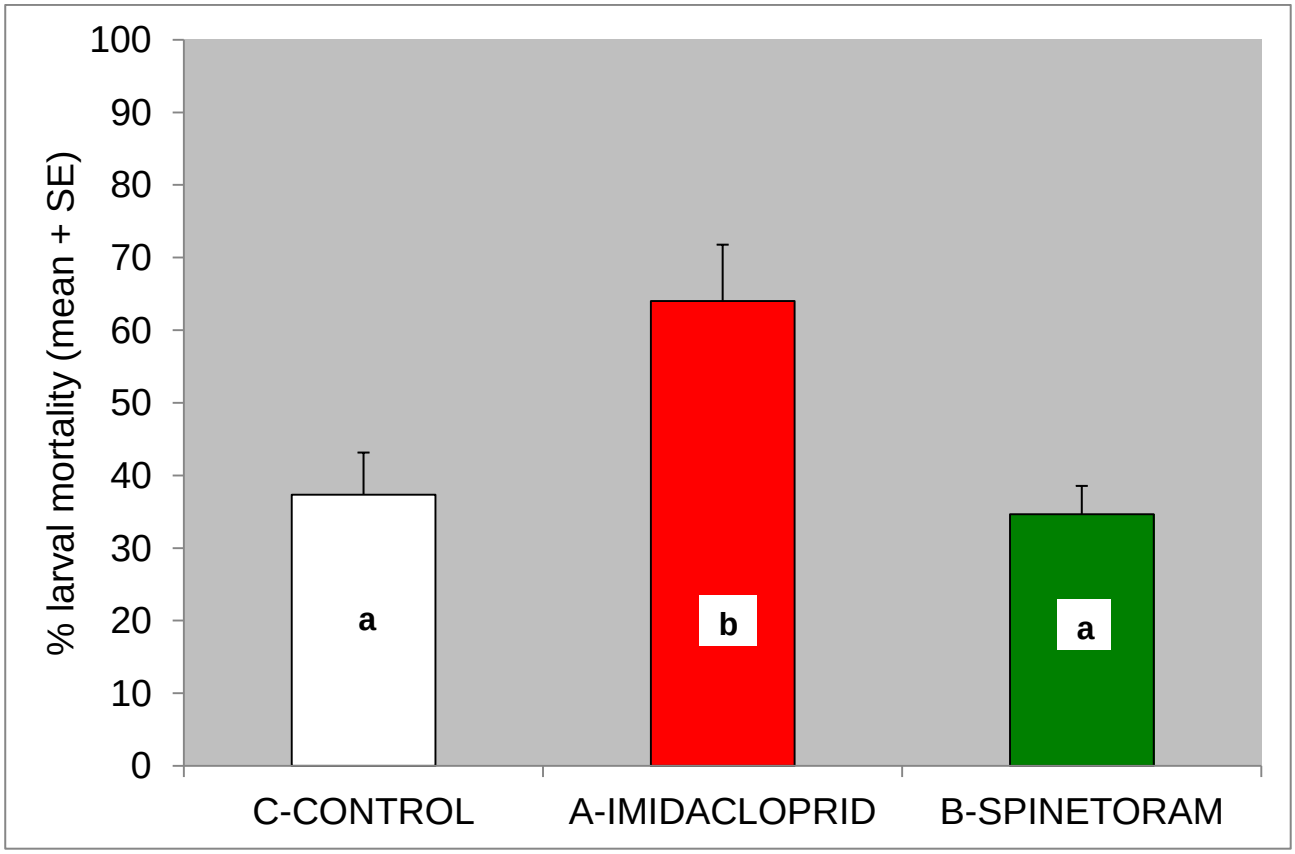


Fig.3 Acute toxicity trial on larvae: effect of the insecticides tested on larval mortality of *A. bipunctata* (expressed in %) at 10 days from the treatment. The data were analyzed by one way ANOVA followed by HSD Tukey test for mean separation ($p \leq 0.05$). The bars represent the standard errors.

The data indicate that, after 10 days, Imidacloprid induced a significantly higher mortality compared with the control, instead Spinetoram did not differ from the control.

Table 4 reports the mean values related to the larval mortality, expressed as pure numbers, of *A. bipunctata* for each thesis and after 10 days from the treatment.

Product	MEAN deaths	ST DEV
C- CONTROL	5,60	1,95
A-IMIDACLOPRID	9,60	2,61
B-SPINETORAM	5,20	1,30

Table 4. Mean values and standard deviations of mortality (expressed as pure numbers) in third instar larvae of *A. bipunctata* after 10 days from the treatment.

Figure 4 reports the mean values of the larval mortality (pure numbers) of *A. bipunctata* for each thesis, after 10 days from the treatment.

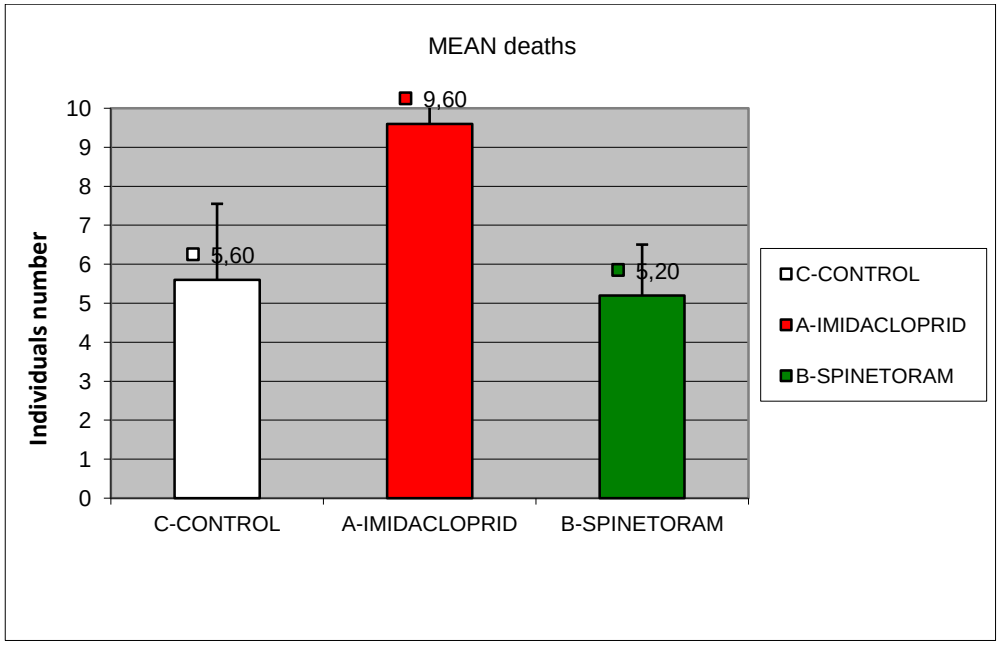


Fig. 4. Mean values (in columns) of mortality of the third instar larvae in *A. bipunctata* after 10 days from the treatment. Bars represent the standard deviations

In Table 4 and in Figure 4 the mean values of *A. bipunctata* larval mortality, expressed as pure numbers, are reported. The data show that the effect of Imidacloprid was distinctly different compared to Spinetoram and the control. On the contrary Spinetoram and the control were similar.

Table 5 reports the mean percentages of larval mortality of *Harmonia axyridis* for each thesis and after 2 days from the treatment.

LARVAE	% mean mortality	ST ERR fra %	ST DEV fra %
C-CONTROL	16,00	4,99	11,16
A-IMIDACLOPRID	69,33	11,47	25,65
B-SPINETORAM	18,67	8,27	18,50

Table 5. Mean percentages (%) of mortality in the third instar larvae of *H.axyridis* after 2 days from the treatment. The percentages were calculated on the original number of larvae.

Figure 5 reports the results of the acute toxicity test carried out on third instar larvae of *H. axyridis*. The data were collected after 2 days from the treatment (speed of action).

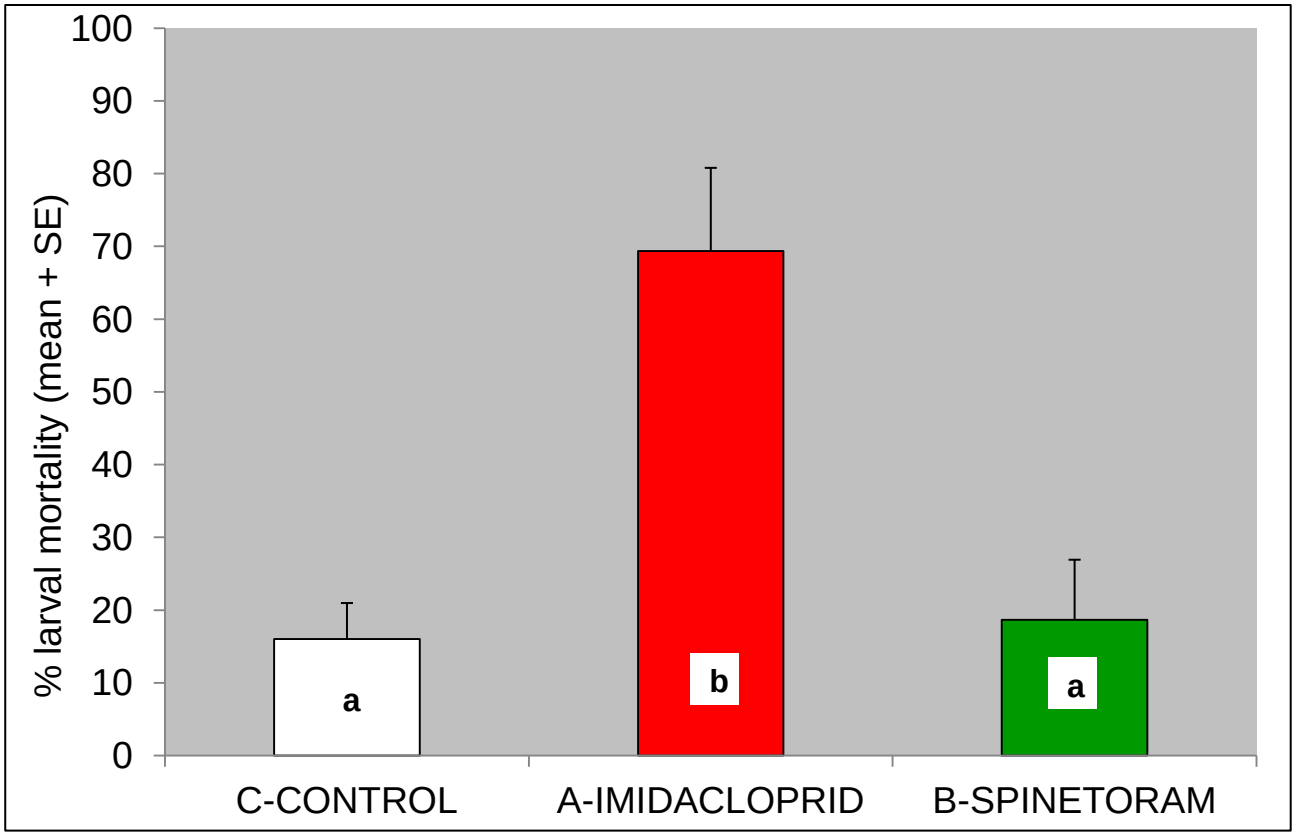


Fig.5. Acute toxicity trial on larvae: effect of the insecticides tested on larval mortality of H. axyridis (expressed in %) at 10 days from the treatment. The data were analyzed by one way ANOVA followed by HSD Tukey test for mean separation ($p \leq 0.05$).The bars represent the standard errors.

The results show that, also for *H. axyridis*, there were not significant differences in % larval mortality between Spinetoram and the control.. Instead, Imidacloprid was significantly different (higher larval mortality) both from Spinetoram and the control.

Table 6 reports the mean values related to the larval mortality, expressed as pure numbers, of *H. axyridis* for each thesis and after 2 days from the treatment.

Product	MEAN deaths	ST DEV
C-CONTROL	2,40	1,67
A-IMIDACLOPRID	10,40	3,85
B-SPINETORAM	2,80	2,77

Table 6. Mean values and standard deviation of mortality (expressed as pure numbers) in third instar larvae of *H. axyridis* after 2 days from the treatment.

Figure 6 reports the mean values of the larval mortality (pure numbers) of *H. axyridis* for each thesis, after 2 days from the treatment.

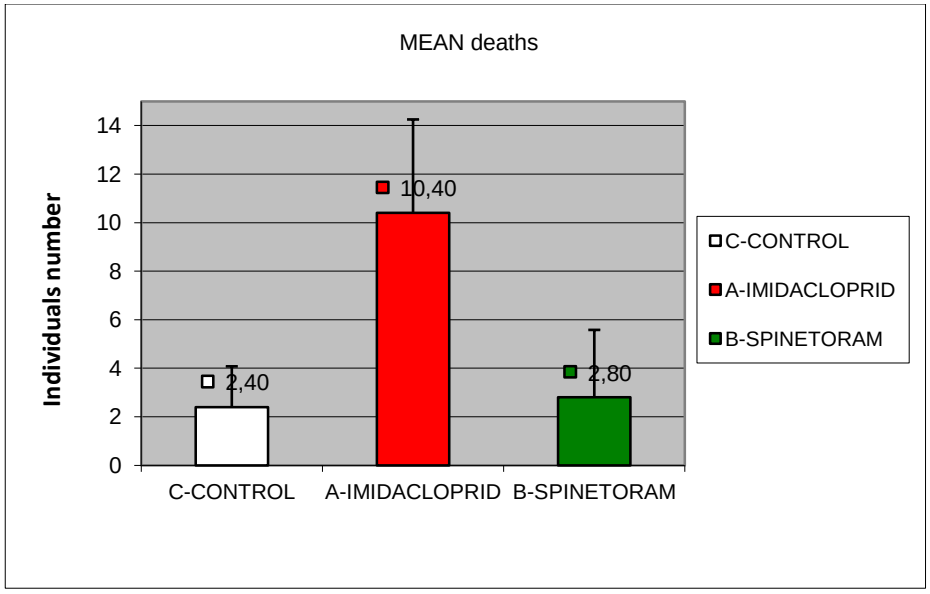


Fig. 6. Mean values (in columns) of mortality of the third instar larvae in *H. axyridis* from the treatment. Bars represent the standard deviations

The mean values reported in table 6 and in figure 6 show that there was a marked difference between Imidacloprid and the control. No great differences were conversely found between Spinetoram and Control.

Table 7 reports the mean percentages of larval mortality of *H. axyridis* for each thesis and and after 10 days from the treatment.

LARVAE	% mean mortality	ST ERR fra %	ST DEV fra %
T-TESTIMONE	26,67	8,16	18,26
A-IMIDACLOPRID	70,67	6,18	13,82
B-SPINETORAM	44,00	5,81	13,00

Table 7. Mean percentages (%) of mortality in the third instar larvae of *H. axyridis* after 10 days from the treatment. The percentages were calculated on the original number of larvae.

Figure 7 reports the results of the acute toxicity test carried out on third instar larvae of *H. axyridis*. The data were collected after 10 days from the treatment (10 days= the necessary time for obtaining the 80% of adult emergence in the control, from the third instar larvae).

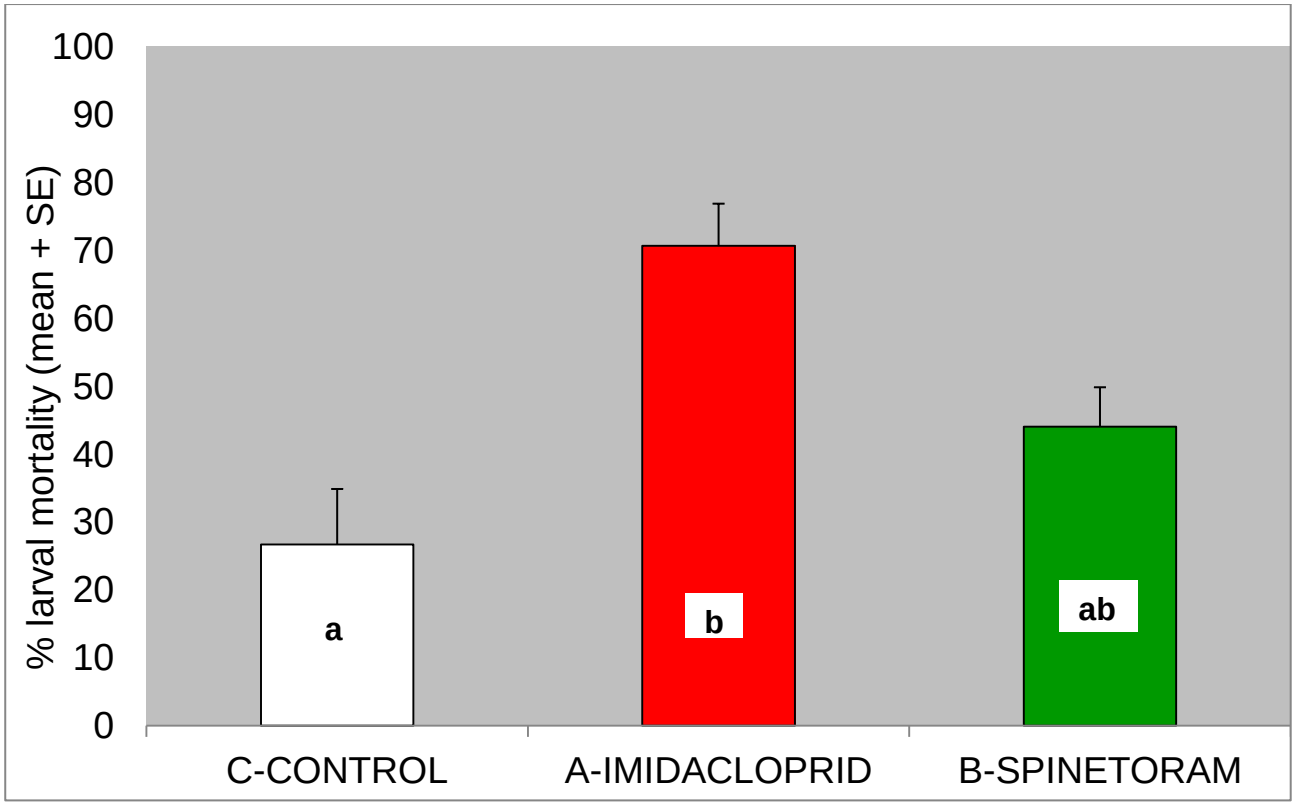


Fig.7. Acute toxicity trial on larvae: effect of the insecticides tested on larval mortality of *H. axyridis* (expressed in %) at 10 days from the treatment. The data were analyzed by one way ANOVA followed by HSD Tukey test for mean separation (p≤0.05). The bars represent the standard errors.

The data indicate that Imidacloprid caused a significantly higher larval mortality compared to the control at 10 days after exposure while Spinetoram did not differ statistically from the other 2 theses treatments (Imidacloprid and Control).

Table 8 reports the mean values related to the larval mortality, expressed as pure numbers, of *H. axyridis* for each thesis and after 10 days from the treatment.

Product	MEAN deaths	ST DEV
C- CONTROL	4,00	2,74
A-IMIDACLOPRID	10,60	2,07
B-SPINETORAM	6,60	1,95

Table 8. Mean values and standard deviations of mortality (expressed as pure numbers)) in third instar larvae of *H. axyridis* after 10 days from the treatment.

Figure 8 reports the mean values of the larval mortality (pure numbers) of *H. axyridis.* The data were collected for each thesis, after 10 days from the treatment.

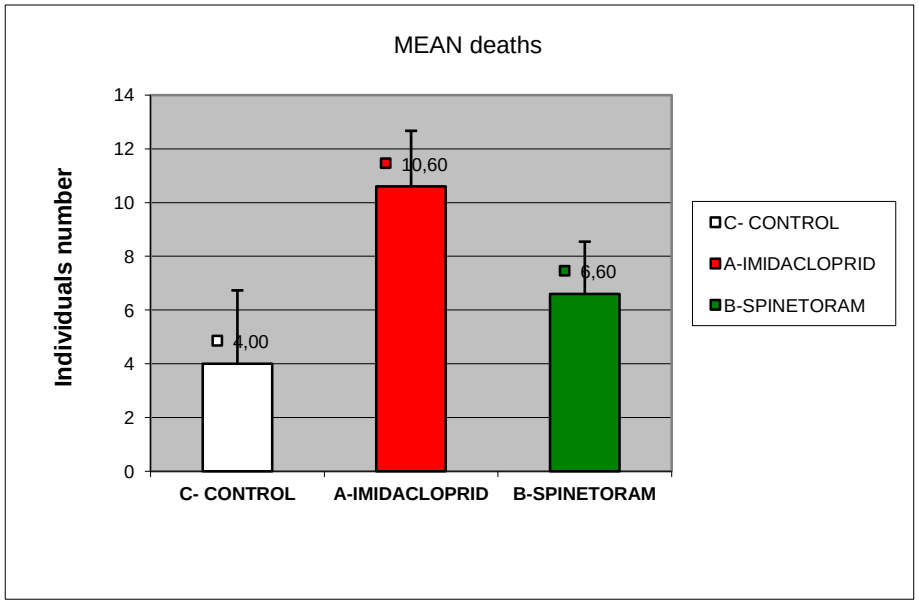


Fig. 8. Mean values (in columns) of mortality of the third instar larvae in *A. bipunctata* after 10 days from the treatment. Bars represent the standard deviations

The mean values reported in the graph (Figure 8) indicate the toxic effect of Imidacloprid on *Harmonia* larvae. In fact the mortality caused by this insecticide was considerably higher than that registered in the control. The effect of Spinetoram was in between that of Imidacloprid and the control.

Table 9 reports the results of the mortality test carried out on adults of *A. bipunctata* . The number of dead individuals was recorded after 2 days from the treatment.

ADULTS	%mean mortality	ST ERR fra %	ST DEV fra %
C-CONTROL	2,67	2,67	5,96
A-IMIDACLOPRID	82,67	5,42	12,11
B-SPINETORAM	6,67	4,22	9,43

Table 9. Mean percentages (%) of mortality in the adults of *A. bipunctata* after 2 days from the treatment. The percentages were calculated on the original number of adults.

In figure 9 the results of the mortality test carried out on adults of *A. bipunctata* are reported. The number of dead individuals was recorded after 2 days from the treatment.

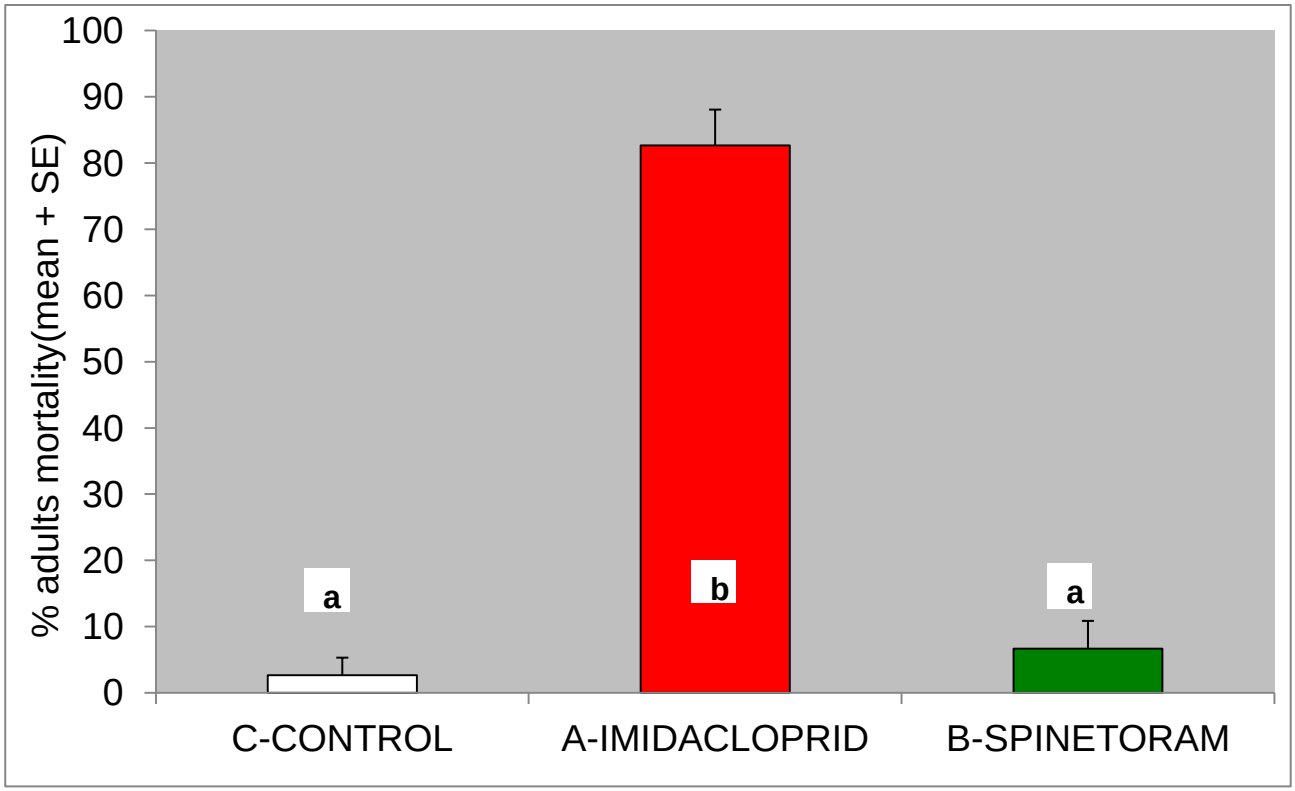


Fig.9. Acute toxicity trial on adults: effect of the insecticides tested on adult mortality of *A. bipunctata* (expressed in %) at 2 days from the treatment. The data were analyzed by one way ANOVA followed by HSD Tukey test for mean separation (p≤0.05). The bars represent the standard error.

The results indicate that the effect of Imidacloprid on *H. axyridis* adult mortality was significantly higher compared to the control. No significant difference was detected between Spinetoram and the control. The effects of the 2 insecticides was thus considerably different from each other.

Table 10 shows the mean values, expressed as pure numbers, of *A. bipunctata* adult mortality for each thesis and after 2 days from the treatment.

Product	MEAN deaths	ST DEV
C-CONTROL	0,40	0,89
A-IMIDACLOPRID	12,40	1,82

B-SPINETORAM	1,00	1,41

Table 10. Mean values and standard deviations of mortality (expressed as pure numbers) in adults of *A. bipunctata* after 2 days from the treatment.

Fig. 10. Figure 6 reports the mean values of the adult mortality (pure numbers) of *A. bipunctata* for each thesis, after 2 days from the treatment.

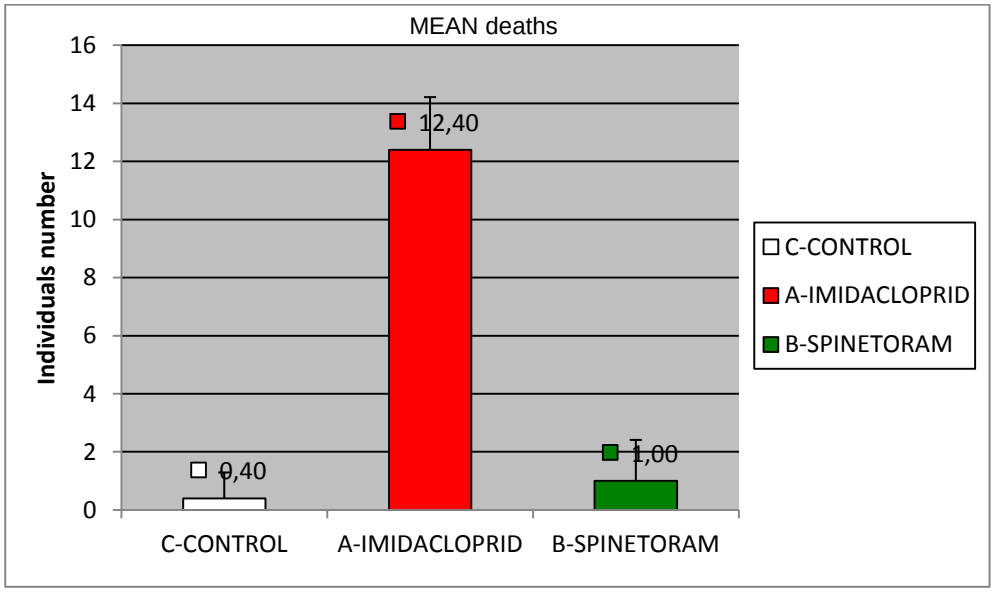


Fig. 10. The graph above reports in columns the mean values of mortality of the adults of *A. bipunctata* after 2 days from the treatment.

The data recorded in table and in graph indicate the great toxicity of Imidacloprid towards the adults of *A. bipunctata*. The effect of this insecticide was higher compared with the control. Instead, for Spinetoram the effect was similar to that of the control.

In table 11 the results of the mortality test carried out on adults of *A. bipunctata* are reported. The , deaths number was recorded after 7 days from the treatment.

ADULTS	% mean mortality	ST ERR fra %	ST DEV fra %
C-CONTROL	4,00	2,67	5,96
A-IMIDACLOPRID	96,00	2,67	5,96
B-SPINETORAM	8,00	5,33	11,93

Table 11. Mean percentages (%) of mortality in the adults of *A. bipunctata* after 7 days from the treatment.

In figure 11 the results of the mortality test carried out on adults of *A. bipunctata* are reported.

The deaths number was recorded after 7 days from the treatment.

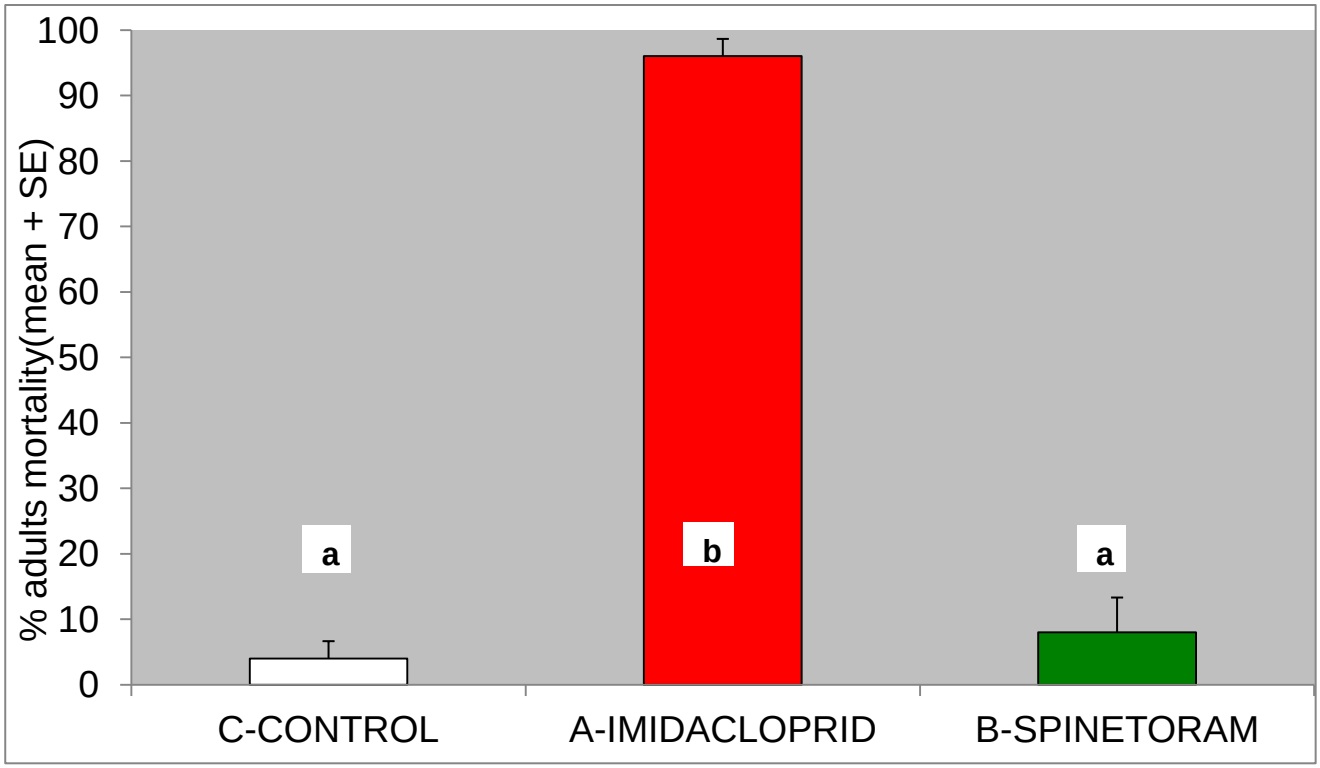


Fig.11. Acute toxicity trial on adults: effect of the insecticides tested on adult mortality of *A. bipunctata* (expressed in %) at 7 days from the treatment. The data were analyzed by one way ANOVA followed by HSD Tukey test for mean separation ($p \leq 0.05$). The bars represent the standard errors.

The data show in table and in graph demonstrate the toxic effect of Imidacloprid on *A. bipunctata* adults with a significant difference compared to the control and to Spinetoram. No significant difference was detected between Sipnetoram and the control. For Spinetoram effects are not noticed significant differences compared to those of the control.

Table 12 shows the mean values, expressed as pure numbers, of *A. bipunctata* adults for each thesis and after 7 days from the treatment.

Product	MEAN deaths	ST DEV
C-CONTROL	0,60	0,89
A-IMIDACLOPRID	14,40	0,89
B-SPINETORAM	1,20	1,79

Table 12. Mean values (obtained by pure numbers) and standard deviation of mortality in adults of *A. bipunctata* after 7 days from the treatment.

Fig. 12. shows the mean values of the adult mortality of *A. bipunctata*, expressed as pure numbers. The data were collected for each thesis, after 7 days from the treatment.

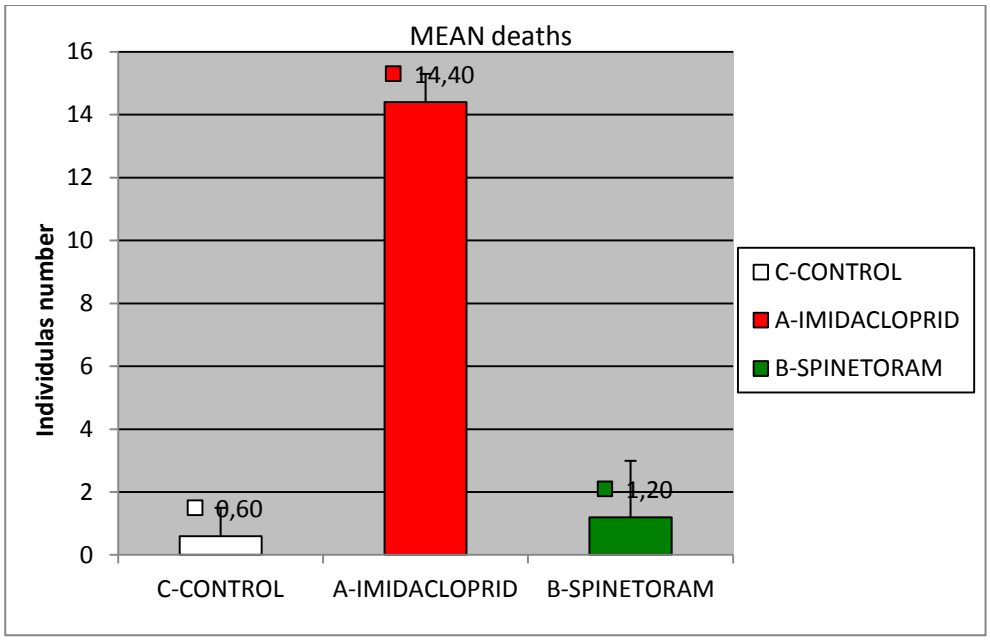


Fig. 12. The graph above reports in columns the mean values of mortality of the adults of *A. bipunctata* after 7 days from the treatment.

The data of death means demonstrate the high acute toxicity of Imidacloprid and the low acute toxicity of Spinetoram on the adults of *A. bipunctata* after 7 days from the treatment.

Table 13 shows the mean values of *H. axyridis* adult mortality (%) for each thesis and after 2 days from the treatment.

ADULTS	% mean mortality	ST ERR fra %	ST DEV fra %
C-CONTROL	5,33	1,33	2,98
A-IMIDACLOPRID	74,67	6,80	15,20
B-SPINETORAM	2,67	1,63	3,65

Table 13. Mean percentages (%) of mortality in the adults of *H. axyridis* after 2 days from the treatment.

In figure 13 the results of the mortality test carried out on adults of *H. axyridis* are reported. The death number was recorded after 2 days from the treatment.

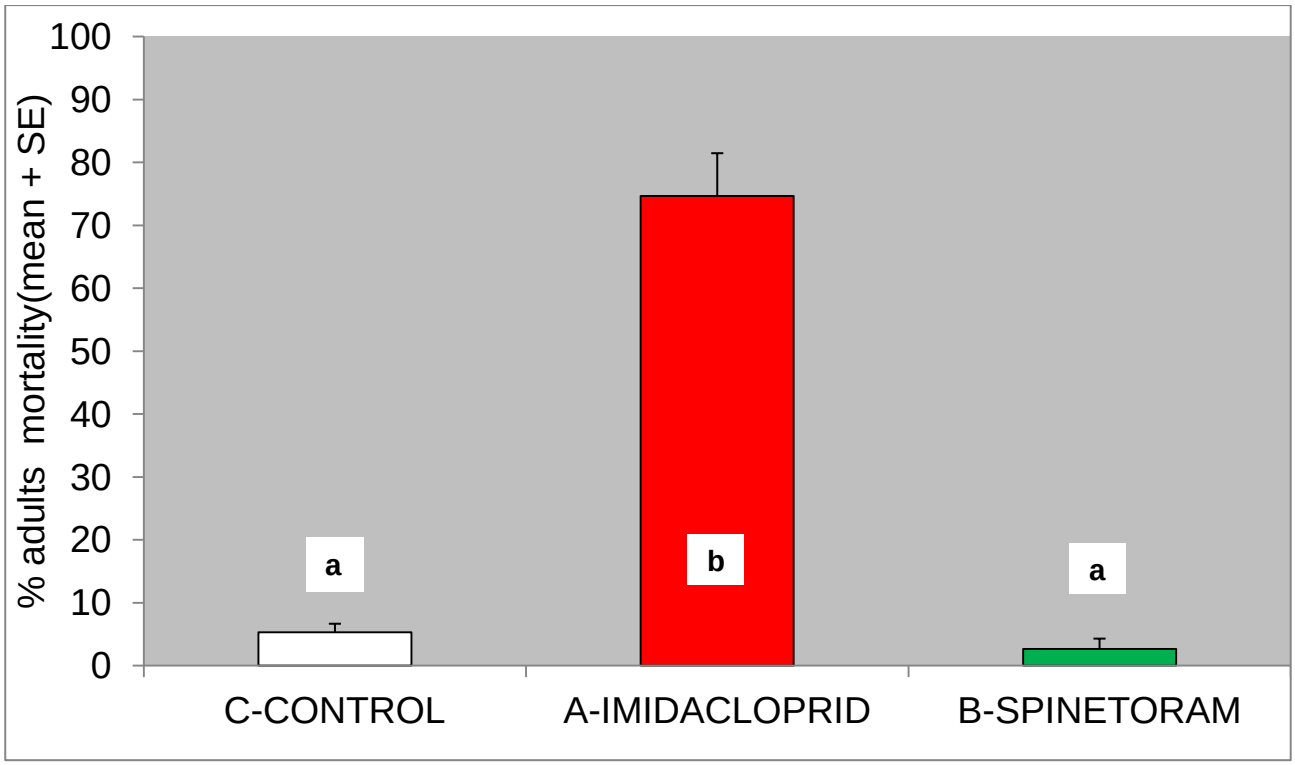


Fig.13. Acute toxicity trial on adults: effect of the insecticides tested on adult mortality (expressed in %) of *H. axyridis* at 2 days from the treatment. The results were evaluated through one way ANOVA followed by HSD Tukey test for the separation of means (p≤0.05). The bars represent the standard error.

Also in *H. axyridis* it is possible to notice the high toxicity of Imidacloprid towards the adults and after 2 days from the treatment, whereas Spinetoram displayed a loweracute toxicity insecticide, considering that its effect was not different from the control.

Table 14 shows the mean values of *H. axyridis* adult mortality (expressed as pure numbers) for each thesis and after 2 days from the treatment.

Product	MEAN deaths	ST DEV
C- CONTROL	0,80	0,45
A-IMIDACLOPRID	11,20	2,28
B-SPINETORAM	0,40	0,55

Table 14. Mean values (expressed as pure numbers) and standard deviation of mortality in adults of *H. axyridis* after 2 days from the treatment.

Fig. 14. In the graph the mean values (expressed as pure numbers) of the adult mortality of *H. axyridis* are reported. The data were collected for each thesis, after 2 days from the treatment.

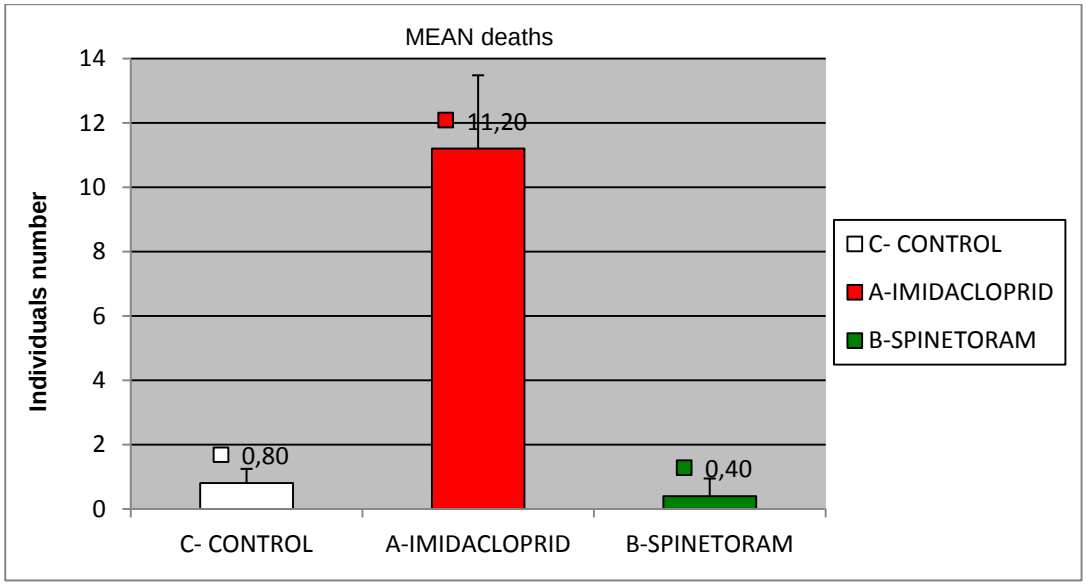


Fig. 14. The graph above reports in columns the mean values of mortality of the adults of *H. axyridis* after 2 days from the treatment.

The mean values of deaths, reported in table and in graph above, show the high toxicity of Imidacloprid compared to the control in adults of *H. axyridis* after 2 days from the treatment. On the opposite the mortality induced by Spinetoram, was similar to that caused by the control.

Table 15 shows the mean values of *H. axyridis* related to the adult mortality (%) for each thesis and after 7 days from the treatment.

ADULTS	% mean mortality	ST ERR fra %	ST DEV fra %
C-CONTROL	9,33	1,63	3,65
A-IMIDACLOPRID	96,00	2,67	5,96
B-SPINETORAM	18,67	8,79	19,66

Table 15. Mean percentages of mortality in the adults of *H. axyridis* after 7 days from the treatment.

In figure 15 the results of the mortality test carried out on adults of *H. axyridis* are reported. The deaths number was recorded after 7 days from the treatment.

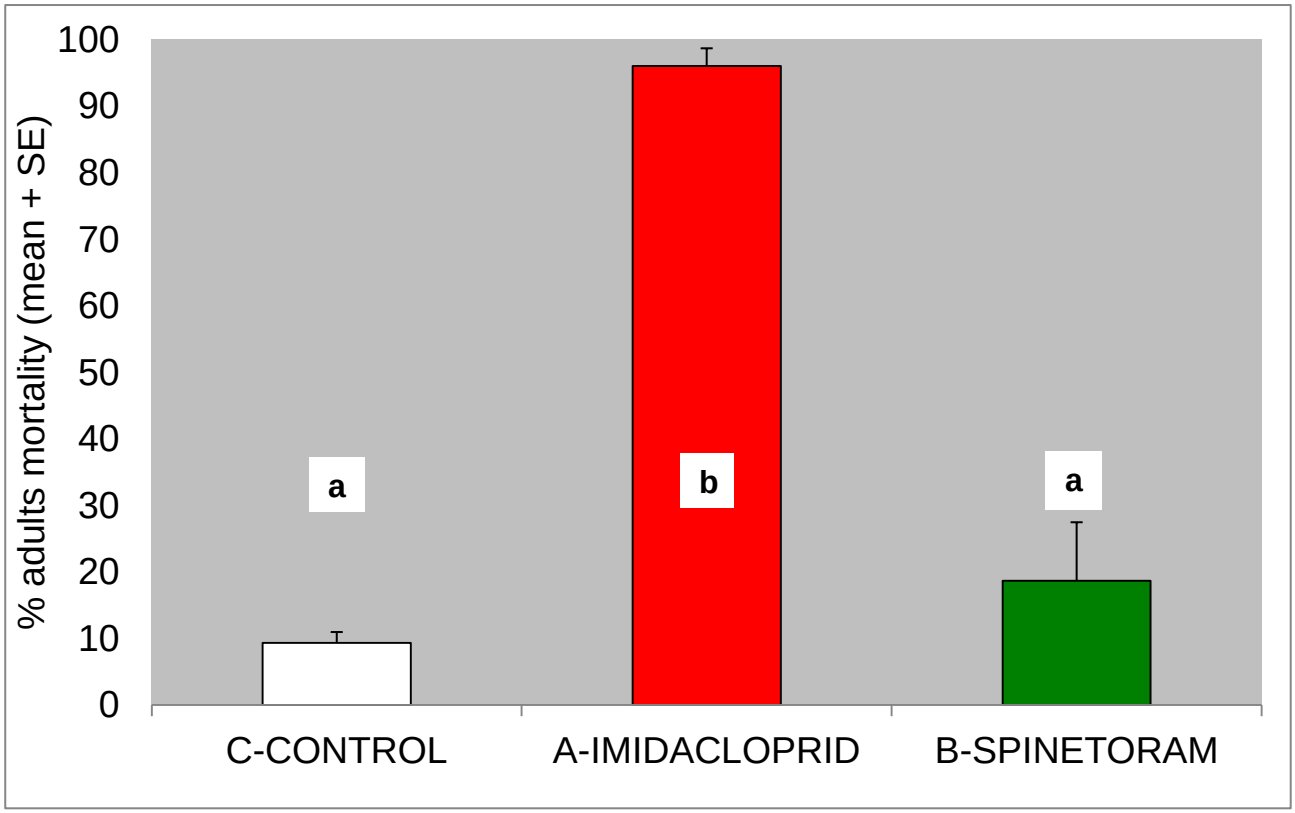


Fig.15. Acute toxicity trial on adults: effect of the insecticides tested on adult mortality (expressed in %) of *H. axyridis* at 7 days from the treatment. The results were evaluated through one way ANOVA followed by HSD Tukey test for the separation of means ($p \leq 0.05$). The bars represent the standard error.

Based on the mortality observed after 7 days from the insecticide treatment, it is possible to confirm that Imidacloprid was much more toxic for the adults of this species compared to the control. Spinetoram is to be considered as non toxic according to the evaluation scale of IOBC (Hassan, 1992; Hassan 1998), since the data related to this last were similar to those of the control.

Table 16 shows the mean values of *H. axyridis* adult mortality (pure numbers) for each thesis and after 7 days from the treatment.

Product	MEAN deaths	ST DEV
C- CONTROL	1,40	0,55
A-IMIDACLOPRID	14,40	0,89
B-SPINETORAM	2,80	2,95

Table 16. Mean values (expressed as pure numbers) and standard deviation of mortality in adults of *H. axyridis* after 7 days from the treatment.

Fig. 16. In the graph the mean values of the adult mortality (pure numbers) of *H. axyridis* are reported. The data were collected for each thesis, after 7 days from the treatment.

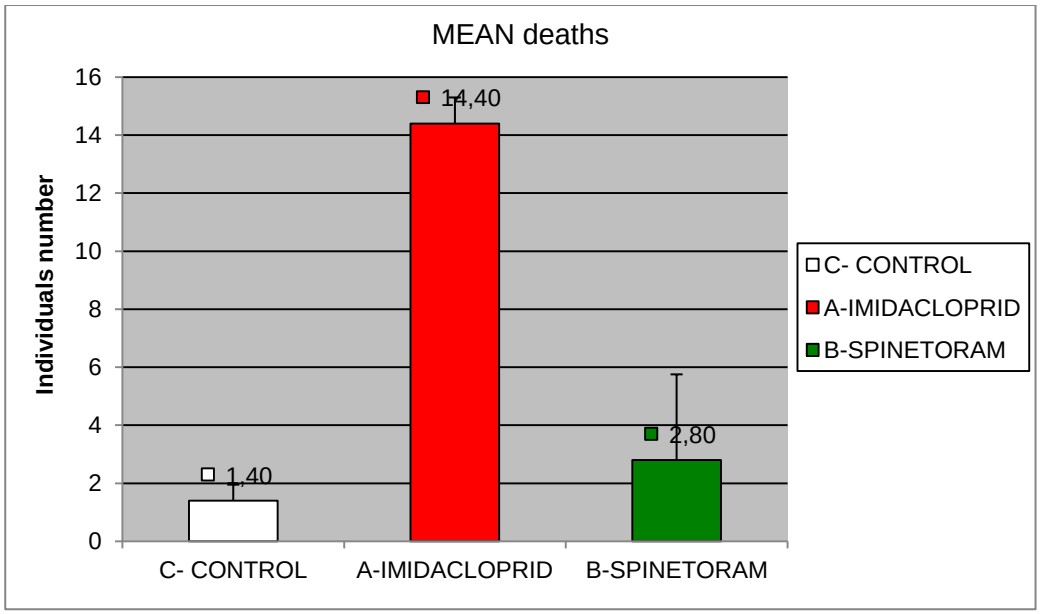


Fig. 16. The graph above reports in columns the mean values of mortality of the adults of *H. axyridis* after 7 days from the treatment.

The data reported in table and graph show the difference of the impact of the two insecticides on adult mortality. In fact, Imidacloprid proved to be highly toxic, instead the acute effect of Spinetoram, was only slightly higher compared to the control.

3.2 EXPERIMENT 2-CHRONIC TOXICITY: sublethal effects on reproduction

Mortality after treatment in the sublethal effects.

Figure 17 shows the results of the mortality of the two coccinellid species after treatment. The image refers to treatments comparison.

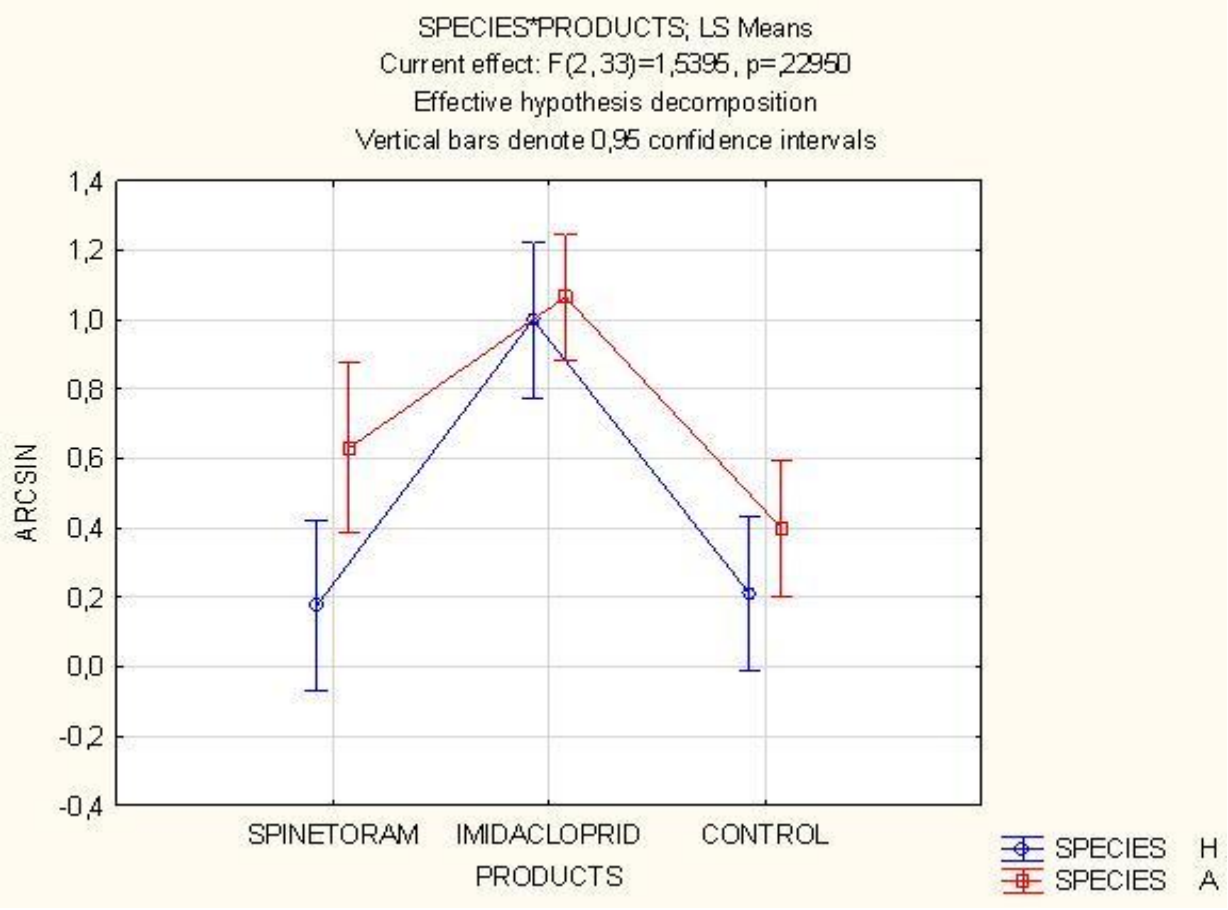


Fig.17. Mortality effects of the selected insecticides on adults of *Adalia* and *Harmonia* after 2 days from the treatment.

the data were analyzed through factorial ANOVA (2*2) followed by Bonferroni test for mean separation (p≤0.05) . The vertical bars represent the 95% confidence interval.

The graph shows that after 2 days from the treatment, in both the species the mortality of adults due to Imidacloprid was much higher than in the other two theses. But the interaction "species x product" was not significant, that is, the species and the products did not produce effect together (they did not influence each other's effect). But about Spinetoram it is possible to note a marked difference between the adult mortality of *Adalia* and *Harmonia*. The species factor and the product factor were individually significant.

Figure 18. In the graph the results of the mortality after treatment with insecticides or in the Control, in the two coccinellid species, are reported. The image refers to the two species comparison.

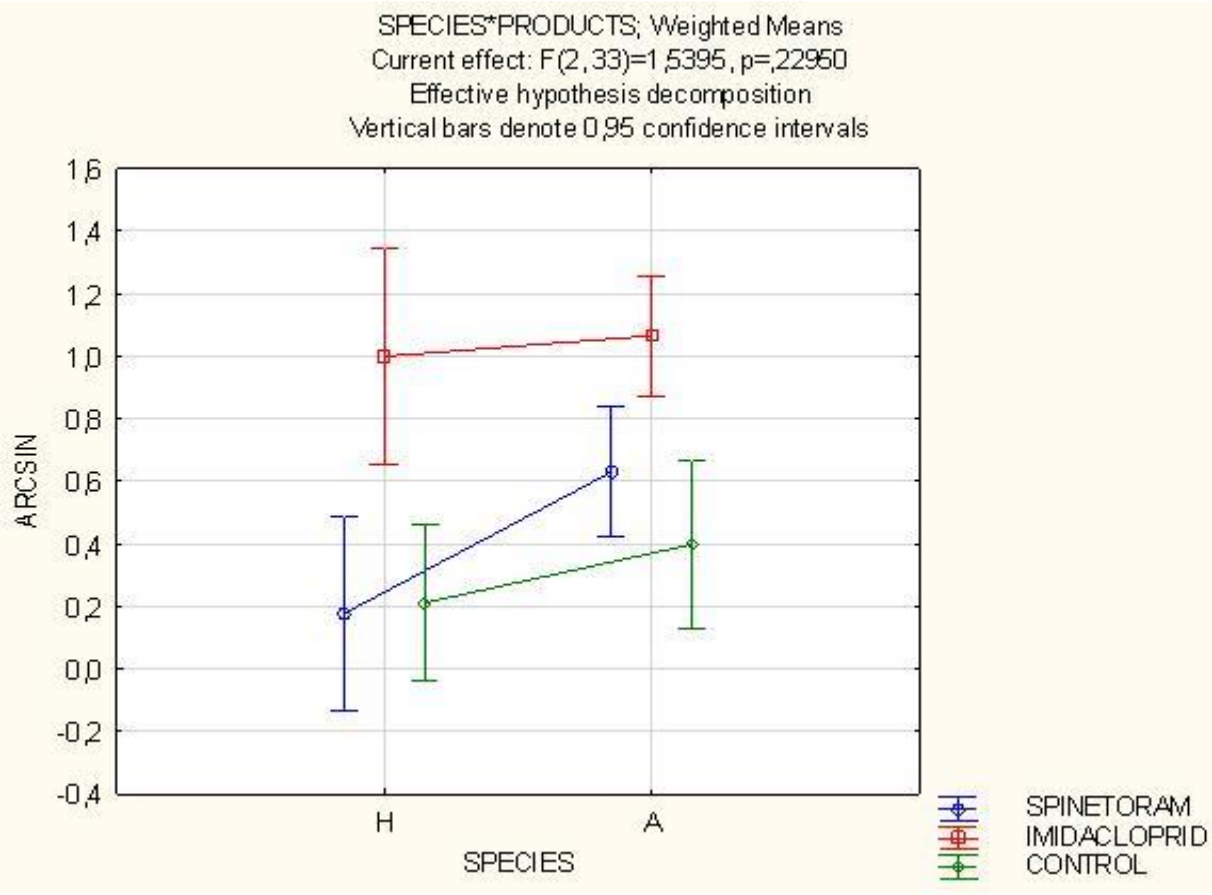


Fig.18. Mortality effects on adults of *Adalia* and *Harmonia* after 2 days from the treatment with the selected insecticides.

The data were analyzed through factorial ANOVA(2*2) followed by Bonferroni test for mean separation (p≤0.05). The vertical bars represent the 95% confidence interval.

The graph shows that Imidacloprid was the most toxic product. In fact it greatly differed from the control. Spinetoram did not differ significantly from the control, but in *Adalia* the mortality was higher than in *Harmonia*. The interaction "species x product" was not significant (*p* value> 0.05).

Figure 19 shows the effect of the two insecticides and the Control.

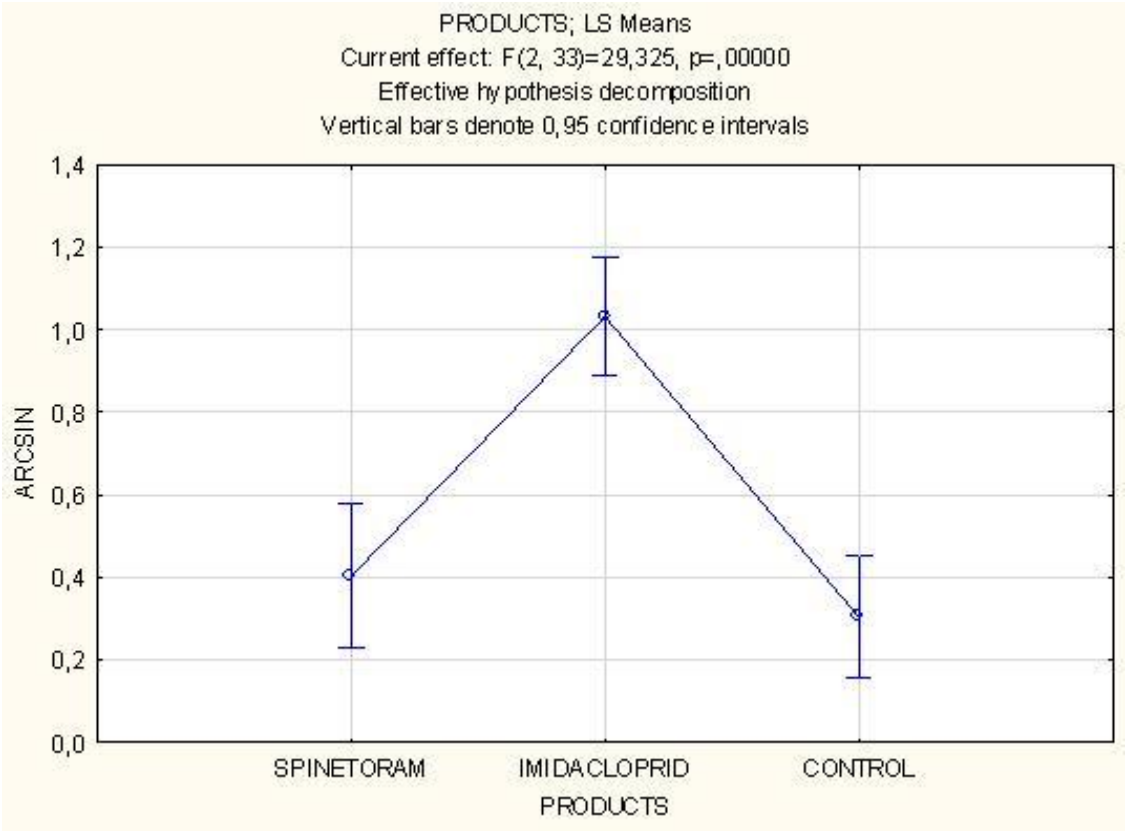


Fig.19. Effects of the two insecticides vs. the Control on the mortality of the two coccinellid species.

The data were analysed by ANOVA (2*2) followed by Bonferroni test for mean separation (p≤0.05)

Figure 19 shows that Imidacloprid was the most toxic product. In fact it differed considerably from the Control and from Spinetoram. Instead, between Spinetoram and the Control there were not significant differences.

Figure 20 shows the mortality of the two species.

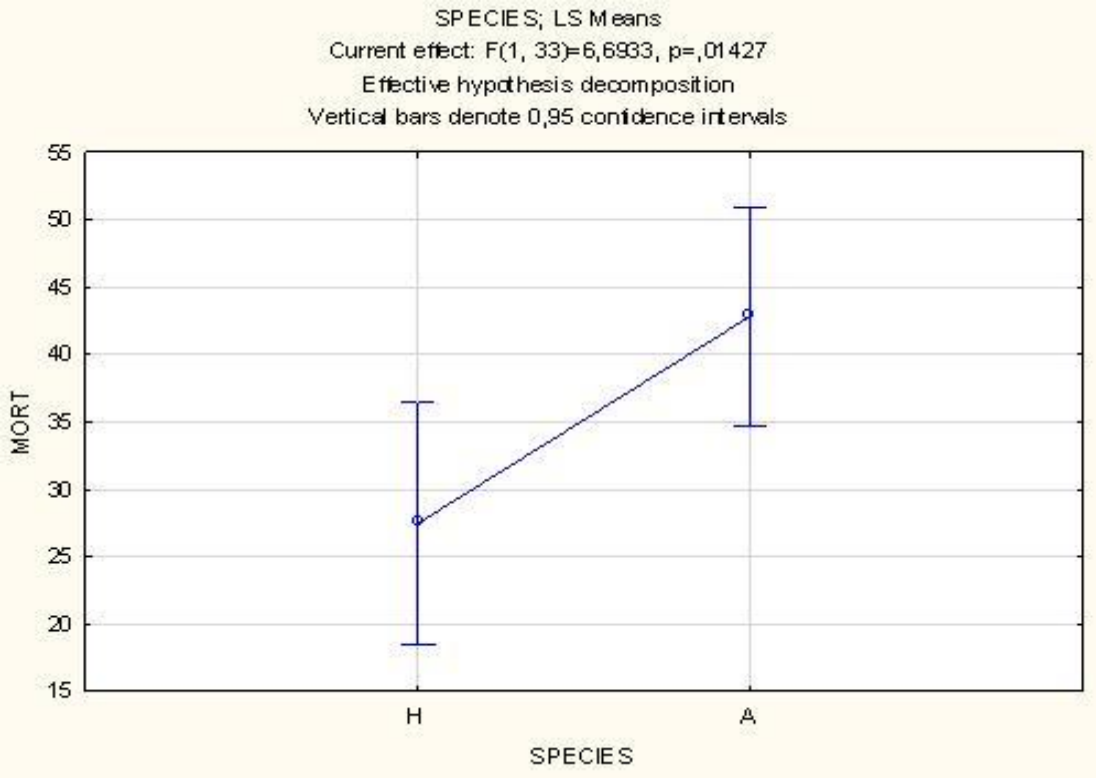


Fig.20. Mortality effects on the two coccinellid species.

The data were analyzed through ANOVA (2*2), followed by Bonferroni test for mean separation ($p \leq 0.05$).

The graph reports the mortality effects of the treatments on *Harmonia* and *Adalia*. A significantly different effect was detected. In detail, the mortality registered in *Adalia* was much higher than in *Harmonia*. The native species seemed to be more susceptible to both products than the exotic.

Survival time (Longevity) of *A. bipunctata* and *H. axyridis*

In table 17 below, the results of Kaplan –Meier Survival Analysis for the treatments on *H. axyridis* are recorded.

Harmonia axyridis	**Survival time (days) (mean)**	**95% C.I.**	**Holm-Sidak test (p≤0.05)**
Imidacloprid	27.4	17.5-37.3	a
Spinetoram	5.2	4.3-6.1	b
Control	31.9	28.4-35.3	a

Table 17. Log-Rank test used for the K-M Survival Analysis for establishing the presence of differences among the three theses of *H. axyridis*.

The results of the Log-Rank test used for the Survival Analysis of *H. axyridis* show that there were significant differences among the three theses. Especially the treatment Spinetoram reported lower values of survival than both the other two theses.

Figure 21. In the graph the same results obtained and reportred in the table above are shown. The Survival time for the three theses of *H. axyridis* are represented.

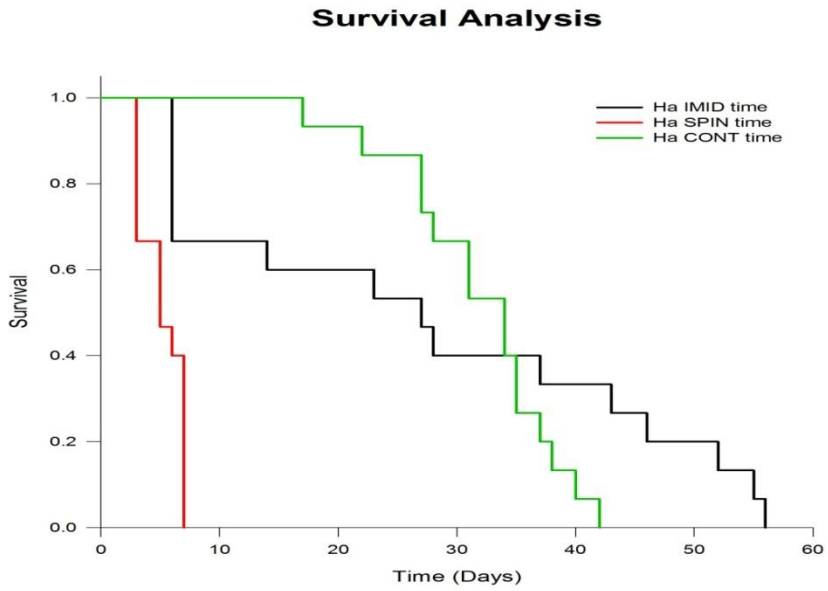


Fig.21. Graph of comparison of the Survival time in *H. axyridis* for the three theses.

The three theses compared in the exotic species show the difference in survival time, particularly short for Spinetoram. Between Imidacloprid and the control there were not significant differences.

In table 18, the results of Kaplan –Meier Survival Analysis for the treatments on *A. bipuncta* are recorded.

Adalia bipunctata	**Survival time (days) (mean)**	**95% C.I.**	**Holm-Sidak test (p≤0.05)**
Imidacloprid	28.3	18.2-38.3	a
Spinetoram	31.3	22.2-40.3	a
Control	25.1	15.6-34.7	a

Table 18. Log-Rank test used for the K-M Survival Analysis for establishing the presence of differences among the three theses of *A. bipunctata*.

The results of the Log-Rank test used for the Survival Analysis of *A. bipunctata* show that there were not significant differences among the three theses. The treatment with Spinetoram resulted in the longer survival time in days, exactly the opposite trend reached in the exotic *Harmonia*.

Figure 22 shows the same results obtained and reported in the table above. The Survival times for the three theses of *A. bipunctata* are represented.

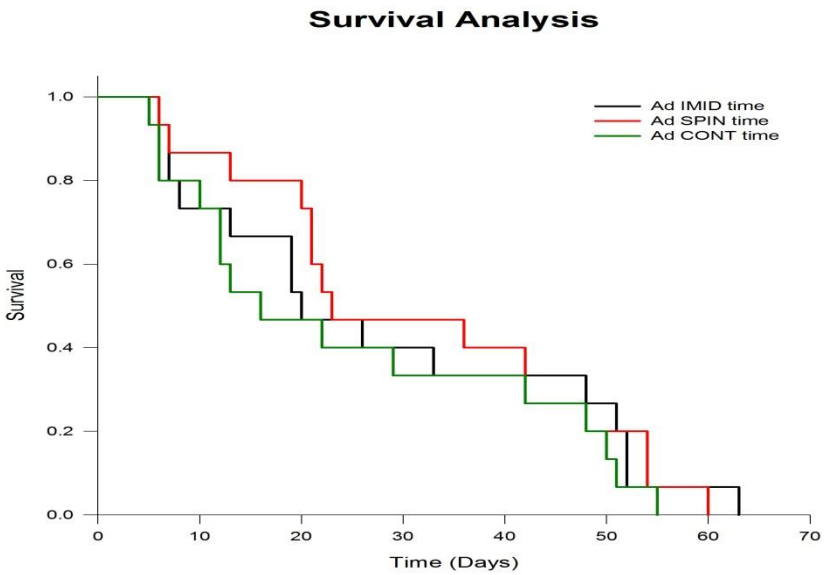


Fig.22. Comparison graph of the Survival times in *A. bipunctata* for the three theses.

For the native species, the comparison of the three theses show no differences. In fact the survival time curves intersect each other. Spinetoram (the red curve) in this case is the thesis with the longer survival time (in days).

Table 19 showes the comparison of Imidacloprid effects on survival time of the two coccinellid species *H.axyridis* and *A. bipunctata*.

Imidacloprid	**Survival time (days) (mean)**	**95% C.I.**	**Holm-Sidak test ($p \leq 0.05$)**
Harmonia axyridis	27.4	17.5-37.3	a
Adalia bipunctata	28.3	18.2-38.3	a

Tab. 19. Comparison of the values of survival time of *Harmonia* and *Adalia* treated with Imidacloprid

The values reported in table 19 show that for the Imidacloprid thesis there were not significant differences in survival time between the two coccinellid species.

Figure 23 represents the comparison of the survival time for the Imidacloprid treatment in the two coccinellids.

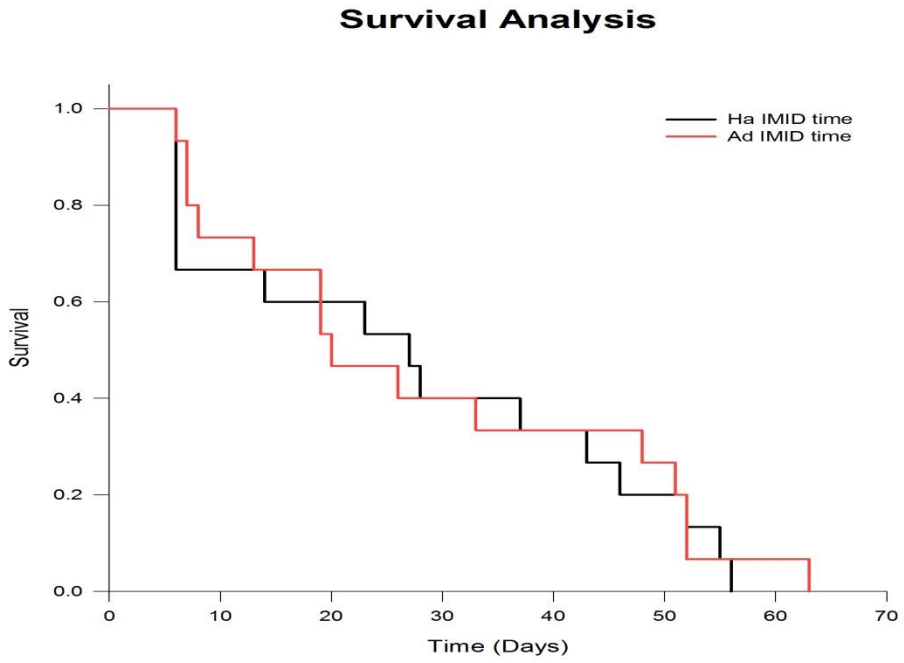


Fig.23. Comparison graph of the Survival time for Imidacloprid treatment in *Harmonia* and *Adalia.*

The curves in graph show that the trend of the survival time in the two species was very similar. In fact they intersect in many points. Between *Adalia* and *Harmonia* the Imidacloprid thesis does not cause any difference in survival time.

Table 20 reports a comparison of Spinetoram effects on survival time of the two coccinellid species *Harmonia* and *Adalia*.

Spinetoram	**Survival time (days) (mean)**	**95% C.I.**	**Holm-Sidak test ($p \leq 0.05$)**
Harmonia axyridis	5.2	4.3-6.1	a
Adalia bipunctata	31.3	22.2-40.3	b

Tab. 20. Comparison of the values of survival time of *Harmonia* and *Adalia* treated with Spinetoram

The Survival time data in table 20 indicate a significant difference on survival between the two species. Spinetoram caused a dramatic survival reduction in the exotic *Harmonia* but did not affect the longevity in *Adalia* so drastically.

Figure 24 represents the comparison of the survival time for the Spinetoram treatment in the two coccinellids.

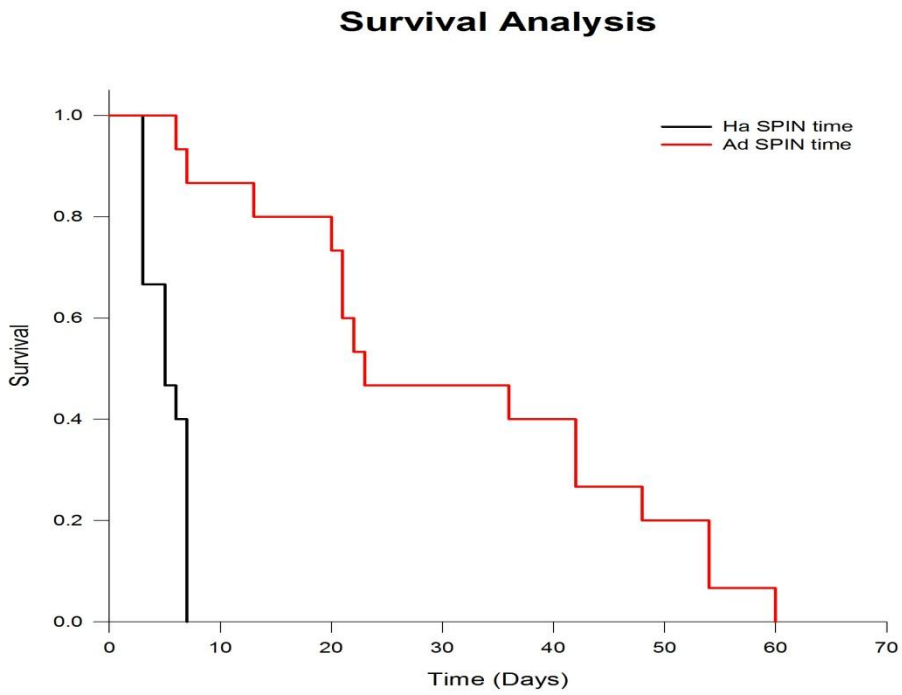


Fig.24. Comparison graph of the Survival time for Spinetoram treatment in *Harmonia* and *Adalia*.

The graph shows a big difference in the trends of the curves. In fact in *Harmonia* the curve immediately falls down, stopping to less than 10 days of survival (see table 4). Instead in *Adalia* the longevity followed a similar trend as that shown in Fig. 18.

Table 21. Comparison of Control theses on survival time of the two coccinellids *Harmonia* and *Adalia*.

Control	**Survival time (days) (mean)**	**95% C.I.**	**Holm-Sidak test ($p \leq 0.05$)**
Harmonia axyridis	31.9	28.4-35.3	a
Adalia bipunctata	25.1	15.6-34.7	a

Tab. 21. Comparison of the survival times of *Harmonia* and *Adalia* in the Control thesis.

The data shown in table 21 indicate that in the Control thesis there were no differences in survival time between the two coccinellid species, although in *A. bipunctata* the adult females lived about one week less.

Figure 25 represents the comparison of the survival time for the Control in the two coccinellids.

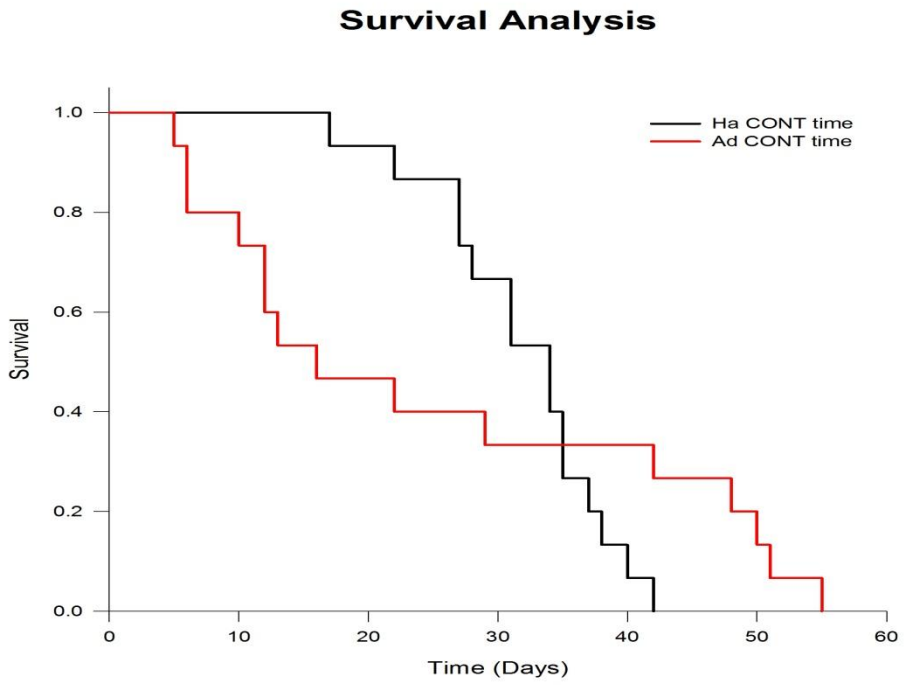


Fig.25. Comparison graph of the Survival time for Control thesis in *Harmonia* and *Adalia*.

The graph shows a not exactly concordant trend of the two survival time curves, but the intersection indicates that the two species did not vary significantly in longevity.

Effects on fecundity and fertility

Figure 26 reports the effects of the three theses on fecundity of the species *Adalia bipunctata.*
(chiedi a Burgio il calcolo che ha fatto, il p non mi viene dello stesso valore)

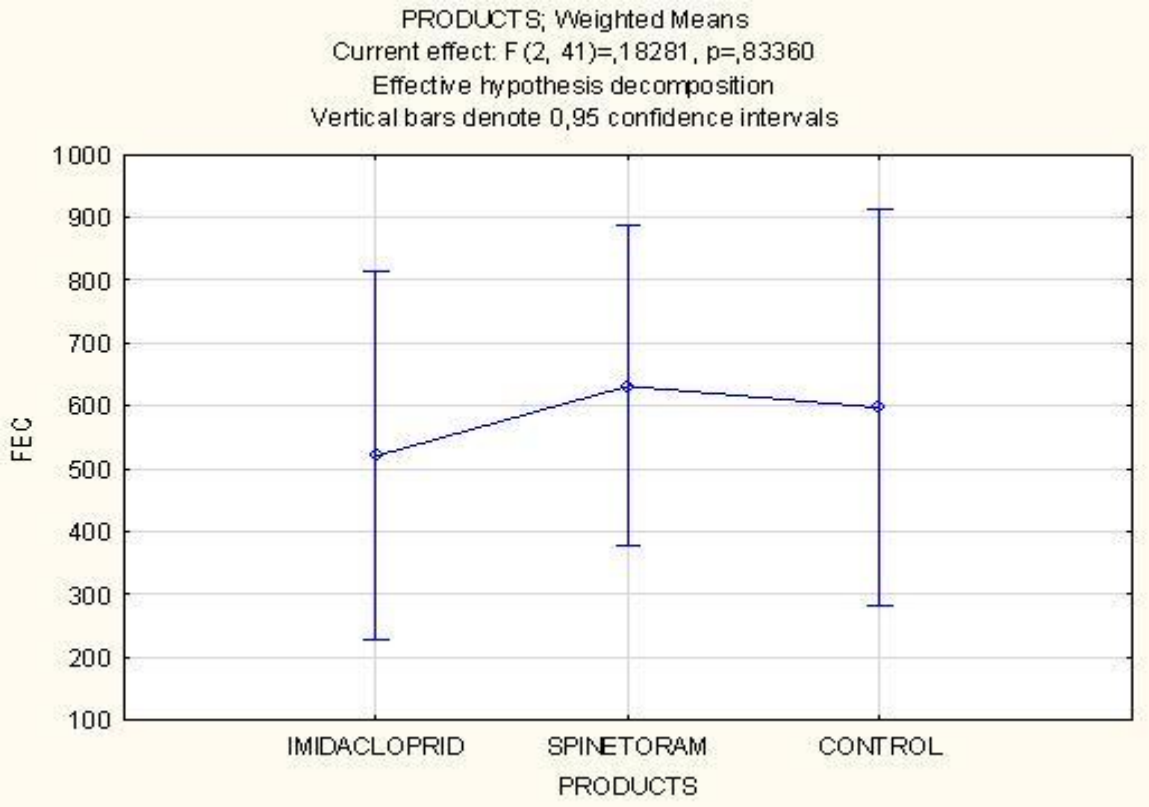


Fig.26 Effects of the three theses on fecundity of adult females of the species *A. bipunctata*.

The data were evaluated through one way ANOVA . Levene's Test for Homogeneity of variances was performed

The graph shows that there were not significant differences among the three theses. The fecundity of adult females after the treatment with Imidacloprid and Spinetoram did not differ from the control. The insecticides did not negatively affect the eggs production in the native species.

	PRODUCTS; Weighted Means (ADALIA FECUNDITY) Current effect: F(2, 41)=,18281, p=,83360 Effective hypothesis decomposition					
Cell No.	PRODOTTI	FEC Mean	FEC Std.Err.	FEC -95,00%	FEC +95,00%	N
1	IMIDACLOPRII	520,866	136,935	227,169	814,563	15
2	SPINETORAM	631,600	118,559	377,314	885,885	15
3	CONTROL	597,285	146,337	281,143	913,428	14

Table 22. Mean values of fecundity of *A.bipunctata* in the three theses.

Figure 27 reports the effects of the three theses on fertility of the species *A. bipunctata.*

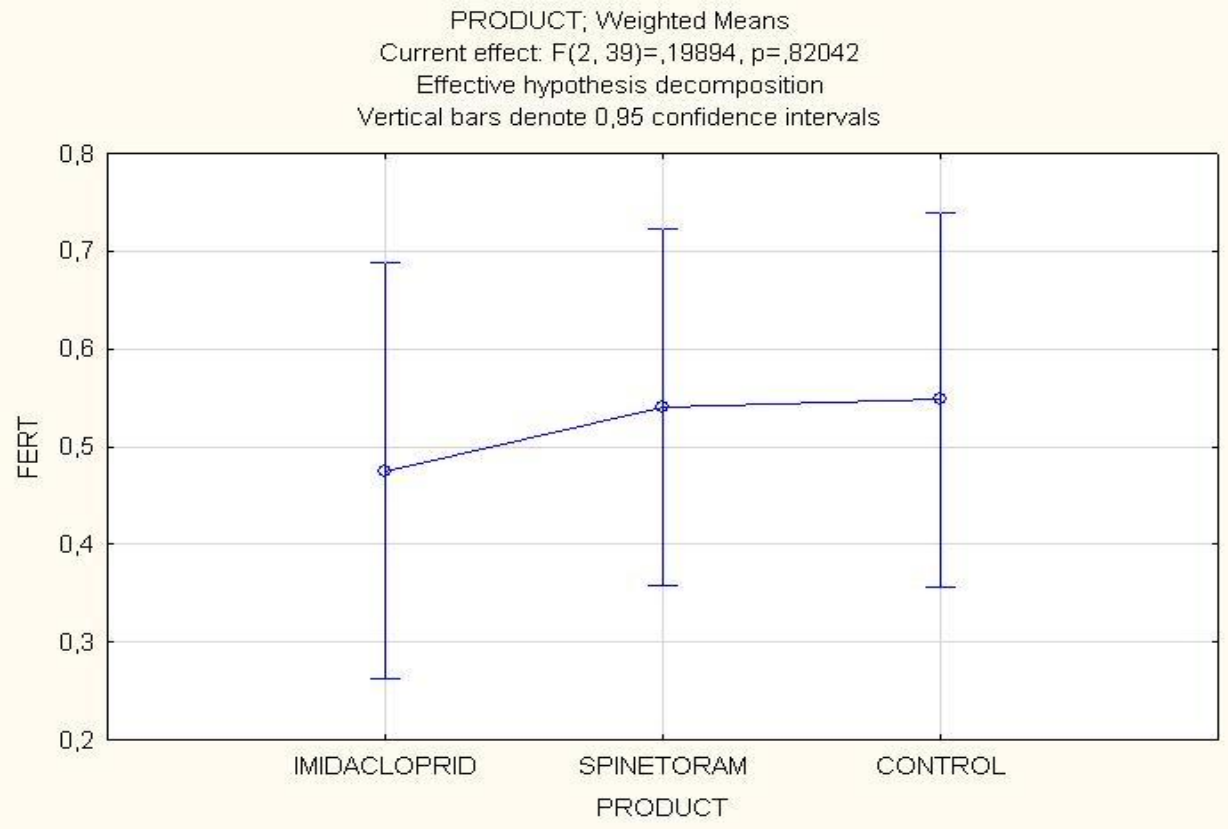


Fig.27 Effects of the three theses on fertility of adult females of the species *A. bipunctata*.

The data were evaluated through one way ANOVA.

Levene's Test for Homogeneity of variances was performed

The graph show that there were not significant differences among the three theses ($p \leq 0.05$). The fertility of adult females after the treatment with Imidacloprid and Spinetoram did not differ from the control. The insecticides did not negatively affect the larval emergence in the native species.

	PRODUCT; Weighted Means (ADALIA FERTILITY) Current effect: F(2, 39)=,09323, p=,91119 Effective hypothesis decomposition					
Cell No.	PRODUCT	FERT Mean	FERT Std.Err.	FERT -95,00%	FERT +95,00%	N
1	IMIDACLOPRII	29,1066	6,71665	14,7008	43,5124	15
2	SPINETORAM	31,4923	5,48161	19,5488	43,4357	13
3	CONTROI	32,7428	6,02678	19,7227	45,7629	14

Table 23. Mean values of fertility of *A.bipunctata* in the three theses.

Oviposition rate.

The oviposition rate was calculated to know how many eggs were laid by each female of *Adalia* and *Harmonia* per day. The calculation was carried out dividing the total number of eggs produced by each female for the total number of days of her life. The calculation was repeated for each thesis.

In table 24 the mean values of oviposition (= eggs/female/day) of females of *Adalia* and *Harmonia* are reported for each thesis.

The missing data related to HB (= *Harmonia* Spinetoram) were due to the impossibility to collect them because all the females died before the beginning of the oviposition.

AA	AB	AC	HA	HC
16.2	18.5	18.7	14.8	20.2

Table 24. Mean values of oviposition of females of *Adalia* and *Harmonia* for each thesis (AA: *Adalia* thesis A, Imidacloprid; AB: *Adalia* thesis B, Spinetoram; AC: *Adalia* thesis C, Control; HA: *Harmonia* thesis A, Imidacloprid; HC: *Harmonia* thesis C, Control).

In Fig. 28, the mean values ov oviposition for each thesis by females of *Adalia* and *Harmonia* are reported.

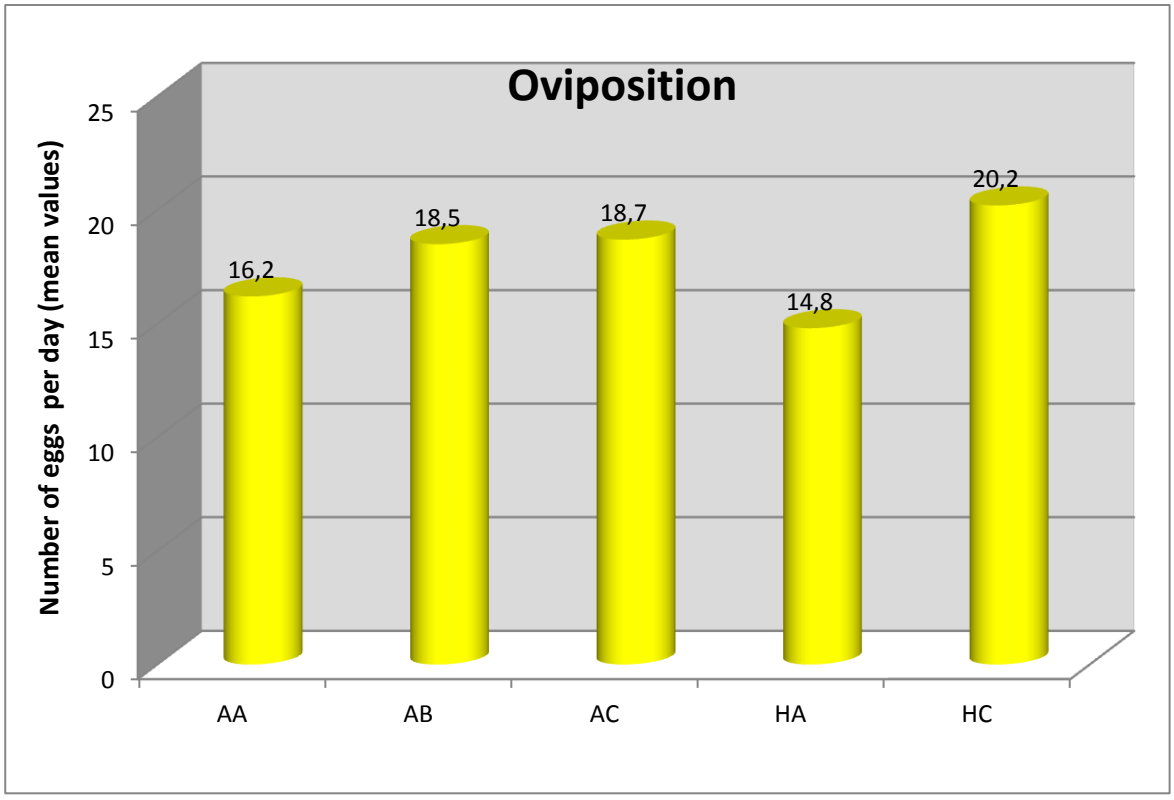


Fig. 28. Number of eggs laid by females of *Adalia* and *Harmonia* per day for each thesis. The mean values reported on the columns were each one obtained from 15 values of oviposition (excluded the data related to HB, as explained above).

In both coccinellids species it is possible to note a lower value of oviposition in the thesis A. The females of *Adalia* and *Harmonia* exposed to the neonicotinoid Imidacloprid showed a lower mean value of eggs laid per day. The difference with the control was more evident in *Harmonia* (~15 eggs laid by a female in the thesis A against ~20 eggs per day in the control) than in *Adalia.* About the thesis B, in *Adalia* Spinetoram was not different from the Control, that is the product seems not to affect oviposition.

Development time.

The first instar larvae were taken, put in a small container with food, kept in a climate cabinet and checked every day until the attainment of the adult stage.

Fig. 29 shows the results of development time (in days) in each thesis for *Adalia* and *Harmonia* larvae. The data related to HB (=*Harmonia* Spinetoram) are not shown for the reason already explained in the oviposition results (see above).

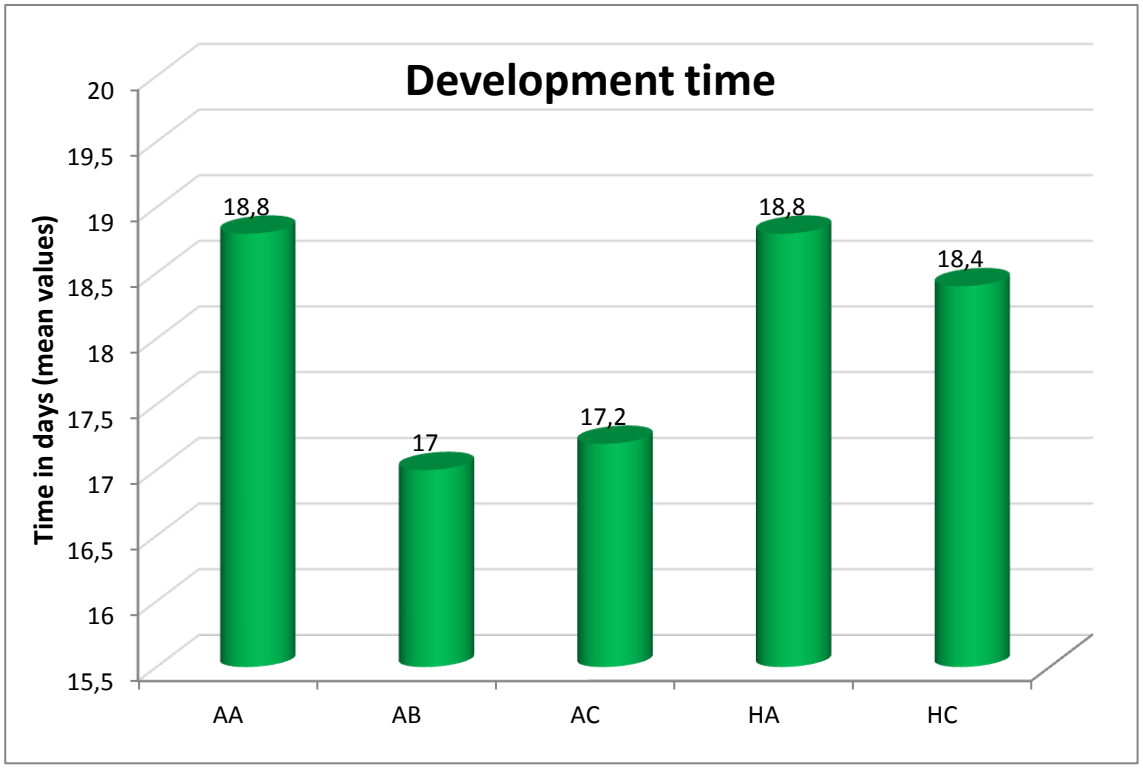


Fig. 29. Number of days (mean values) necessary for the pre-imaginal development for each thesis. The time was calculated from the day 0=egg hatching to the day n= adult.

The development time in *Adalia* showed differences in thesis A, where the time necessary for the complete development until the adult stage was 1.6 days longer than the control. The Imidacloprid treatment delayed the development of more than one day. The thasis B, instead, was very similar to the control. In *Harmonia* there was a minimal difference between the thesis A and the control.

Emerged adults

Table 25 reports The 2×2 contingency tables for testing the independence of the complete development from egg hatching to adult among the theses. The missing data related to HB (= *Harmonia* Spinetoram) are explained in the paragraph related to the oviposition (see above).

Thesis	Emeged adults		Non emerged adults			χ^{2*}	Df	*P*
	N	%	N	%				
AA	40	66.6	20	33.4	(aa-ab)	0.65	1	0.42
AB	45	75.0	15	25.0	(ab-ac)	0.44	1	0.51
AC	49	81.6	11	18.4	(aa-ac)	2.78	1	0.095
HA	51	85	9	15	(ha-hc)	0.07	1	0.79
HC	53	88.3	7	11.7				

*Yates corrected χ^2 values are presented (sample size <100)

Table 25. The 2×2 contingency tables values show the χ^2 results obtained comparing the theses of *Adalia* and *Harmonia* in pairs. The data related to HB are excluded (see above).

Fig. 30 reports the percentages of adults emerged for each thesis for both the coccinellid species. The data of HB (= *Harmonia* Spinetoram) are not shown, as explained in the paragraph related to the oviposition (see above).

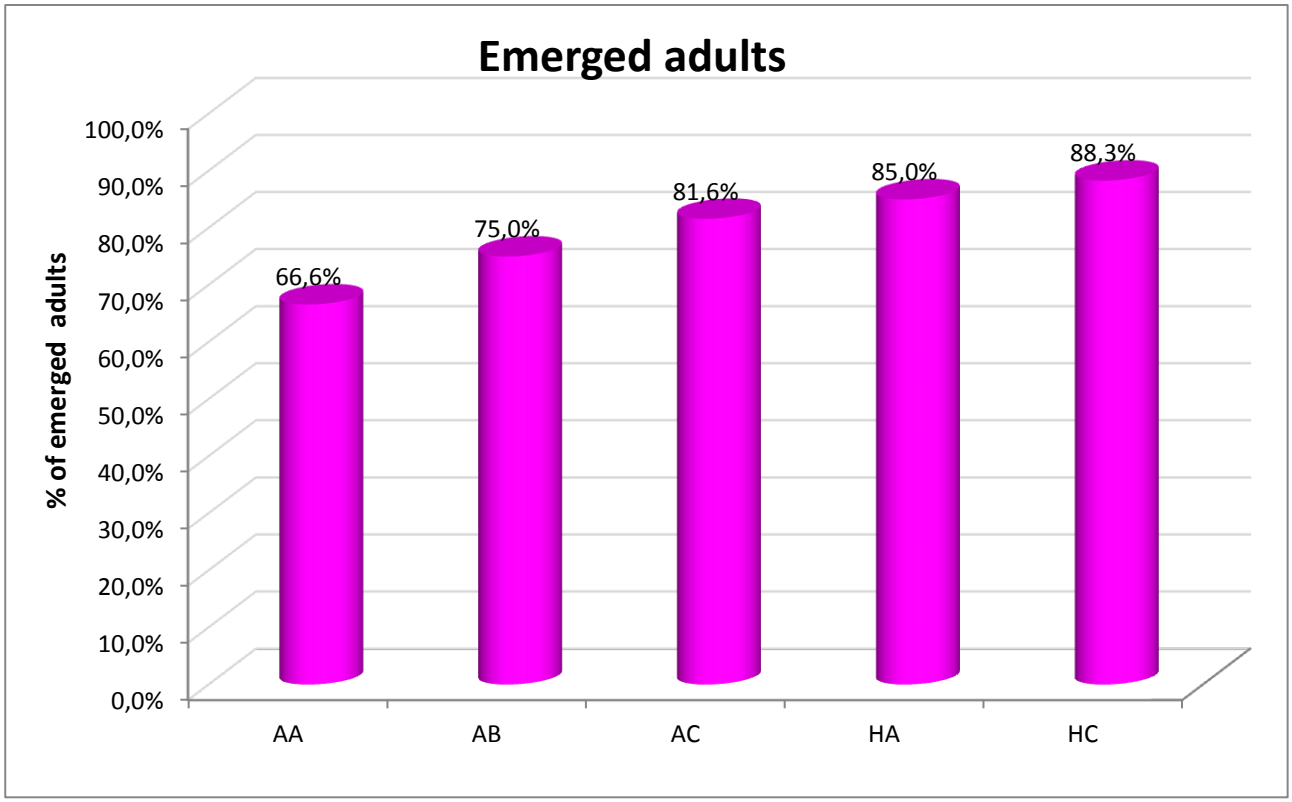


Fig. 30. In this graph each column represents the % of adults emerged, starting from the 60 eggs considered for each thesis in *Adalia* and *Harmonia*.

The graph shows a difference between *Adalia* and *Harmonia* in the percentage of adult emergence. In *Adalia* the percentages were lower than in *Harmonia*. In the native species the thesis A, Imidacloprid treatment, registered a lower percentage than the Control, instead in the exotic species, the thesis A was not so different from the Control. About the thesis B, in *Adalia* Spinetoram treatment reaches a lower percentage than the control.

The native species was more susceptible to insecticides as regards the completion of development, in terms of necessary time to achieve the adult phase and the percentage of adult emergence from a starting condition of egg.

Sex-ratio.

All the adults emerged from the 60 eggs considered were sexed in order to know the number of females and males found in each treatment of the two species.

Fig. 31 reports the percentages of females and males of *Adalia* and *Harmonia* obtained. The data related to HB(= *Harmonia* Spinetoram) are not shown (see the explanation in the paragraph related to the oviposition).

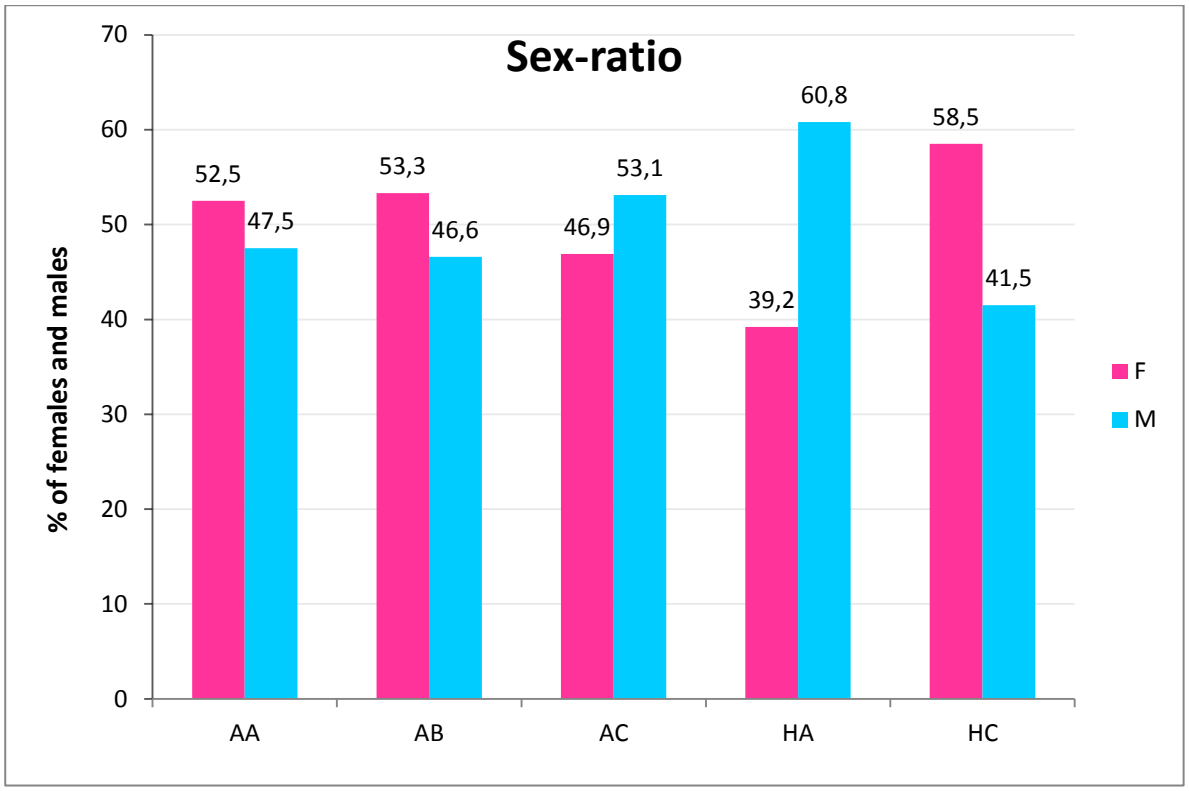


Fig. 31. Sex-ratio of the adults of *Adalia* and *Harmonia* for each tested thesis. The data related to HB are not shown (see above).

In the graph, the percentages of females and males on the total number of adults emerged, indicate that there were difference between the two coccinellid species. In fact for *A. bipunctata* the values of females and males are very similar in all the three theses.

The difference, instead, was visible for *H. axyridis*. The thesis A and the Control showed an opposite trend between females and males. For the thesis A, Imidacloprid, the percentage value of females obtained resembled the percentage value of the males obtained in the Control and vice versa. As reaffirmed, it was impossible to complete the study on the exotic species treated with Spinetoram, because all the females died before starting the oviposition, thus no data could be collected.

Table 26 reports the 2×2 contingency tables for testing the independence of the sex- ratio among the tested theses. The data related to HB (= *Harmonia* Spinetoram) are not shown as explained in the paragraph related to the oviposition (see above).

Thesis	**F**		**M**			χ^{2*}	**df**	***P***
	N	**%**	**N**	**%**				
AA	21	52.5	19	47.5	(aa-ab)	0.02	1	0.88
AB	24	53.3	21	46.6	(ab-ac)	0.17	1	0.68
AC	23	46.9	26	53.1	(aa-ac)	0.10	1	0.76
HA	20	39.2	31	60.8	(ha-hc)	3.15	1	0.77
HC	31	58.5	22	41.5				

*Yates corrected χ^2 values are presented (sample size <100)

Table 26. In this table the 2×2 contingency tables values show the χ^2 results obtained comparing the theses of *Adalia* and *Harmonia* in pairs.The data related to HB are excluded (see above).

Fig. 32 consists of a pie chart below reporting the percentages of females obtained in *Adalia* and *Harmonia* for all the theses, HB (= *Harmonia* Spinetoram) excluded for the reason explained in the paragraph related to the oviposition(see above).

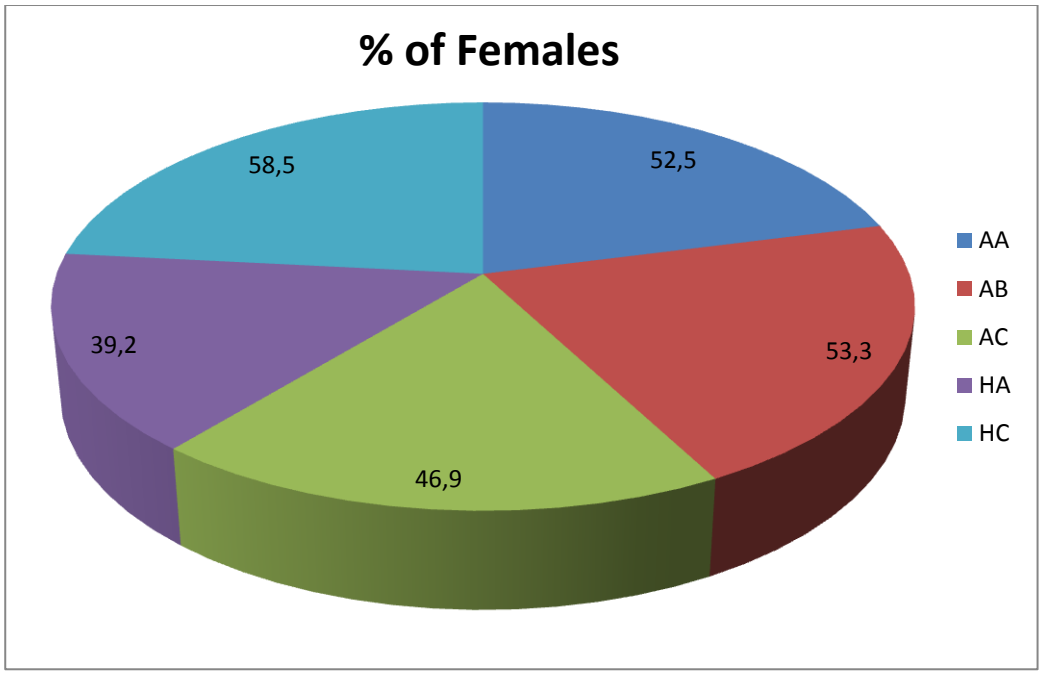


Fig. 32. The percentages of females emerged in all the theses considered are represented in different colors. The data related to HB are not shown (see above).

In the pie chart the percentages of females obtained are better highlighted and show that, among the theses, the lowest value reported concerns the Imidacloprid treatment for the species *H. axyridis* (purple portion, indicating a percentage of 39.2%). Instead the others were all more or less close to 50% and for *Harmonia* the percentage of females in the control was even close to 60%.

4. CONCLUSIONS

The aim of this study was to evaluate in the laboratory the acute and sublethal effects of two different insecticides on the indigenous species *A. bipunctata* and its exotic competitor, *H. axyridis*, in order to compare Imidacloprid with more modern insecticide, Spinetoram, considered to be more environmentally-friendly and to have less impact on the bio-ecosystems.

The insecticides used in the tests were Imidacloprid, a neonicotinoid, one of the most sold insecticides in the world (eur-lex.europa.eu, website) and Spinetoram, a spinosyn derived by a soil bacterium *Saccharopolyspora spinosa.*

About the acute effects on third instar larvae and adults, the results show that Imidacloprid has a toxic effects on both the coccinellid species. The toxicity was expressed both at 2 and 10 days from the treatment for larvae and both at 2 and 7 days from the treatment for adults. Spinetoram, instead, had a non toxic effect. The results, in fact, were not different from those obtained in the Control. Only in the third instar larvae of *Harmonia* the spinosyn had an effect similar to Imidacloprid and the Control, after 10 days from the treatment.

In the sublethal effects, the survival analysis calculated with Kaplan-Mayer estimator showed that the effects of the two insecticides on *H. axyridis* was exactly the opposite than that seen above. In fact, Spinetoram induced a dramatic mortality effects in the exotic species after two days from the treatment; therefore, it was not more possible to evaluate the other parameters (fecundity, fertility, oviposition rate, development time, sex-ratio) for this species treated with Spinetoram. For *A. bipunctata*, the two insecticides did not affect longevity in a significant way.

In *Adalia* neither Imidacloprid nor Spinetoram induced significant differences in fecundity and fertility that is, they did not negatively affect oviposition and hatching eggs. But the oviposition was slightly lower in the Imidacloprid thesis for both the coccinellids.

The development time of larvae was also evaluated. The 60 larvae per thesis were randomly collected and observed until the adult stage. At the emergence of the adult, the sex- ratio was also considered.

The results showed for *Adalia* a longer development time from egg hatching to adult in the Imidacloprid thesis, instead the development time in Spinetoram thesis resembled that of Control. Also in *Harmonia*, in the Imidacloprid thesis, the development time was similar to that of the Control.

The lowest percentage of larvae that completed the development and reached the adult stage belonged to the Imidacloprid thesis of *Adalia*.

The sex-ratio in *Adalia* adults showed reports a well balanced ratio of females and males in all the three theses, instead in *Harmonia* the percentages of the two sexs were inverted between the Imidacloprid thesis and the control, that is that the percentage of females in Imidacloprid thesis was almost the same value as the percentage of males in the Control and vice versa.

In conclusion, considering the acute effects, Imidacloprid was found to be the most toxic insecticide both for larvae and adults of the two coccinellid species. In particular, according to the IOBC evaluation, Imidacloprid would fall in the category as "slightly harmful" (30-79%) for larval mortality of both the species. Instead for the adult mortality of *A. bipunctata,* this insecticide would result "moderately harmful" (80-99%). In adult mortality of *H. axyridis,* Imidacloprid would fall in the "moderately harmful"category only at 7 days from the treatment.

Spinetoram instead, would result "slightly harmful" in case of adult mortality of *H. axyridis* at 10 days from the treatment. This insecticide in most of the cases was found not to be different from the control.

In contrast with the above mentioned results, in the sublethal effect Spinetoram negatively affected the survival of *H. axyridis*. All females died, starting from two days from the treatment, so that it was not possible to record any data of laid eggs and larvae emerged. The survival of the exotic species was drastically reduced in the Spinetoram treatment, but in the Imidacloprid treatment it does not differ from the Control.

In *A. bipunctata,* neither Imidacloprid nor Spinetoram reduced the survival of the the native species, being both treatments not different from the Control.

Therefore, in the acute effects, *A. bipunctata* resulted to be the most susceptible species to the insecticides, especially to Imidacloprid treatment.

Harmonia axyridis, instead, resulted to be the most susceptible species in the sublethal effects. It was particularly affected by Spinetoram treatment, so that the survival was dramatically reduced and consequently, it was not possible to perform the evaluation of fecundity and fertility.

Previous experiments reported the susceptibility of *H. axyridis* to selective insecticides. According to a study carried out by Galvan et al (2005) on two different selective insecticides, Indoxacarb decreased survival of first instars and adults, extended the developmental time for first instars to

become adults, reduced the fecundity of females. Spinosad decreased survival of first instars, extended the time for first instars to become adults, decreased weight gain and reduced the fertility of females. Moreover, in another study of Galvan et al. (2006), in which were also investigated Indoxacarb and Spinosad, the exposure to these insecticides through topical application, residues and treated prey, caused mortality to third instars. The mortality was higher when *H. axyridis* was exposed to both insecticides via residues. The adults resulted more susceptible to Indoxacarb, instead they were tolerant to Spinosad via all routes of exposure.

Jansen and Hautier (2006), comparing the effects of five different products on four coccinellid species, showed that most of the time *A. bipunctata* was the most susceptible species, except for some case, such as Confidor (Imidacloprid), in which *Harmonia axyridis* was such.

As Benelli et al. 2015 reported, in the larval mortality, *Harmonia* was less susceptible than *Adalia* to the piretroyd λ-Cyhalothrin. Benelli et al. (2015) also reported that most of the studies concerning pesticide effects just analyzed the mortality effects. Weissenberger et al., 1997 also studied the sublethal effects of Imidaclorid on *H. axyridis* and the results showed a direct effect of the neonicotinoid on fecundity of this coccinellid.

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Chapter 4

1. DEVELOPMENT OF ENTOMOPHAGOUS INSECTS ON ISOGENIC OR TRANSGENIC POTATO PLANTS INFECTED BY *PHYTOPHTHORA INFESTANS* DE BARY: LABORATORY EXPERIMENTS AT WAGENINGEN UNIVERSITY (NL)

INTRODUCTION

This last part of the PhD program was carried out in an international framework and, more specifically, in the Netherlands (Wageningen University, Department of Radix, Plant Science Group) where I spent six months for the experimental trials. This work has been performed within the European "AMIGA" project (Assessing and Monitoring the Impacts of Genetically Modified plants in Agro-Ecosystems) with the supervision of prof. Joop van Loon. The experimental results obtained are reported in this thesis upon his approval (obtained on February 26, 2025).

1.1 THE STUDY OF TRANSGENIC POTATO PLANTS EFFECTS (AMIGA PROJECT)

The project is subdivided into 11 work packages (WPs) about different aspects of this theme (AMIGA Collaborative project proposal 2011). Part of the task of the AMIGA project is to assess the protocols of the European Food and Safety Authority (EFSA) on environmental risk assessment of genetically modified organisms (GMOs). The focus is to provide scientific data in relation to the possible impact of GMOs (in this case, plants) in the European environmental context. The investigation deals with maize and potato, the only two GM plants admitted to be cultivated in Europe and just in some countries (GMO-Compass 2010). Several institutions are involved in this

study including The *Alma Mater Studiorum*, University of Bologna (UNIBO) and The University of Wageningen (WUR).

The WUR Laboratory of Entomology is involved with one of these WPs, i.e. "Trophic structure analysis in agro-ecosystems". The aim of this WP is the development and the evaluation of standard protocols for the risk assessment of possible effects of GM crops on NTO (non target organisms, such as insect and other arthropod communities in agro-ecosystems). The objective is pursued through laboratory, greenhouse and field bioassays (AMIGA Collaborative project proposal 2011). The assessment is focused on possible different effects of GM plants versus their isogenic genotypes on NTO (e.g. aphids and their natural enemies), in order to test their survival, development and reproduction, when exposed to these different conditions. In the present study the GM plants were genetically modified potatoes resistant to the pathogenic fungus *Phytophthora infestans* (Mont.) de Bary (Peronosporales: Pythiaceae), the causative agent of the famous desease "late blight"(AMIGA Collaborative project proposal 2011).

1.2 THE FUNGUS *PHYTOPHTHORA INFESTANS* DE BARY AND THE DuRPh (GM) PLANTS

Solanum tuberosum L. is a tuber belonging to Solanaceae with other plants such as tomato, pepper and eggplant. This species is one of the most cultivated crops in the world, just after wheat, rice and maize (Spooner and Bamberg, 1994), and the second in Europe after wheat (Haverkort et al., 2008). Besides *S. tuberosum* there are other six cultivated species that are only grown in the Andes region. In addition, there are 199 wild species (Hijmans and Spooner, 2001) which have been collected to contribute to potato gene banks and have been used in breeding programs to improve the cultivated potato (Hijmans and Spooner, 2001).

Nowadays, *S. tuberosum* is grown almost in all world. In 2005, the total cultivation area was almost 20 million hectares with a production of 300 Mt. The consumption per capita per year is by far the largest in Europe with 31kg per person per year (Haverkort et al., 2009). In the Netherlands the 25 % of the land is used for this crop, e.g. about 160 hectares with a production of 7 million tons (Haverkort et al., 2009).

Potato is susceptible to different pests and deseases, including the worst, the late blight, caused by *P. infestans* (Haverkort et al., 2009). This oomycete fungus can affect the plants in a short time (about two weeks) when weather conditions are favorable to its reproduction. It infect leaves, stems and tubers, and it was the causative agent of the famous Irish famine between 1845 and 1849. The

strong adaptation and virulence of this microorganism is due to its complex sexual and asexual reproductive cycle, its rapid growth in plant tissues and an ability in overcoming plant resistance (Fry, 2008; Vleeshowers et al., 2011). An asexual reproduction can start from a persistent fungal oomycete in infected tubers in the field of the previous year or from dispersion through the wind from an infected field (Aylor et al. 2001). The sporangia are mobile for a short time before to encyst into the plant and the effect is visible with a necrosis just after a couple of days. Thousands of sporangiophores in each lesion can disperse through water, air and infected tubers (Fry, 2008). In the less common sexual reproduction, each thallus can absolve the male or female function, producing either antheridia or oogonia for the oospores creation through sexual recombination. In this case the fungus can undergo genetic variation and show a better survival (Judelson, 1997). In fact, the oospores are resilient and can persist in the soil for long time, also several years, and can produce new infections (Turkesten et al., 2000). This plant destroyer can cause over $ 3 billion economic damage per year worldwide in production losses and control (Fry, 2008). In the Netherlands, this problem is reduced through fungicides treatments, but this method implies monetary and environmental costs (AMIGA Collaborative project proposal 2011). The Netherlands annually spends 115.5 million euro for the spray treatments, and other 9.4 million are considered for harvest losses and crop damage (Haverkort et al., 2008; Haverkort et al., 2009).

As the fungus eradication involves annually worldwide spending large sums of money, the need to create resistant cultivars is becoming bigger and bigger, so the use of techniques for the introduction of resistance genes. In these last years techniques of molecular biology and genetics are improving in developing interactions between plant and oomycete, using resistance genes (traditionally one gene) and effector proteins. But the breeding experiments in resistance obtaining, scarce effects and with much time and work has had. About the "late blight" desease, in hundred years of traditional breeding no progress there were including only a single resistance –gene on potatoes from wild species (Haverkort et al., 2009).

In addition to crossing and mutation, the plant varieties can also be improved through genetic modification. Usually, the transgenes come from non-crossable species, but nowadays it is possible to isolate cisgenes from DNA plant sequences, which are genes from crop plants themselves or from crossable species (Jacobsen and Schouten, 2007).

In 2006 the Dutch government started a project aimed at creating potato plants resistant to *P. infestans*, which is still in progress. This project is called DuRPh (Durable resistance against *Phitophthora* through cisgenetic marker-free modification) and it has been developed by a research group of Wageningen UR. In the cisgenesis case, the introduction of cisgenes, from crossable

species, is possible without leaving gene markers, such as genes for antibiotic or herbicide tolerance in its genome, as instead occurs in the transgenesis case (Jacobsen and Schouten, 2008).

1.3 EFFECTS OF GM POTATO PLANTS IN THE THRITROPHIC INTERACTION (POTATO-APHID-PARASITOID/PREDATOR)

A matter related to GM crops concerns their placing into the environment and the relationship established with all the species in contact with them. Non target organisms (NTOs) are defined as all species directly and/or indirectly exposed to the GM plant, which are not the target organisms of the newly expressed metabolites in these plants (EFSA, 2010).

About the potato cultivation, a considerable group of organisms such as several pest species and their natural enemies (predators and parasitoids), as well as pollinators, decomposers and plant symbionts visit and interact whit this crop. The most dangerous pests for potato are aphids as sup-sucking herbivores, and the Colorado potato beetle, *Leptinotarsa decemlineata* Say, leaf-chewer herbivore (Gosset et al., 2009).

The insect predators of aphids belongs to different families, in particular Anthocoridae and Miridae (Hemiptera), Chrysopidae (Neuroptera), Cecidomyiidae and Syrphidae (Diptera) and Coccinellidae (ladybirds, e.g. *Adalia bipunctata* (L.) (Coleoptera) (Yano et al., 2006). Also hymenopteran (e.g. Aphidiidae, Aphelinidae and Encyrtidae) or dipteran (Cecidomyiidae) parasitoids can attack aphids (Mackauer, 1968).

2. MATERIALS AND METHODS

2.1 EXPERIMENT 1:

Aphidius colemani Viereck performance on aphids reared on ISO, CIS, TRANS-genic potato plants (Desirèe variety) with and without *Phytophthora infestans* De Bary

The aim of this experimental trial was to evaluate the impact of ISO-genic, CIS-genic, TRANS-genic potato plants, infected by *Phytophthora infestans*, towards the performance of *Aphidius colemani* Viereck, a parasitoid of different species of aphids, including *Myzus persicae* Sulzer. Both insects were NTOs in this study.

2.1.1 INSECTS USED FOR THE EXPERIMENT

2.1.1.1 *Aphidius colemani* Viereck

Aphidius colemani (Hymenoptera: Braconidae: Aphidiinae) is a brown and tiny parasitoid (2-3 mm length), pan-tropical and subtropical species (Takada, 1998) probably of Indian origin. Now it is widely available from commercial insectaries in Europe (www.bioplanet.it; www.koppert.com), where it is used in protected cropping systems for the biological control of the two aphids *Myzus persicae* (Sulzer) (Homoptera: Aphididae), the green peach aphid, and *Aphid gossypii* Glover, the

cotton-melon aphid (Grasswitz and Reese, 1998). Most of aphid braconid parasitoids are either oligophagous or polyphagous. All the species belonging to the Aphidiinae subfamily are solitary endoparasitoids of aphids. Usually the female lays a single egg in the host, but superparasitism may happen in host deficiency conditions. The larva develops inside the aphid body and the remains of its esoskeleton takes the appellative "mummy". Most of them are arrhenotokous and bisexual, but some species are thelytokous. In bisexual species, the females select the offspring sex ratio on the basis of the host quality, respectively laying fertilized (female) eggs in high-quality (= larger) hosts and unfertilized (male) eggs in lower-quality (=smaller) hosts (Cloutier et al., 1991; Mackauer and Volkl, 1993). In a favorable environmental condition, a female selects a preferred host and the host acceptance involves three steps: 1) host recognition, with a directed response towards the aphid; 2) host evaluation, in which antennation and ovipositor probing are involved; 3) host acceptance when the oviposition is effective (Storeck et al., 2000).

2.1.1.2 *Myzus persicae* (Sulzer) (see the description of the species in chapter 1)

2.1.2 EXPERIMENTAL TRIAL

2.1.2.1 Description

Three different lines of *S. tuberosum*, all clones of Desirèe cultivar, were used for the experiment. One was isogenic and the other two were genetically modified through an insertion of the gene *Rpi-vnt* 1 that confers resistance to the causative agent of "late blight", the fungus *Phytophthora infestans*. This R gene was identified and cloned from the wild species *Solanum venturii* Hawkes & Hijert. The cis-genic line, in which this R-gene was inserted, was named clone A 15-31. For the trans-genic line, the R-gene *Rpi-vni 1* was inserted with the other R-gene *Rpi-sto* 1, the latter cloned from the wild species *Solanum stoloniferum* Schltdl & Bouchè. Moreover, in the trans-genic line a marker gene *npII* resistant to kanamycin (an antibiotic) was also inserted and the clone was named A16-02. This engineered technique was developed by a research group of Wageningen UR (NL). The Desirèe clones were obtained from the Laboratory of Plant Breeding of Wageningen Research

Centre (WUR). The three lines of plants used for the experiment were maintained in small boxes in climatic chambers of the Laboratory of Plant Breeding at 21°C, 70% UR, and 16L : 8D photoperiod. Plants were grown on agar.

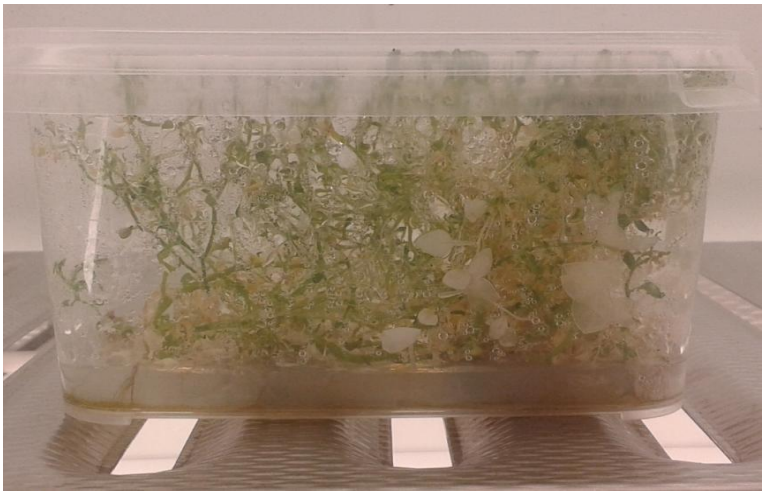


Photo 13 Potatoes grown in agar (Desirèe variety) in small plastic container by Laboratory of Plant Breeding (Radix, Department of Plant Sciences). Photo Di Vitantonio C.

Photo 14. Seedlings obtained from the transfer of small fragments of potato plants (see picture above) in new container with agar. Photo Di Vitantonio C.

On request of each line, small fragments (2 cm L) of plant were cut in sterile conditions (working under laminar flow hoods) and transferred in plastic containers for vitro cultivation containing sterile medium (agar). After this process, the containers were kept in climatic chambers at the above mentioned conditions for about two weeks, the necessary time to obtain seedlings of the right size for the transplanting in sterile soil, into small plastic pots. The transplanting was carried out in the same climatic chamber. The experiment was perfomed when the potato plants were 17-20 cm high (about 30 days after the transplanting).

Photo 15. Trans (label red), Cis (label yellow), Control (label green) potato plants. Photo Di Vitantonio C.

At the same time, in a separate climatic chamber (with the same conditions described above), the aphids were reared on radishes (*Raphanus sativus* L.) and then transferred on Bintje plants, a different potato cultivar. The purpose was to accustom them to potato, avoiding to rear them on the cultivar used for the experiment. The Bintje plants were provided by Unifarm, a supplier company of Wageningen UR.

a) *Phytophthora* maintenance

The *Phytophthora* culture needs a weekly maintenance, in which both leaves and tubers are infected to always obtain new infected material for experimental trials. Leaves and tubers were taken from potato plants cv. Bintje, weekly made available from Unifarm. The Bintje plants were kept in the greenhouse close to the Entomology Laboratory, in Radix Department, Plant Science Group. For the leaves infection about 5/6 leaves were used. They were put in ventilated petri dishes with sterile agar (purified agar : 1L tap water,15 g agarose, autoclaved for 20 minutes at 121°C), to keep them in good conditions and not to make them dry, because the fungus needs humidity to sporulate. On each leaf, about 5 droplets of tap water were deposited using a pipette; in each droplet, the *Phytophthora* mycelium, from infected tubers of the previous week, was mixed with a sterile needle. For infecting tubers, 2-3 tubers were washed in tap water and left for ten minutes under a napkin wet with alcohol to keep them cleaned and to avoid molds and other contaminations. They were then cut under sterile conditions and infected through contact with leaves covered by *Phytophthora* mycelium. Infected tubers were then transferred into petri dishes. Both tubers and leaves were maintained in small climate cabinets at 15°C, 70% RH and 8L: 16D photoperiod.

b) Inoculation for the experiment

The *Phytophthora* inoculation on ISO, CIS and TRANS potato plants was carried out 24 hours before starting the experiment, because the fungus needs this time to sporulate (at the conditions described below). The desired concentration for the experiment was 10000 spores/mL. In order to be sure to cover all the plant leaf surface, 2.5 mL solution/plant were sprayed, using a sprayer containing 10 mL solution. The procedure was performed in the laboratory, in sterile conditions. For each Desirèe line (ISO, CIS and TRANS), 10 plants were considered (30 plants in total). For each line, 5 plants were treated with the inoculum of the fungus and 5 just with water. All plants were kept in the greenhouse close to the Entomology lab at the same conditions, e.g. 15°C, 75%

RH, and 0L: 24D photoperiod. The infected and non-infected plants were kept separately in big plastic trays 110 cm(l), 55cm(w), 5 cm(h), with the pots covered until half of their highness with water, to make sure to have enough humidity. The trays were closed with a dark plastic bag, to facilitate the development of the spores. After one day the potted plants were removed from these containers and transferred to climate cells (at 22°C, 68-70% RH, and 16L: 8D photoperiod) to dry. Each pot with a plant was placed in a bigger plastic pot. In about 2 hours the plants were dry and thus ready for the aphid introduction. Twenty *M. persicae* nymphs were placed on each plant with a tiny brush. The plant was then covered with white synthetic fabric to allow the transpiration. The aphids were left alone for 48 hours, so as to accustom them to the plants while avoiding their fast reproduction and a possible alteration of the experiment due to their excessive number. The *A. colemani* specimens were provided as mummies by the company Koppert (NL), in small plastic bottles "Aphipar". The mummies were kept in a small cage for parasitoid emergence, which occurred in about 24-36 hours. The wasps were fed with paper soaked in a solution of water and honey (25% honey).

It was necessary to wait about two days to allow mating. Then, on each plant infested by aphids 1 parasitoid female was introduced and left for 24 hours to permit parasitization. After this time all the females were removed and the aphids daily checked until the mummy formation. After 4-5 days the *Phytophthora* effects could also be noticed, that is necrosis, leaf yellowing and fall. The time requested to have mummies from the parasitization was about 9 days. After that time they were removed and maintained in petri dishes. From the mummification it was important to check the emergence of new wasps every day. As soon as the new adult parasitoids emerged, they were frozen; after 24 hours they were sexed and weighed with a digital scale in the Entomology Lab. The experiment was carried out in a climatic chamber at 22°C, 68-70% RH and 16L:8D photoperiod. The replicates per treatment were 5 (for each plant line, either infected or not, each replicate corresponded to a single plant).

2.1.2.2 Parameters considered in the study

The parameters considered in this experiment were:

-% mummies formed by aphids exposed to parasitoids

-% parasitoids emerged from mummies

- development time from mummy formation to parasitoid adult emergence (days)

- weight of parasitoids emerged (mg)

- sex- ratio

2.3 DATA ANALYSIS

The data concerning the sex ratio were analysed by 2 x 2 contingency tables. All the other data were analysed by a factorial analysis of variance (Zar, 1984) (3 × 2 factors tested for the effects of the plant line [Desirée, CIS and TRANS] and of *Phytphthora* infection (Minus, Plus). Means were compared using the Tukey's HSD multiple range test where significant difference ($P<0.05$) occurred. The statistical tests were done with STATISTICA 10.0 (StatSoft, 2010). The percentage values were transformed for the analysis using an arcsine transformation (Mosteller & Youtz, 2006).

3. RESULTS AND DISCUSSION

The results are illustrated in the following tables and graphs.

Table 1. *Myzus persicae* mummies (%) formed by aphids exposed to *A. colemani* parasitoids, as related to the combination of the factors "plant line" and "*Phytphthora* infection". The percentages were calculated on the original aphid number. The ANOVA results report the values for "Plant line effect", "Infection effect" and the "Interaction" between plant line and infection. Number of replicates (= number of plants) is given in parentheses above the means (± SE).

Parameters	**Plant line**	***Phytophtora* infection**		**ANOVA Results**		
		Minus	**Plus**	**Plant effect**	**Infection effect**	**Interaction**
Mummy (%)	Desirée	**(5) 15±10.7**	**(5) 17±9.8**			
	Trans	**(5) 33±10.3**	**(5) 20±7.6**			
				F= 2.32	**F=0.09**	**F=0.83**

				df= 2,24	**df= 1,24**	**df= 2,24**
				P= 0.12	**P=0.77**	**P=0.45**
	Cis	**(5) 27±4.6**	**(5) 28±4.6**			

The values reported in Table 1 show that there were not significant differences between plants infected by *Phytophthora* and plants non-infected by the fungus. The pathogen didn't affect the percentage of mummies formed. Also the plant line did not affect this parameter. The interaction between plant effect and infection effect was also not significant.

Table 2. Parasitoid adults (%) emerged from mummies, as related to the combination of the factors "plant line" and "*Phytphthora* infection". The percentages (± SE) were calculated on the number of mummies. The ANOVA results report the values for "Plant line effect", "Infection effect" and the "Interaction" between plant line and infection. Number of replicates (= number of plants) is given in parentheses above the means (± SE). When no mummy formed, that plant was not considered as a replicate.

Parameters	**Plant line**	***Phytophtora* infection**		**ANOVA Results**		
		Minus	**Plus**	**Plant effect**	**Infection effect**	**Interaction**
Parasitoid adults (%)	Desirée	**(2) 59.1±31.8**	**(4) 27.3±12.9**			
	Trans	**(5) 36.4±9.5**	**(4) 29.6±9.4**			
				F= 0.02	**F=2.89**	**F=0.49**
				df= 2,19	**df= 1,19**	**df= 2,19**
				P= 0.75	**P=0.11**	**P=0.63**
	Cis	**(5) 45.5±13.5**	**(5) 32.7±9.4**			

The data in Table 2 show that, about the % of parasitoid adults, there were not significant differences between plants infected and non-infected and among the three plant lines. That means that the adult emergence of parasitoids didn't change in relation to infection and plant genotype. In the case of Desirée without inoculation of *Phytophthora* (minus) the value was higher than the others, but adult wasps were obtained from only two plants. No significant differences were however found among treatments.

Table 3. Development time (days) from mummy formation to parasitoid adult emergence. The values reported in the table refer to the days necessary for the development of the adult parasitoids from the mummy formation (aphids successfully parasitized) in all the three plant lines with and without *Phythophthora* infection. The ANOVA results report the values for "Plant effect", "Infection effect" and the "Interaction" between plant and infection. Number of replicates (= number of plants) is given in parentheses above the means (± SE). When no mummy formed (and no adult parasitoid was obtained), that plant was not considered as a replicate. The grand means followed by the same letter (uppercase in a row; lowercase in a column) are not significantly different ($P < 0.05$, Tukey HSD multiple range test).

Parameters	**Plant line**	***Phytophtora* infection**		**Grand mean**	**ANOVA Results**		
		Minus	**Plus**		**Plant effect**	**Infection effect**	**Interaction**
Development time	Desirèe	**(2) 3.0±0.00**	**(4) 4.54±1.0**	**(6) 3.77±0.44a**			
	Trans	**(5) 3.0±0.00**	**(4) 3.62±0.48**	**(9) 3.31±0.15a**			
					F= 1.25	**F=21.49**	**F=1.25**
					df= 2,19	**df= 1,19**	**df= 2,19**
					***P*= 0.31**	***P*=0.0002 ****	***P*=0.31**
	Cis	**(5) 3.0±0.35**	**(5) 3.93±0.48**	**(10) 3.46±0.21a**			
	Grand mean	**(12) 3±0.21A**	**(13) 4.02±0.74B**				

The values related to the development time of the parasitoid (from mummy formation to adult) show a significant between infected and non-infected plants (Table 3). On non-infected plants (minus) the parasitoids emerged faster than on infected plants (e.g. the *Phytophthora* delayed the development time of the parasitoid). Conversely, there weren't any differences among plant lines. The interaction between the plant genotype and the infection was also not significant.

Table 4. Weight in milligrams (mg) of the adult parasitoids emerged from mummies in the three plant lines, with and without *Phytophthora* infection. The ANOVA results report the values for "Plant line effect", "Infection effect" and the "Interaction" between plant line and infection. Number of replicates (= number of plants) is given in parentheses above the means (± SE). When no mummy formed (and no adult parasitoid was obtained), that plant was not considered as a replicate.

Parameters	**Plant** line	***Phytophtora* infection**		**ANOVA Results**		
		Minus	**Plus**	**Plant effect**	**Infection effect**	**Interaction**
Adult Weight (mg)	Desirèe	**(2) 104.6±22.9**	**(4) 93.9±24.1**			
	Trans	**(5) 97.4±9.2**	**(4) 98.6±12.5**			
				F= 0.17	**F=0.81**	**F=0.32**
				df= 2,19	**df= 1,19**	**df= 2,19**
				***P*= 0.84**	***P*=0.38**	***P*=0.73**
	Cis	**(5) 108.8±8.1**	**(5) 96.9±10.8**			

The data reported in Table 4 indicate that there weren't significant differences among the theses; neither the plant genotype or the infection, or the interaction affected the difference in the weight of adult parasitoids.

Table 5. Sex- ratio percentage values (F= females; M= males) of adult parasitoids emerged from infected (Plus) or non-infected (Minus) plants. The data related to the three plant lines were jointed. The percentages of males and females related to Minus-plants and Plus-plants were respectively calculated over the total number of adults obtained on non infected (57) or infected plants (45)

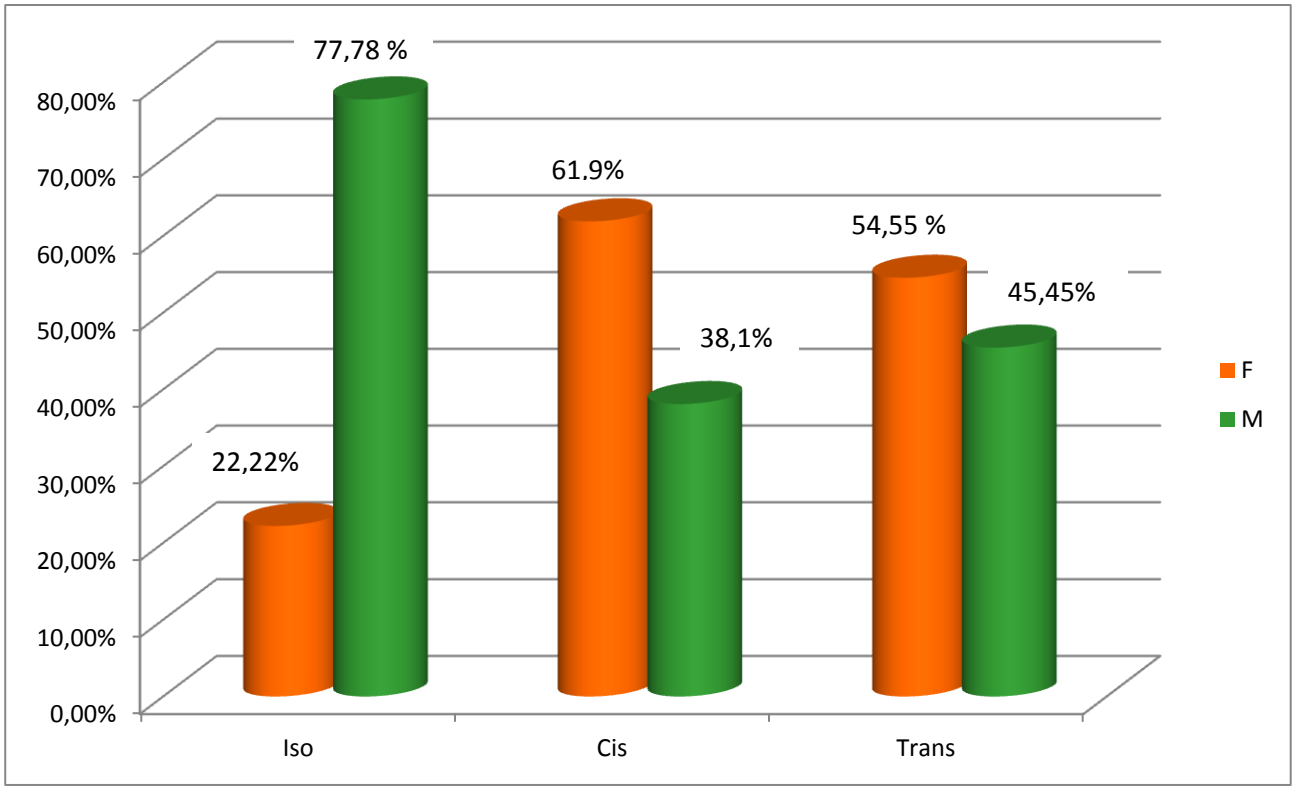
Parameters	**Sex**	***Phytophtora* infection**	
		Minus	**Plus**
Sex-ratio	*F*	50.87%	46.66%
	M	49.13%	53.34%

Considering the data related to the three plant lines jointed, the percentages of males and females obtained from plants with *Phytophthora* inoculation and infection and plants without *Phytophthora* (treated only with water) indicate that the percentage of males was higher for the Plus-plants compared to the Minus-plants (Table 5). The difference is however not significant (χ^2= 0.05, df =1; *P*= 0.82). The percentage of males, calculated on the total number of males obtained on infected and non-infected plants, was however higher on the minus-plants. Similar results were obtained for the females (Table 6)

Table 6. Sex- ratio percentage values (F= females; M= males) of adult parasitoids emerged from infected (Plus) or non-infected (Minus) plants. The data related to the three plant lines were jointed. The percentages of males orfemales obtained on Minus-plants and Plus-plants were calculated on the total number of males (= 52) or the total number of females (50) emerged.

***Phytophtora* infection**	**Sex ratio**	
	M	**F**
Minus	53.84%	58%
Plus	44.23%	42%

Figure 1. Sex –ratio of adult parasitoids emerged from the mummies formed on ISO, CIS and TRANS potato plants. Within each plant line, Infected and non-infected plants were considered together. The percentage values were calculated on the total number of adults emerged from each plant line.


77,78 %
61.9%
54,55 %
45,45%
38,1%
22,22%
80,00%
70,00%
60,00%
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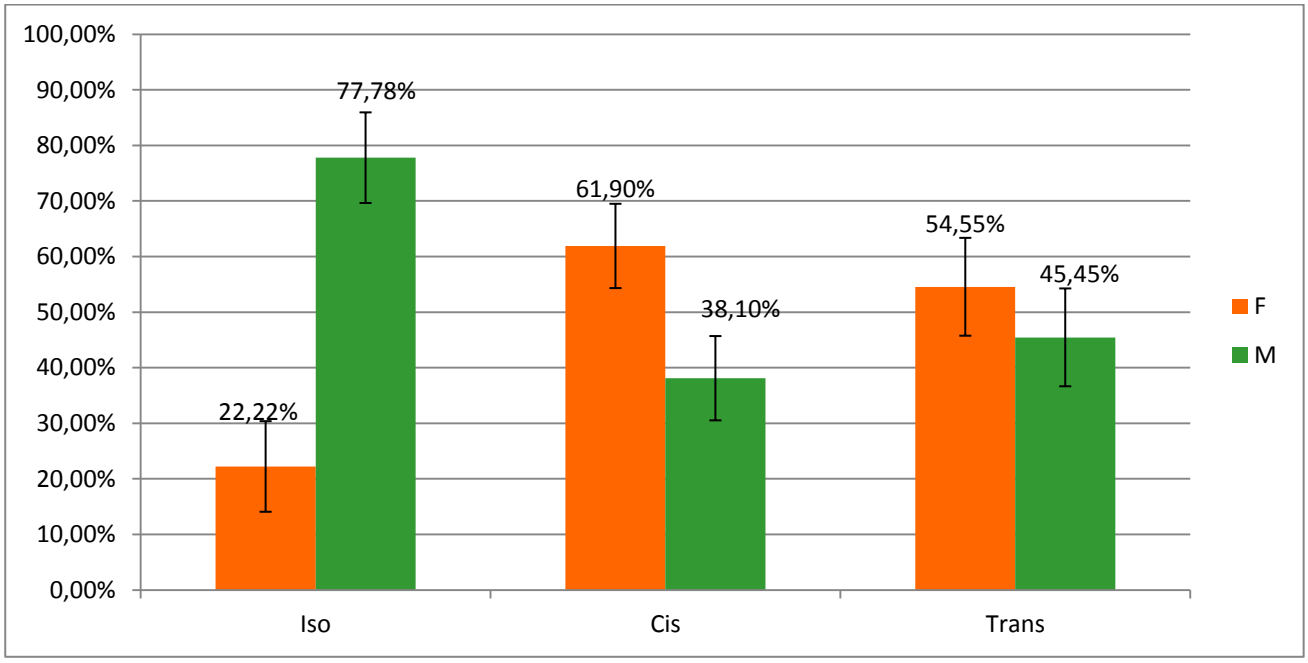
100,00%
90,00%
80,00%
70,00%
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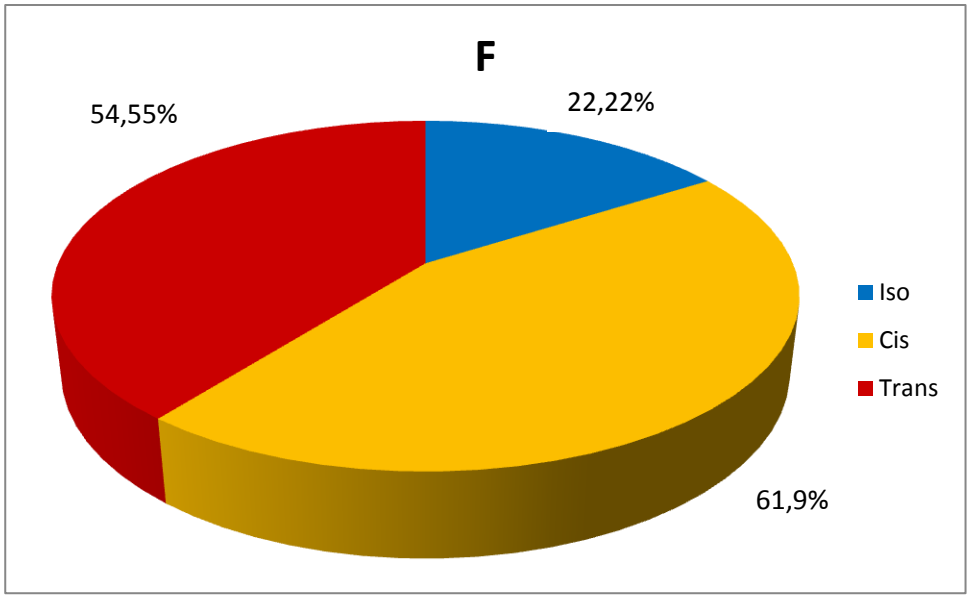
50,00%
40,00%
30,00%
20,00%
10,00%
0,00%
77,78%
61,90%
54,55%
45,45%
38,10%
22,22%
Iso
Cis
Trans
F
M

Table 7. Number and percentage of females and males in each plant line. Data referred to infected and non-infected plants were considered together. The percentage values were calculated on the total number of adults emerged from each plant line. See Table 7 for statistics.

Plant line	**Females**		**Males**	
	Number	**%**	**Number**	**%**
Iso	6	22.22	21	77.78
Cis	26	61.9	16	38.1
Trans	18	54.55	15	45.45

In Figures 1 and 2, and in Table 6, data concerning numbers and percentages of female and male parasitoids emerged from each plant line are shown. The main difference was found between females and males emerged from Iso-genic plants. Since both infected and non-infected plants were considered together, it may be hypothesized that the overall data were influenced by *Phytophthora* infection, which may have affected the development of parasitoids in the sex-ratio determination. In fact *Aphidius colemani* displays arrhenotoky (as it is common among hymenopteran parasitoids) and more males may be produced under stress environmental conditions (Godfray, 2004). Infection could represent a biotic stress.

Figure 2. Percentage of females emerged from each thesis in all the three theses considered



The % of Females in the Iso plants was much lower compared with the two other theses

Table 8. The 2 x 2 contingency tables for testing the independence of plant line (ISO, CIS, TRANS) and parasitoid sex-ratio (% of females). Yates corrected χ^2 values are presented (sample size <100) .

Plant lines	χ^2	***P***
Iso/Cis	8.87	0.003
Iso/Trans	5.19	0.03
Trans/Cis	0.17	0.69

About the number and % of females and males for each plant line (Iso-genic, Cis-genic,Trans-genic), the results in Table 7 indicate that there was a significant difference between the sex-ratios (% females) of the Iso-genic and Cis-genic genotypes. We observe a statistic difference also between Iso-genic and Trans-genic lines. Conversely, for the last comparison, between Cis-genic and Trans-genic wasn't any significant difference (*P*-value>0.05).

2.2 EXPERIMENT 2:

Larval development of *Adalia bipunctata* (L.) reared on potato plants (Bintje variety) infected and non-infected by *Phytophthora infestans* De Bary

The aim of this study was the evaluation of *Phytophthora* impact on the larval development, until pupal stage, of the indigenous (in Europe) aphidophagous predator *Adalia bipunctata*. In this experiment non-GM potato plants, cv. Bintje, were used in order to investigate only the effect of the fungus on the larval development.

2.2.1 INSECTS USED FOR THE EXPERIMENT

2.2.1.1*Adalia bipunctata* (L.) (see the description of the species in chapter 1)

2.2.1.2 *Myzus persicae* Sulzer (see the description of the species in chapter 1)

2.2.2.EXPERIMENTAL TRIAL

2.2.2.1 Description

For this experiment, 40 Bintje potato plants (35-40 cm high) were used. This variety was provided by the Unifarm, a supplier company next to the Plant Science Department (WUR). About these plants, 20 were sprayed with *Phytophthora* and 20 with only water (as control) with the same technique, and then kept at the same climate condition and photoperiod and for the same time, as above mentioned in the first experiment (15°C, 75% RH, and 0L: 24D photoperiod). At the same time, the rearing of aphids was maintained in a different climate- room, in net cages, at 22°C, 70% R.H., 16 L: 8 D hours. After 24 hours from the *Phytophthora* inoculation, the plants were kept in the climate- room at 22°C, 68-70% R.H 16 L: 8 D hours to make them dry and for the whole

experimental trial. After two hours it was possible to introduce the aphids. The aphids were inserted by cutting leaves of previously infested plants. The aphids were then left on the plants for 48 hours before introducing the coccinellid larvae. The plants were then covered with a white synthetic tissue and closed with an elastic band for the transpiration maintaining. They were watered to avoid the water stress.

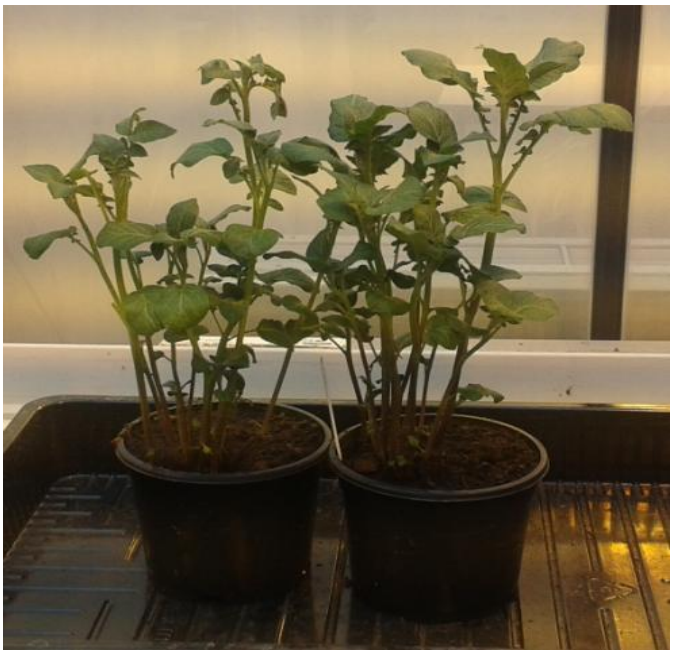


Photo 16. Bintje potato plants (no GM) for experiment with *A. bipunctata*. Photo Di Vitantonio C.

Adalia bipunctata "APHIDALIA" was supplied by the Dutch company Koppert (www.Koppert.com), in a small bottle containing one hundred larvae in L3 stage. Two larvae per plant were introduced, with a small brush with a total of 40 larvae per thesis. These were checked every day until the pupae formation. The newly-formed pupae were removed from the support (these pupae are always attached to a support to complete the metamorphosis) with metallic tweezers. They were then transferred to petri dishes, frozen and weighed within 24 hours.

Photo 17. Bintje potato plants covered for the experiment. Photo Di Vitantonio C.

2.2.2.2 Parameters considered in the study

The parameters evaluated were:

- Developing time from L3 stage to pupal stage (in days)
- Pupal weight (in mg)

2.3 DATA ANALYSIS

As statistical analysis a simple t-test was carried out.

3. RESULTS AND DISCUSSION

The results are presented in Table 1 and Figures 1 and 2.

Table 1. *P*- values of the t-test analysis related to two parameters: *Adalia bipunctata* development time and weight**.** The coccinellids were obtained on infected and non-infected Bintje potato plants.

Parameters	**T-test**
Development time	*P*= 0.30
Weight	*P*= 0.28

A total number of 16 larvae (infected plants) and 18 larvae (non infected plants) survived and pupated.

In this t-test analysis the data didn't show any significant difference for both these two parameters. That is the plants infected by *Phitophthora*, did not affect the development time and the pupal weight of the coccinellid.

Figure 1. Development time (days) of *Adalia bipunctata* larvae on Bintje potato plants with and without *Phytophthora* (number of coccinellids = 16 B+; 18 B-).

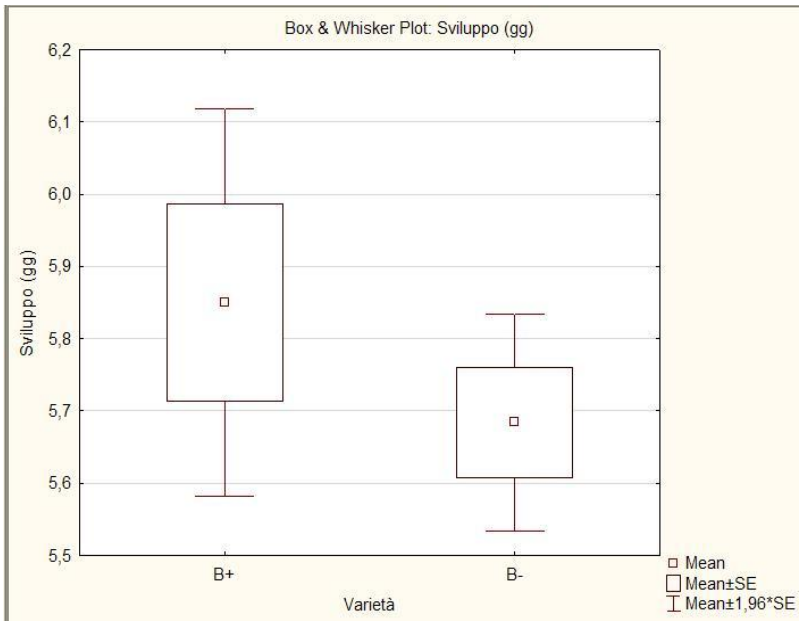


Figure 1 shows the data on development time for the pupae grown on Bintje potato plants with the inoculum of the fungus vs. those without the pathogen. On the plants without the pathogen, the development was faster, although the difference was not significant

Figure 2. Weight (mg) of *Adalia bipunctata* pupae grown on Bintje potato plants with and without *Phytophthora* inoculum. (number of coccinellids = 16 B+; 18 B-).

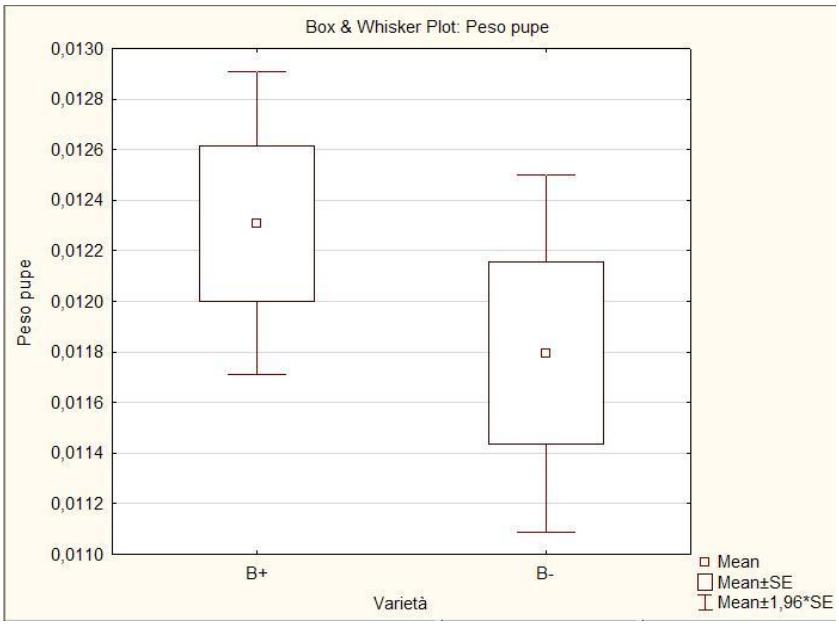


Figure 2 shows the data concerning the weight of the pupae obtained from the Bintje potatoes affected by *Phytophthora* and those maintained as control. On the infected plants, heavier pupae were obtained than on non-infected plants, although the difference was not significant. The longer larval development might be related with the higher pupal weight of the ladybirds maintained on the infected plants.

4. CONCLUSIONS

4.1 EXPERIMENT 1

Phytophthora did not cause significant effects either on percentage of *A. colemani* mummies formed, or on percentage of parasitoid adults emerged, or on the weight of parasitoids in all the three plant lines considered.

The fungus effect produced a significant delay in the emergence of the parasitoids from infected plants compared to those obtained from non-infected plants

As regards the sex-ratio, the comparison Iso/Cis and Iso/Trans was significant (independently of *Phytophthora* infection. In detail, The percentage of females in the iso-genic plants was much lower compared with the two other theses.

4.2 EXPERIMENT 2

Phytophthora infestans did not affect either the larval development time until the pupal stage or the pupal weight of the indigenous aphidophagous coccinellid *Adalia bipunctata*. However, a trend (though not significant) of longer development and higher pupal weight was observed in the ladybirds obtained on the infected plants.

These experiments are part of a bigger project in which different research groups are involved. In order to evaluate the risk assessment of these trans-genic and cis-genic potato plants, other laboratory and greenhouse trials should be carried out before testing them in the field. Despite the encouraging results till now achieved about the effect of transgenes and cisgenes (no negative consequences were found on the third trophic level), this GM crop (either CIS or TRANS) should be longer investigated in the thritrophic relationship context.

ACKNOWLEDGEMENTS

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REFERENCES CITED

AMIGA Collaborative project proposal, 2011. “Assessing and monitoring impact of genetically modified plants in agro-ecosystem”.

A.M.R. GATEHOUSE et al., 1996. Transgenic potato plants with enhanced resistance to the peach-potato aphid *Myzus persicae*. Entomologia Experimentalis et Applicata 79: 295-307.

AYLOR D.E., FRY W.E., MAYTON, H. and ANDRADE-PIEDRA, J.L., 2001. Quantifying the rate of release and escape of *Phytophthora infestans* sporangia from a potato canopy. Epidemiology, 91:1189–1196.

BALDUF W.V. (1926). The bionomics of Dinocampus coccinellae (Schrank). Annals of the Entomological Society of America 19: 465-498.

BELLA S., 2013. The box three moth *Cydalima perspectalis* (Walker, 1859) continues to spread in Southern Europe: New records for Italy (Lepidoptera Pyraloidaea Crambidae). Redia, XCVI: 51-55.

BENELLI M. et al., 2015. Effect of two temperatures on biological traits and susceptibility to a pyrethroid insecticide in an exotic and native coccinellid species. Bullettin of Insectology 2015, 68(1): 23-29.

BERKVENS et al., 2010. *Dinocampus coccinellae* as a parasitoid of the invasive ladybird *Harmonia axyridis* in Europe. Biological Control 53: 92-99.

BROWN P.M.J. et al., 2011. Decline in native ladybirds in response to the arrivalof *Harmonia axyridis*: early evidence from England. Ecological Entomology (2011), 36: 231-240.

BROWN P.M.J. et al., 2011. The global spread of *Harmonia axyridis* (Coleoptera:Coccinellidae): distribution, dispersal and route of invasion. BioControl 56: 623-641.

BURGIO G. et al., 2008. *Harmonia axyridis* recordings in Northern Italy. Bulletin of Isectology 61 (2) : 361-364.

BURGIO et al., 2002. On intra-guild predation and cannibalism in *Harmonia axyridis* (Pallas) and *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae) Biological Control 24: 110-116.

CERRETTI P., TSCHORSNIG, H. P., 2010. Annotated host catalogue for the Tachinidae (Diptera) of Italy. Stuttgarter Beiträge zur Naturkunde A. Neue Serie 3: 305-340.

CERYNGIER P. ROY H., POLAND R.L., 2012. Natural enemies of ladybird beetles. In HODEK I., H.F. van EMDEN, HONEK A., 2012. Ecology and Behaviour of the Ladybird Beetles (Coccinellidae) Wiley-Blackwell pp: 391-399.

CHAPIN E.A., 1956. Coleoptera, Coccinellidae. Insects of Micronesia. Honolulu, Hawai, USA: Bernice P. Bishop Museum.

CHAPIN J.B., BROU V.A., 1991. *Harmonia axyridis* (Pallas), the third species of the genus to be found in the United States (Coleoptera Coccinellidae). Proceedings of the Entomological Society of Washington 93: 630-635.

CHENG S.P., 2005. Studies on the box tree caterpillar, *Diaphania perspectalis* (Walker). Anhui Agri. Sci. Bull. 11: 107-108 (in Chinese).

CHOO H.Y. et al., 1991. Laboratory evaluation of entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* against some forest insect pests. Kor. J. Appl. Entomol. 30: 227-232.

ClOUTIER C., LEVESQUE CA., EAVES DM., MACKAUER M (1991). Maternal adjustment of sex ratio in response to host size in the aphid parasitoid *Ephedrus californicus*. Can J Zool 69: 1489-1495.

BALLETTO E., CASALE A., 1991. Mediterranean insect conservation. In COLLINS N.M., THOMAS J.A., 1991. The conservation of insects and their habitats. 15th Symposium of the Royal Entomological Society of London, 14,15 September 1989. Academic Press Inc pp.: 135.

CSOKA G., LESKO K., AMBRUS A., 1989. Biology and damage of *Dendrolimus pini* L. (Lepidoptera: Lasiocampidae) in Hungary. Novenyvedelem 25 (2), 61-65.

DE BACH P., ROSEN D., 1991. Biological control by natural enemies. Second edition. Cambridge University Press, 443 pp.

DE BACH P., 1964. Biological control of insect pests and weeds. De Bach P: 844 pp.

DE CLERCQ P., 2004. Culture of natural enemies on factitiuos food and artificial diets. Encyclopedia of Entomology, Volume 1, 1133-1136.

DECRETO LEGISLATIVO n.194 del 17 marzo 1995. Attuazione della direttiva 91/414/CEE in materia di immissione in commercio di prodotti fitosanitari. G.U. n.122 del 27 maggio 1995, S.O. www.isprambiente.gov.it/files/sostanze-pericolose/dlgs194-95.pdf

DEPALO L., DINDO M. L., EIZAGUIRRE M., 2012. Host location and suitability of the armyworm larvae *Mythimna unipuncta* for the tachinid parasitoid *Exorista larvarum*. BioControl 57, 471-479.

DEPALO L. et al., 2010. Location, acceptance and suitability of *Spodoptera littoralis* and *Galleria mellonella* as hosts for the parasitoid *Exorista larvarum*. Bulletin of Insectology 63: 65-69.

DESNEUX N. DECOURTYE A., DELPUECH J.M., 2007. The sublethal effects of pesticides on beneficial arthropods. Annu. Rev. Entomol., 52: 81-106.

DI DOMENICO F., LUCCHESEA F., MAGRI D., 2012. *Buxus* in Europe: late Quaternary dynamics and modern vulnerability. Perspect. Plant Ecol. Evol. Syst. 14: 354-362.

DINDO M.L., GRENIER S., 2014. Mass Production of Beneficial organisms: Invertebrates and Entomopathogens. 4 : 101-143.

DINDO M.L. et al., 2013. Acceptance and suitability of *Cacyreus marshalli* (Lepidoptera: Lycaenidae) as host for three indigenous parasitoids. Biocontrol Science and Technology. 23 (11): 1342-1348.

DINDO, M. L., 2011. Tachinid parasitoids: are they to be considered as koinobionts? BioControl 56 (3), 249-255.

DINDO, M. L.; MARCHETTI, G.; BARONIO, P., 2007. *In vitro* rearing of the parasitoid *Exorista larvarum* (Diptera: Tachinidae) from eggs laid out of host. Journal of Economic Entomology 100: 26–30.

DINDO M.L. et al., 2003. Rearing of *Exorista larvarum* (Diptera Tachinidae): simplification of the *in vitro* technique. Bulletin of Insectology 56 (2): 253-257.

DINDO M.L., FARNETI R., SCAPOLATEMPO M., GARDENGHI G., 1999. *In Vitro* Rearing of the parasitoid *Exorista larvarum* (L.) (Diptera: Tachinidae) on meat homogenate-based diets. Biological Control 16: 258-266.

DINDO M.L., 1987. Effetti indotti da parassitoidi Imenotteri nei loro ospiti. Boll. Ist. Ent. "G. Grandi" Univ. Bologna, 42: 1-46.

DI VITANTONIO C., DINDO M.L., MARCHETTI E., FRANCATI S., MAINI S., 2014. Effetti indotti dal parassitoide *Dinocampus coccinellae* (Schrank) sul coccinellide esotico *Harmonia axyridis* (Pallas) e confronto con la specie indigena *Adalia bipunctata* (L.). Poster presentato al XXIV Congresso Nazionale Italiano di Entomologia - Orosei (Sardegna), 9-14 giugno 2014 – (pag. 48).

DOBZHANSKY T., 1933. Geographical variation in ladybeetles. The American Naturalist 67: 97-126.

DRIPPS J. et al., 2008. Spinetoram: How artificial intelligence combined natural fermentation with synthetic chemistry to produce a new Spynosin insecticide. Plant management network (www.plantmanagementnetwork.org)

EFSA. 2010. Guidance on the environmental risk assessment of genetically modified plants". Panel on Genetically Modified Organisms (GMO); EFSA Journal 2010; 8(11):1879. [111 pp.].

EISNER T., EISNER M., 1992. Operation and defensive roles of "Gin Traps" in a coccinellid pupa (*Cycloneda sanguinea*). Psyche, A Journal of Entomology 99: 265-273.

ELBERT et al., 1991. Imidacloprid- a new systemic insecticide. http://agris.fao.org/agris-search/search.do?recordID=DE92U0152

EPPO, 2014. http: // www.eppo.int (website)

EUR-Lex-32004D0304- EN-EUR-Lex. EU law and publications. eur-lex.europa.eu(website)

FERRAN A. et al., 1998. An artificial non-flying mutation to improve the efficiency of the ladybird *Harmonia axyridis* in biological control of aphids. BioControl 43:53-64.

FILATOVA I.T., 1974. The parasites of Coccinellidae (Coleoptera) in West Siberia. In KOLOMIYETZ N.J. The Fauna and Ecology of Insects from Siberia. Novosibirsk, Siberia URSS: Siberian Branch RAS Publishing, pp: 173-185.

FIRLEJ A., BOIVIN G., LUCAS E., and CODERRE D., 2005. First report of Harmonia axyridis Pallas being attacked by Dinocampus coccinellae Schrank in Canada. Biological invasions 7: 553-556.

FRANCATI S., 2013. New associations between native parasitoids and exotic insects introduced in Italy and in Europe. PhD thesis in Agricultural Entomology, University of Bologna, Italy, cycle XXV.

FRY W., 2008. *Phytophthora infestans*: the plant (and R gene) destroyer. Molecular Plant Pathology 9 (3): 385-402.

GALVAN T.L., KOCH R.L., HUTCHISON W.D., 2006. Toxicity of Indoxacarb and Spinosad to the multicolored Asian lady beetle, *Harmonia axyridis* (Coleoptera: Coccinellidae), via three routes of exposure. Pest Manag Sci 62: 797- 804.

GALVAN T.L., KOCH R.L., HUTCHISON W.D., 2005. Effects of Spinosad and Indoxacarb on survival, development, and reproduction of the multicolored Asian lady beetle (Coleoptera: Coccinellidae). Biological Control 34: 108-114.

GENTZ M.C., MURDOCH G., KING G.F., 2010. Tandem use of selective insecticides and natural enemies for effective, reduced-risk pest management. Biological Control 52: 208-215.

GERWICK B.C., SPARKS T.C., 2014. Natural products for pest control: an analysis of their role, value and future. Society of Chemical Industry. Pest Manag Sci 70: 1169-1185.

GMO-Compass 2010. Genetically modified food and feed: Authorization in the EU.
http://www.gmo-compass.org/eng/gmo/db/

GOSSET V., et al., 2009. Attacks by a piercing-sucking insect (Myzus persicae Sultzer) or a chewing insect (Leptinotarsa decemlineata Say) on potato plants (Solanum tuberosum L.) induce

differential changes in volatile compound release and oxylipin synthesis. Journal of Experimental Botany 60 (4): 1231-1240.

GRASSWITZ T.R., REESE B.D.,1998. Biology and host selection behaviour of the aphid hyperparasitoid Alloxysta victrix in association with the primary parasitoid Aphidius colemani and the host aphid Myzus persicae. BioControl 43: 261-271.

GURLAY E.S., 1930. Some parasitic Hymenoptera of economic importance in New Zealand. New Zealand Journal of Science and Technology 39: 339-343.

HAFEZ, M.; TAWFIK, M.F.S.; AZAB, A.K.; IBRAHIM, A.A. (1976). Survey and economic importance of parasites of the cotton leaf worm, *Spodoptera littoralis*(Boisd.), in Egypt. Bulletin of the Entomological Society of Egypt 60: 179-189.

HAFEZ, M. (1953). Studies on *Tachina larvarum* L. (Diptera, Tachinidae). Bulletin of the Entomological Society of Egypt 37: 255-335.

HASSAN S.A., 1998. The initiative of the IOBC/WPRS Working Group on Pesticides and Beneficial Organisms. Ecotoxicology, pp: 22-27.

HASSAN S.A., 1992. Guidelines for testing the side- effects on beneficial organisms: description of test methods. IOBC/ WPRS Bullettin, 15(3): 18-39.

HASSAN S.A., 1988. Working Group " Pesticides and Beneficial Organisms", Guidelines for testing the effects of pesticides on beneficials: short description of test methods. IOBC/WPRS Bullettin XI/4, 143 pp.

HASSAN S.A., OOMEN P.A.,1985. Testing the side effects of pesticides on beneficial organisms by OILB Working Party. In: " Biological Pest Control- the Glasshouse Experience" (Hussey N.W. and Scopes N.). Blandford Press, Poole, Dorset., pp 145-152.

HAVERKORT A.J. et al., 2009. Applied biothecnology to combat late blight in potato caused by *Phytophthora infestans*. Potato Research (2009) 52: 249-264.

HAVERKORT A.J. et al., 2008. Societal costs of late blight in potato and prospects of durable resistance through cisgenic modification. Potato Research (2008) 51: 47-57.

HERTING B., 1960. Biologie der westpalaarktischen Raupenfliegen (Diptera:Tachinidae). Monographien zur angewandten Entomologie (Nr. 16), 188 pp.

HIJMANS R.J., SPOONER D.M., 2001. Geographic distribution of wild potato species. American Journal of Botany 88(11): 2101-2112.

HEJBICH K., 2003. Producers in Ontario and northern U.S. bugged by bad odors in wine. Wine spectator 15 May: 16.

HODEK I., HONEK A., 2013. Ecology of Coccinellidae. Springer- Science+ Business Media, B.V., 463 pp.

HODEK I., HONEK A., van EMDEN H.F., 2012. Ecology and Behaviour of the Ladybird Beetles (Coccinellidae). Wiley-Blackwell: 561 pp.

HODEK I., 1973. Life history and biological properties. In HODEK I., 1973. Biology of Coccinellidae. Tha Hauge Holland: Dr. V. JunkN.V., Publishers: pp 70-76.

HONEK A., 1993. Intra-specific variation in body size and fecundity in insects - a general relationship. Oikos 66: 483-492.

HUKUSIMA S., KAMEI M., 1970. Effects of various species of aphids as food on development, fecundity and longevity of Harmonia axyridis Pallas (Coleoptera Coccinellidae). Research Bulletin of the Faculty of Agriculture, Gifu University 29: 56-66.

HULME P.E., ROY D.B., 2010 – DAISIE and arthropod invasions in Europe p. 1-3. In: Roques A. et al. (Eds), Alien terrestrial arthropods of Europe. – BioRisk, 4(1).

IABLOKOFF-KHNOZORIAN, S.M., 1982. Les Coccinelles, Coléoptères- Coccinellidae. Paris, France : Nouvelle Edit Boubé.

IPERTI G., 1964. Les parasites des Coccinelles aphidiphades dans les Alpes-Maritimes et les Basses- Alpes. Entomophaga 9: 153-180.

JACOBSEN E., SCHOUTEN H.J., 2007. Cisgenesis strongly improves introgression breeding and induced translocation breeding of plants. Trends in Biotechnology 25 (5): 219–223.

JACOBSEN E., SCHOUTEN H.J., 2008. Cisgenesis, a new tool for traditional plant breeding, should be exempted from the regulation on genetically modified organisms in a step by step approach. Potato Research 51 (1): 75–88.

JALALI M.A., TIRRY L., ARBAB A., DE CLERCQ P., 2010. Temperature–dependent development of the two- spotted ladybeetle, *Adalia bipunctata*, on the green peach aphid, *Myzus persicae*, and a factitious food under constant temperatures. Journal of Insect Science. Vol.10 Article 124: 1-14.

JALALI M.A., VAN LEEUWEN T., TIRRY L., DE CLERCQ P., 2009. Toxicity of selected insecticides to the two-spot ladybird *Adalia bipunctata.* Phytoparasitica, 37: 323-326.

JANSEN J.P., HAUTIER, H., 2006. Comparative sensitivity of four ladybird species to five pesticides. Pesticides and Beneficial Organisms IOBC/wprs Bulletin 2006. Vol. 29 (10): 95-103

JOSEPH S.B., SNEYDER W.E., MOORE A.J., 1999. Cannibalizing Harmonia axyridis (Coleoptera: Coccinellidae)larvae use endogenous cues to avoid eating relatives. Journal of Evolutionary Biology 12: 792-797.

JUCKER C., LUPI D., 2011. Exotic insects in Italy: an overview on their environmental impact. In www.researchgate.net, 4: 51-73.

JUDELSON, H.S., 1997. Expression and inheritance of sexual preference and selfing potential in *Phytophthora infestans*. Fungal Genetics and Biology, 21:188–197.

KATSAROU J., MARTINOU A., PAPACHRISTOS D., ZOAKI D., 2009. Toxic effects of insecticide residues on three aphidophagous coccinellid species. Hellenic Plant Protection Journal, 2: 101-106.

KATSOYANNOS P. et al., 1997. Establishment of *Harmonia axyridis* on citrus and some data on its phenology in Greece. Phytoparasitica 25: 183-191.

KEANE R.M., CRAWLEY M.J., 2002. Exotic plant invasions and the enemy release hypothesis. Trends in Ecology and Evolution. 17 n.4 April 2002.

KENIS et al., 2013. The box tree moth, Cydalima perspectalis, in Europe: horticultural pest or environmental disaster? Aliens 33: 38-41.

KENIS M., et al. 2008. Current and potential management strategies against *Harmonia axyridis*. BioControl 53: 235-252.

KENIS M. , LOPEZ VAAMONDE C., 1998. Classical biological control of the gypsy moth, *Lymantria dispar* (L.) in North America: prospects and new strategies. Proceedings: population dynamics, impact and integrated management of forest defoliating insects, USDA Forest Service General Technical Report NE-247, 213–221.

KIRPICHNIKOVA V.A., 2005. Pyralidae. In: Key to the insects of Russian Far East 5 (2). Ed. By Ler P.A., Dalnauka, Vladivostok, 526-539 (in Russian).

KOCH R.L., 2003. The Multicolored Asian lady beetle, Harmonia axyridis: A review of its biology, uses in biological control, and non-target impacts. Journal of Insect Science, 3: 32, 16 pp.

KOJAMA S., MAJERUS M.E.N., 2008. Interactions between the parasitoid wasp Dinocampus coccinellae and two species of coccinellid from Japan and Britain. BioControl 53: 253-264.

KOVACH J., 2004. Impact of the multicolored Asian lady beetle as a pest of fruit and people. Am Entomol 50: 165-167.

KRUGER E.O., 2008. *Glyphodes perspectalis* (Walker, 1859) Neu-f ur die Fauna Europas (Lepidoptera: Crambidae). Entomol Z, 118: 81-83.

LaMANA M.L., MILLER J.C., 1998. Temperature dependent development in an Oregon population of *Harmonia axyridis* (Coleoptera: Coccinellidae). Environmental Entomology 27: 1001-1005.

LaMANA M.L., MILLER J.C., 1996. Field observations on *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) in Oregon. Biological Control 6: 232-237.

LANZONI A., ACCINELLI G., BAZZOCCHI G.G., BURGIO G., 2004. Biological traits and life table of the exotic *Harmonia axyridis* compared with *Hippodamia variegata*, and *Adalia bipunctata* (Col., Coccinellidae). Journal of Applied Entomology, 128 (4): 298-306.

LEUTHARDT F., GLAUSER G., BAUR B., 2013. Composition of alkaloids in different box tree varieties and their uptake by the box tree moth *Cydalima perspectalis.* Chemoecology 23: 203-212.

LI S.G. et al., 2004. Biology and microbial control of box tree caterpillar, Diaphania perspectalis (Walker). Forest. Ins. 6, 584-587 (in Chinese).

LOCKWOOD J.A., 1993. Environmental Issues Involved in Biological Control of Rangeland Grasshoppers (Orthoptera: Acrididae) with Exotic Agents. Environmental Entomology 22: 503-518.

LOMBAERT E., ESTOUP A., FACON B., JOUBARD B., GREGOIRE J. C., JANNIN A., BLIN A., GUILLEMAUD T., 2014.- Rapid in-crease in dispersal during range expansion in the invasive ladybird *Harmonia axyridis*.- Journal of Evolutionary Biol-ogy, 27 (3): 508-517.

LOPEZ-VAAMONDE et al., 2010. Lepidoptera, chapter 11, p. 603-668. In : Roques A. et al. (Eds.), Alien terrestrial arthropods of Europe. BioRisk, 4 (2).

MACKAUER, M. 1968. "Insect parasites of the green peach aphid, *Myzus persicae* Sulz., and their control potential." *BioControl* 13 (2): 91–106.

MACKAUER M., VOLKL W., 1993. Regulation of aphids population by aphidiid wasps: does parasitoid foraging behaviour or hyperparasitism limit impact? Oecologia 94: 339-350.

MAETA Y., 1969. Some biological studies on the natural enemies of some coccinellid beetles. Tohoku Konchu Kenyu 4: 1-6.

MAHMOUD M.F. et al., 2009. Efficiency of Spinetoram as a biopesticide to Onion Thrips (*Thrips tabaci* Lindeman) and Green Peach Aphid (*Myzus persicae* Sulzer) under laboratory and field conditions. Journal of Biopesticides 2009, 2 (2): 223-227.

MAINI S. et al., 2010. Insetti e globalizzazione: controllo sostenibile di specie esotiche in ecosistemi agro-forestali. PROGETTO PRIN, prot. 2010CXXHJE, 2010/2011.

MALLY R., NUSS M., 2010. Phylogeny and nomenclature of the box tree moth, *Cydalima perspectalis* (Walker, 1859) comb. n., which was recently introduced into Europe (Lepidoptera: Pyraloidaea: Crambidae: Spilomelinae). Eur. J. Entomol. 5759: 393-400.

MARCHETTI, E., 2006. Possibili effetti secondari di batteri epifiti modificati con geni di *Bacillus thuringiensis* (Bt) su *Exorista larvarum* (L.) (Diptera Tachinidae). Dottorato di Ricerca in Entomologia Agraria, 18th Ciclo, 82 p.

MASETTI A., DE LUIGI V., BURGIO G., 2008. Effects of nucleopolyhedrovirus based product on *Spodoptera littoralis*. Bulletin of Insectology 61(2): 299-302.

MASUTTI L., ZANGHERI S., 2001. Entomologia generale e applicata. Cedam 978 pp.

MELLINI, E. & CAMPADELLI, G., 1996a. Analisi del superparassitoidismo di *Exorista larvarum* (L.) nell'ospite di sostituzione *Galleria mellonella* (L.).Bollettino dell'Istituto di Entomologia "Guido Grandi" dell'Università di Bologna, 51:1-11.

MELLINI, E., 1991. Sinossi di biologia dei ditteri larvevoridi (studi sui *Ditteri larvevoridi* L. contributo). Bollettino dell'Istituto di Entomologia "Guido Grandi" dell'Università di Bologna 45: 1-38.

MICHALKOVA V. et al., 2009. Larval Morphology and Anatomy of the Parasitoid *Exorista larvarum* (Diptera: Tachnidae), with an Emphasis on Cephalopharyngeal Skeleton and Digetsive Tract. The Journal of Parasitology , 95 (3): 544-554.

MICHAUD J.P., 2003. A comparative study of larval cannibalism in three species of ladybirds. Ecological Entomology 28: 92-101.

MILLS N.J., 1981. Some aspects of the rate of increase of a coccinellid. Ecological Entomology, Vol.6, Issue 3, pp: 293-299.

MOSTELLER F., Youtz C., 2006. Tables of the Freeman- Tukey. Transformations for the Binomial and Poisson Distributions. Selected Papers of Frederick Mosteller. Springer Series in Statistics 2006, 337-347.

MULLINS J.W., 1993. Imidacloprid. A new nitroguanidine insecticide. http://agris.fao.org/agris-search/search.do?recordID=US19940056309

NACAMBO S. et al., 2013. Development characteristics of the box-tree moth *Cydalima perspectalis* and its potential distribution in Europe. Journal of Applied Entomology, 138: 14-26.

NALEPA et al., 1996. Biology of *Harmonia axyridis* (Coleoptera: Coccinellidae) in Winter Aggregations. Annals of the Entomological Society of America.

OBRYCKI J.J., KRING T.J., 1998. Predaceous coccinellidae in biological control. Annu.Rev.Entomol. 43: 295-321

OBRJCKI J.J., 1989. Parasitization of native and exotic coccinellids by Dinocampus coccinellae (Schrank) (Hymenoptera: Braconidae). Journal of the Kansas Entomological Society. 62: 211-218.

OBRJCKI J.J., TAUBER M.J., 1978. Thermal requirements for development of Coleomegilla maculata (Coleoptera: Coccinellidae) and its parasite Perilitus coccinellae (Hymenoptera: Braconidae). The Canadian Entomologist 110: 407-412.

OGLOBIN A., 1924. Le role du blastoderme extraembryonnaire du Dinocampus terminatus Nees pendant l'etat larvaire. Věstník Královské české společnosti náuk 2: 1-27.

ONGAGNA P. et al., 1993. Cycle de développement d'*Harmonia axyridis* (Col. Coccinellidae) dans son aire d'introduction: le sud-est de la France. Entomophaga 38: 125-128.

OSAWA N., 2000. Population field studies on the aphidophagus ladybird beetle *Harmonia axyridis* (Coleoptera: Coccinellidae): resource tracking and populatin characteristics. Researches on Population Ecology 42: 115-127.

POLLINI A. Entomologia applicata, 2013. Edagricole: 410-413.

REGIONE EMILIA ROMAGNA. Il corretto impiego dei prodotti fitosanitari. http://www.dinamica-fp.it/centri/reg/guidapatentini/modulo_01/REV2013_MOD1_UD1_prodotti.pdf

REPUBBLICA ITALIANA, Legge 3 febbraio 2011 n.4. Disposizioni in materia di etichettatura e di qualità dei prodotti alimentari. G.U. n.41 del 19 febbraio 2011.

RICHERSON J.V., DeLOACH C.J., 1972. A world list of parasitoid of Coccinellidae. Journal of Entomological Society of British Columbia 65: 834-839.

RIDDICK, E.W., 2009. Benefits and limitations of factitious prey and artificial diets on life parameters of predatory beetles, bugs and lacewings: a mini-review. Biocontrol 54: 325-339.

RIMOLDI F., SCHNEIDER M.I., RONCO A.E., 2012. Short and Long-Term Effects of Endosulfan, Cypermethrin, Spinosad, and Methoxyfenozide on Adults of *Chrysoperla externa* (Neuroptera: Chrysopidae)

ROY H., WAJNBERG E., 2007. From biological control to invasion: the ladybird Harmonia axyridis as a model species. BioControl (2008) 53: 1-4.

SABROSKY C.W., REARDON R.C., 1976. Tachinid parasites of the gypsy moth, *Lymantria dispar*, with keys to adults and puparia. Miscellaneous publications of the Enomological Society of America 10 (2), 126.

SAKURAI H., HAWAY T., TAKEDA S, 1992. Physiological changes related to diapause of the ladybeetle, Harmonia axyridis (Coleoptera: Coccinellidae). Applied Entomology and Zoology 27: 479-487.

SALERNO G. et al., 2014. La piralide del bosso preoccupa anche in Italia centrale. Difesa delle piante. L'Informatore Agrario. 42: 62-65.

SANTI F., RADEGHIERI P., INGA SIGURTA' G., MAINI S., 2015. Sex pheromone traps for detection of the invasive box tree moth in Italy. Bulletin of Insectology 68 (1): 158-160.

SHE D.S., FENG F.J., 2006. Bionomics and Control of *Diaphania perspectalis* (Walker). J. Zhejiang Forest. Sci. Tech. 26: 47-51 (in Chinese).

SHI H.Z., HU K.F., 2007. Occurrence regularity and control techniques of *Diaphania perspectalis* (Walker). Hubei Agric. Sci. 46: 76-78 (in Chinese).

SIGHINOLFI L. et al., 2008. Biological and Biochemical Characteristics for Quality Control of *Harmonia axyridis* (Pallas) (Coleoptera, Coccinellidae) Reared on a Lived-Based Diet. Archives of Insect Biochemistry and Physiology 68: 26-39.

SOLIANI L., 2008. Statistica applicata. Il principio di Bonferroni e il metodo Dunn- Sidak. UniNova, 10: 61-67.

SPINETORAM, TECHNICAL BULLETIN, 2006. Dow AgroSciences.

SPOONER D.M., BAMBERG J.B., 1994. Potato genetic resources: Sources of resistance and systematics. American Potato Journal. Volume 71, Issue5, pp 325-337.

STATSOFT, 2010. STATISTICA data analysis software system, version 10. StatSoft Inc., Tulsa, USA.

STERK et al., 1999. Results of the seventh joint pesticide testing programme carried out by IOBC/WPRS Working Group "Pesticides and Beneficial Organisms". BioControl, 44: 99-117.

STORECK A., POPPY G.M., van EMDEN H.F. & POWELL W., 2000. The role of plant chemical cues in determining host preference in the generalist aphid parasitoid *Aphidius colemani*. Entomologia Experimentalis et Applicata 97: 41-46.

SUCHAIL S., GUEZ D., BELZUNCES L.P., 2000. Characteristics of Imidacloprid toxicity in two *Apis mellifera* subspecies. Environmental toxicology, Vol. 19, Issue 7: 1901-1905.

SUMITOMO Chemical Co., 2012. Development of the Novel Insecticide Spinetoram (DIANA®). http://www.sumitomo-chem.co.jp/english/rd/report/theses/docs/2012E_1.pdf

TAKADA H.,1998. A review of *Aphidius colemani* (Hymenoptera:Braconidae; Aphidiinae) and closely related species indigenous to Japan. Appl.Entomol.Zool.33 (1): 59-66.

TESCARI E. et al., 2014. Spinetoram (Delegate®, Radiant®), nuovo insetticida ad ampio spettro d'azione. ATTI Giornate fitopatologiche, 1: 11-20.

TIMBERLAKE P.H., 1918. Note on some of the immigrant parasitic Hymenoptera of the Hawaiian Islands. Proceedings of the Hawaiian Entomological Society 3: 399-404.

TURKENSTEEN, L.J., FLIER, W.G., WANNINGEN, R. and MULDER, A., 2000. Production, survival and infectivity of oospores of *Phytophthora infestans*. Plant Pathology, 49: 688–696.

UEZUMI Y., 1975. Lepidoptera harmed the trees and the pest control. Plant Prot. 29: 349-354.

VALIGUROVA, A.; MICHALKOVA, V.; KONIK, P.; DINDO M.L.; GELNAR, M.; VANHARA, J., 2014. Penetration and encapsulation of the larval endoparasitoid *Exorista larvarum* (Diptera:Tachinidae) in the factitious host *Galleria mellonella* (Lepidoptera:Pyralidae). Bulletin of Entomological Research 104: 203-212.

VINSON S.B., IWANTSCH G.F., 1980. Host regulation by insect parasitoids. Quarterly Review of Biology 55: 143-165.

VLEESHOWER V.G., RAFFAELE S., VOSSEN J.H., CHAMPOURET N., OLIVA R., SEGRETIN M.E., RIETMAN H., CANO L.M., LOKOSSOU A., KESSEL G., PEL M.A. and KAMOUN S., 2011. Understanding and exploiting late blight resistance in the age of effectors. Annual Review of Phytopathology, 49: 507–531.

WALKER M.F., 1961. Some observations on the biology of the ladybird parasite Perilitus coccinellae (Schrank) (Hym.: Braconidae), with special reference to host selection and recognition. Entomologist's Monthly Magazine 97: 240-244.

WAN H. et al., 2014. Biology and natural enemies of *Cydalima perspectalis* in Asia: Is there biological control potential in Europe? Journal of Applied Entomology, 138: 715-722.

WANG L.Y., 1986. Mass rearing and utilization in biological control of the ladybeetle *Leis axyridis* (Pallas). Acta Entomologica Sinica 29: 104.

WEISSENBERGERA., BRUN J., HEROLD D., 1997. Essais de toxicite d'un aphicide (Confidor) sur la Coccinelle Harmonia axyridis Pallas. Mededelingen van de Faculteit Land-bouwwetenschappen, Rijksuniversiteit Gent, 62: 589-593.

YANO E., 2006. Ecological considerations for biological control of aphids in protected cultures. Population Ecology 48: 333-339.

ZAR J.H., 1984. Biostatistical analysis. Prentice Hall, Englewood Cliffs, NJ, USA.

ZAR J.H., 2010. Biostatistical analysis. Fifth Edition. Prentice Hall, Englewood Cliffs, NJ, USA.