EVALUATION OF *MACROLOPHUS PYGMAEUS* [HETEROPTERA : MIRIDAE] AS BIOCONTROL AGENT AGAINST APHIDS

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This study was realised with the entomology department of the University of Gembloux Agro Bio Tech in collaboration with the company of Biobest.

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Abstract

This study is aimed at evaluating *Macrolophus pygmaeus* (Rambur) as a biocontrol agent against aphid. *M. pygmaeus* has already been identified as an aphid predator but never used to control them. The advantage of this predator is its ability to survive feeding on plant tissues in the event of prey scarcity and without damaging the plant.

This study is divided into two sections; the first evaluated the predation of *M. pygmaeus* on *Myzus persicae* and the second investigated the plant defence induction of the phytophagous predator.

For the first part, three trials were realised on bell peppers (*Capsicum anuum*) in large cages containing 4 plants. The first one evaluated the predation of the predator on aphid after a rearing of *M. pygmaeus* for a month and the impact of a second diet made of *Ephestia kuehniella* eggs or *Trialeurodes vaporariorum*. The two last trials evaluated the effectiveness of different predator densities on small (10 individuals) and large (50 and 200 individuals) aphid colonies in a curative application.

The experiments of the second section were realised in net individual cages on bell peppers. The first experiment evaluated the impact of a pre-exposition of the plant to *M. pygmaeus*, and *Myzus persicae* on the aphid reproduction, behaviour and suitability. The two last experiments were made to verify the results of the first one. They separately investigated the impact of pre-exposition of the plant to aphids and to *M. pygmaeus*.

The first section revealed that 4 *M. pygmaeus* per plant were enough to control 75% of the small aphid colonies. The same results were encountered for a density of 8 predators per plant. This is probably correlated with the aphid development rate differences. Only a density of 12 *M. pygmaeus* per plant allows getting over any development rate. Obviously, this density is too high to be applied to a crop. However, applying a density of 4 predators per plant, it is likely that in greenhouses, the predators will move from the healthy plants to the infested ones and this way increase their density. On a large aphid colony, the predator helped to slightly reduce the aphid number. A second diet didn’t influence the predation of *M. pygmaeus*.

A pre-exposition to *M. pygmaeus* male or female had an impact on the aphid reproduction rate. Males increased in rate compared to females. This section permits to make a supposition about the plant defence manipulation by the aphid. Their development rate was strongly enhanced by the first infestation. It also seemed that the plant was able to recover its defences, suppressed by the first infestation, during the period between the two infestations forcing the aphids to adopt a different distribution on the plant.

Résumé

Cette étude a pour but l'évaluation de *Macrolophus pygmaeus* (Rambur) [Heteroptera: Miridae] en tant qu'agent de lutte biologique contre les pucerons. *M. pygmaeus* est reconnu par d'autres études comme prédateur de pucerons mais n'a jamais été utilisé en lutte biologique contre ce ravageur. Son avantage par rapport aux agents de lutte utilisés contre le puceron est sa capacité à se nourrir directement des tissus de la plante et ainsi à se maintenir sur une culture entre deux infestations.

Cette étude a été divisée en deux parties, toutes deux réalisées sur *Capsicum anuum*. La première a pour but d'évaluer la prédation de *M. pygmaeus* sur les pucerons. La deuxième partie s'attèle à mettre en évidence une éventuelle induction des défenses de la plantes par *M. pygmaeus*.

Pour la première partie, trois expériences ont été réalisées dans de grandes cages contenant quatre plantes. La première évalue la prédation de *M. pygmaeus* sur *Myzus persicae* en application préventive et l'impact d'une diète alternative constituée d'œufs d'*Ephestia kuehniella* ou de *Trialeurodes vaporaiorum* sur cette prédation. Les deux expériences suivantes évaluent l'efficacité de différentes densités de prédateurs en application curative sur des petites (10 individus) et des grandes (200 et 50 individus) colonies de pucerons.

Trois expériences ont également été réalisées dans le cadre de la deuxième partie du travail. La première a pour but d'évaluer l'impact d'une pré-exposition des plantes aux prédateurs et aux pucerons sur le comportement et la reproduction des pucerons ainsi que sur la prédation de *M. pygmaeus*. Cette expérience a ensuite été divisée en deux nouvelles expériences évaluant l'impact d'une pré-exposition des plantes respectivement aux pucerons et aux prédateurs.

Il a été démontré qu'une densité de 4 *M. pygmaeus* par plante était suffisante pour contrôler une population de 10 pucerons dans 75% des cas. Les mêmes chiffres ont été trouvés pour une densité de 8 *M. pygmaeus* par plante. Ce pourcentage est probablement du à une différence du taux de développement des colonies de pucerons. Seule une densité de 12 prédateurs par plante permettait de s'affranchir de cette différence de rapidité de développement. Sur le terrain, il est impossible d'appliquer une telle densité de prédateur. Par ailleurs, une application de 4 prédateurs par plante est sans doute efficace en supposant que les prédateurs bougeront des plantes saines vers les plantes infestées, augmentant ainsi leur densité locale.

Les pucerons introduits sur les plantes pré-exposées aux prédateurs mâles se développent significativement plus vite que sur les plantes pré-exposées aux prédateurs femelles et présentent une distribution différente sur la plante. Étant donné que les populations sont naturellement composées de femelles et de mâles, leurs effets respectifs devraient s'annuler et ne pas interférer avec la lutte biologique.

Par ailleurs, plusieurs phénomènes survenus suite à une pré-exposition des plantes aux pucerons ont pu être observés. Effectivement, une première infestation de pucerons améliore considérablement le taux de reproduction d'une infestation suivante. Il semble également que la plante parvienne à rétablir et à exprimer ses défenses, inhibées par la première infestation de pucerons, forçant la seconde infestation à adopter une distribution différente sur la plante.

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Introduction

The biological pest control has become an important alternative to the chemicals in the greenhouse productions. Unfortunately, this strategy of Integrated Pest Management is less effective than the chemicals, and sometimes not sufficient enough to avoid the destruction of the entire crop. The subject of this study has been chosen because of the urgeto improve the biocontrol by the development of new biocontrol agents more efficient. The experiments related to this study were carried out at Biobest in Westerloo, Belgium. Biobest is a worldwide company specialised in pollination and biocontrol. Several biocontrol agents are commercialised in tube as larvae, adults or mummies, but also biopesticides, beneficial nematodes, scouting and monitoring material. Working in a company such as Biobest offers the advantage of accessing some specific material and space required to lead experiments in semi-field conditions. The work has been supervised by Felix Wäckers the Director R&D of the Biobest Group.

This document presents the experiments made on *Macrolophus pygmaeus* (Rambur) [Heteroptera: Miridae] and the results. A discussion about all the results will conclude the work. But, first, the objectives of the study and an overview of the current research and knowledge on biocontrol, *Macrolophus*, its predation on aphids and plant induced defences will be displayed.

Figure 1 : *Macrolophus pygmaeus* (De backer L.)

State of the art

Biological control

Biological control is a method used to reduce pest populations below damaging levels by using their natural enemies (BARBERCHACK, 2011) which are predators or parasitoids. The aim of biocontrol is to totally or partially reduce the use of chemicals. It is a component of an integrated pest management (LEFORT, 2010). Besides the reduction of chemicals, the advantages of biocontrol are specificity, safety for the environment and human health and the avoiding of the resistance issues. From a commercial point of view, the development of a biocontrol agent is cheaper than an insecticide (LEFORT, 2010) but the mass rearing remains expensive and transport and storage are often an issue (VERHEGGEN, 2011). Unfortunately, biocontrol is often not as efficient as chemicals especially when it is used in curative and the effectiveness is not always constant. Also, the pest population decreasing is delayed, chemicals are faster effective (LEFORT, 2010).

It is a very old concept but only applied in the first half of the 19th century (VERHEGGEN, 2011). The term "biocontrol" sometimes includes the microbiological control using fungi, bacteria, viruses or nematodes (FRAVAL, 1999). The biological control has developed to offer an alternative to insecticides. Indeed, the first reason is that the use of insecticides is increasingly outlawed. In the year 2003, 410 molecules have been removed from the annexe I of the directive 91/414/CEE on commercially available molecules (LEFORT, 2010). Moreover, the insects are becoming resistant to some chemicals, especially fruit and vegetable insect pests. Also, chemicals affect non-target organisms and are damageable for human health and the environment (HOFFMANN & FRODSHAM, 1993). For now, the biological control is especially used in greenhouse fruit and vegetable crops. Greenhouses are closed systems providing barriers against dispersal agents and are less vulnerable to pest infestations. In this special environment, pest densities can be easily monitored and the conditions can be adapted to the biocontrol agent. Moreover, the pests of the greenhouse crops are the most susceptible to chemical resistance issues and the products are eaten fresh thus subject to chemical residue restrictions (PERDIKIS, *et al.*, 2008).

Figure 2 : One of the main greenhouse pest, the aphids (*Myzus persicae*) (De Backer L.)

The biocontrol agents can be used in several ways.

- The first way is the classical biocontrol, where an exotic agent is introduced and acclimatized, usually to control exotic pests. The agent is established and becomes permanent (FRAVAL, 1999; HUFBAUER, 2010). Sometimes, the predator is already present on the crop and the environment has to be manipulated to keep or increase the natural population of the predators (HUFBAUER, 2010). The environment handlings consist in conserving the host plants, the beneficial organisms and the natural vegetation providing alternative food to the agent. The use of non-crop plants to assist biocontrol has been investigated and applied in cereal crops among others (PERDIKIS, *et al.,* 2008). This is called conservation biocontrol.
- The second method is the augmentation biocontrol where the agent has to be released on the crop at critical periods (FRAVAL, 1999). This biocontrol method includes two different application ways depending on the number of agent released and the period of the release.
    - The inundative release is a curative application of a large number of agents, when the pests are already established on the crop.
    - The agent can also be released according to a preventive application called inoculation, and be reared on the crop before the pest arrives.

  In both cases, the agent is not permanent and has to be released at each critical period (HUFBAUER, 2010; VERHEGGEN, 2011).
- The last method is the autocidal biocontrol; or "Steril Insect Technique"; where pest males are sterilised by x-rays and released in the population to control. The number of the released males is a determining factor for the pest control. If there are 9 times more sterilised males than normal males, and if the females need only one copulation to produce an egg, 9 females out of 10 will not produce any eggs (FRAVAL, 1999).

Frequently, the crop is infested with several pest species simultaneously. In this case, the use of more than one enemy can be considered and is expected to increase the effectiveness of the control (PERDIKIS, *et al.,* 2008). Some enemies are even commercialized together, such as the aphid parasitoids *Aphidius ervi* and *Aphidius colemani* contained in the "Aphidius-mix-System" sold by Biobest. The use of more than one predator or more than one parasioid can have a synergist effect whereas the simultaneous use of a predator and a parasitoid together can be negative. First because predators cannot discriminate parasitized and healthy pests, thus the predator may eat some parasitoid larvae making the parasitoid population decrease and it is eating a condemned pest instead of killing a healthy one. Instead of killing two pests, one is killed twice. Moreover, the parasitoid can be sensitive to the predatory action (PERDIKIS, *et al.,* 2008).

Biocontrol approaches have been criticised because of the establishment risk of an exotic predator and the adverse effects it may cause to the local fauna after its establishment (PERDIKIS, *et al.,* 2008). The case happened for instance with *Harmonia axyridis* [Coleoptera: Coccinelidae] introduced to control aphids. It has been introduced because of its high

predatory rate. Unfortunately, it also has a high predatory rate on local predator such as *Adalia bipunctata* (VERHEGGEN, 2011).

The evaluation of a new biocontrol agent has to consider in first, its predation or parasitism rate and its specificity. Its life cycle, the number of generations per year and its sensibility to predation are also important (VERHEGGEN, 2011). A good biocontrol agent has to present some characteristics. It cannot interfere with other beneficial insects (UNRUH, 1993; VERHEGGEN, 2011). It has to be adapted to the climate and its cycle has to be synchronised with its host or prey specie cycle (PERDIKIS, *et al.,* 2008). Finally it has to find its hosts or preys at relatively low densities (UNRUH, 1993; PERDIKIS, *et al.*, 2008). From a commercial point of view, an agent must be easily reared, stocked and transported. Obviously, the mass rearing must keep it unchanged (VERHEGGEN, 2011).

Figure 3 : *Aphidius colemani* from Aphidius-System sold by Biobest (De Backer L.)

Predators

The predators decrease the pest population by catching and eating them (BARBERCHACK , 2011). For instance, lady bugs, lacewings or pray mantis are predators. They have chewing mouthparts, and eat the entire prey; or piercing-sucking mouthparts, and they suck the fluids out of the prey leaving some dead bodies behind (MCPARTLAND, *et al*., 2000). The predators can be generalists, they eat a different prey species; or specifics, and they eat only one prey species. The inconvenience of the generalists is that they can eat other natural enemies of the pest or they can prefer another prey species than the one to be controlled.

They present the advantage to be autonomic and mobile for the prey search and they are more persistent than chemicals (VERHEGGEN, 2011). The behaviour of the predator is very similar for different species. First it has to locate the host plant and the prey. Than it has to recognise and select a prey, and finally attack it (VERHEGGEN, 2011). The selection of a prey

depends on several characteristics such as size, vulnerability, mobility and caloric value. The predator decides to attack the available prey or shift to more preferable prey type. This discrimination mechanism acts to optimize the fitness of the predator. As a consequence, when many preys are available, the predator is more selective than when the preys are scarce (FANTINOU, *et al.*, 2009).

However, the predator species are responding differently to the prey density. In 1965, C. S. Holling developed the concept of prey-predator dynamic. According to him, when the prey density increases or decreases, it has an impact on the predator population. This dynamic results from two effects, the predatory rate and the predator density. These two effects are two kinds of responses of predator population to prey density: the functional and the numerical responses.

Three models have been established to describe the different kinds of functional response explaining the predatory rate variation depending on the prey density. The type I functional response is found in passive predators (SHAROV, 1996). Prey mortality due to predation is constant until a certain value where the predator has reached the satiation (BERRYMAN, 1998). At this moment, the predator stops eating and the mortality falls to zero. The type II functional response is the most typical. The search rate is constant while the attack rate increases at a decreasing rate. The prey mortality declines with prey density (SHAROV, 1996). This response is typical of predators specialized on one or a few prey (BERRYMAN, 1998). The type III functional response occurs when the predators increase their search activity while the prey density is increasing. The search activity increases when it is related to kairomone emissions from the prey, for instance (SHAROV, 1996). The attack rate first increases at an increasing rate then at a decreasing rate (BERRYMAN, 1998).

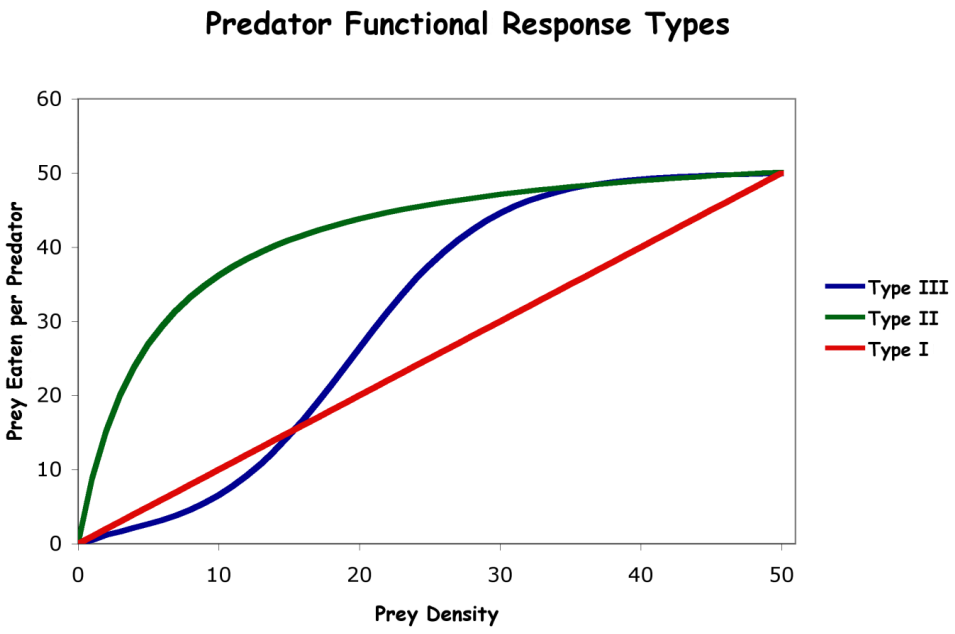


Figure 4 : Functional response types (GANTER & PETERSON, 2006).

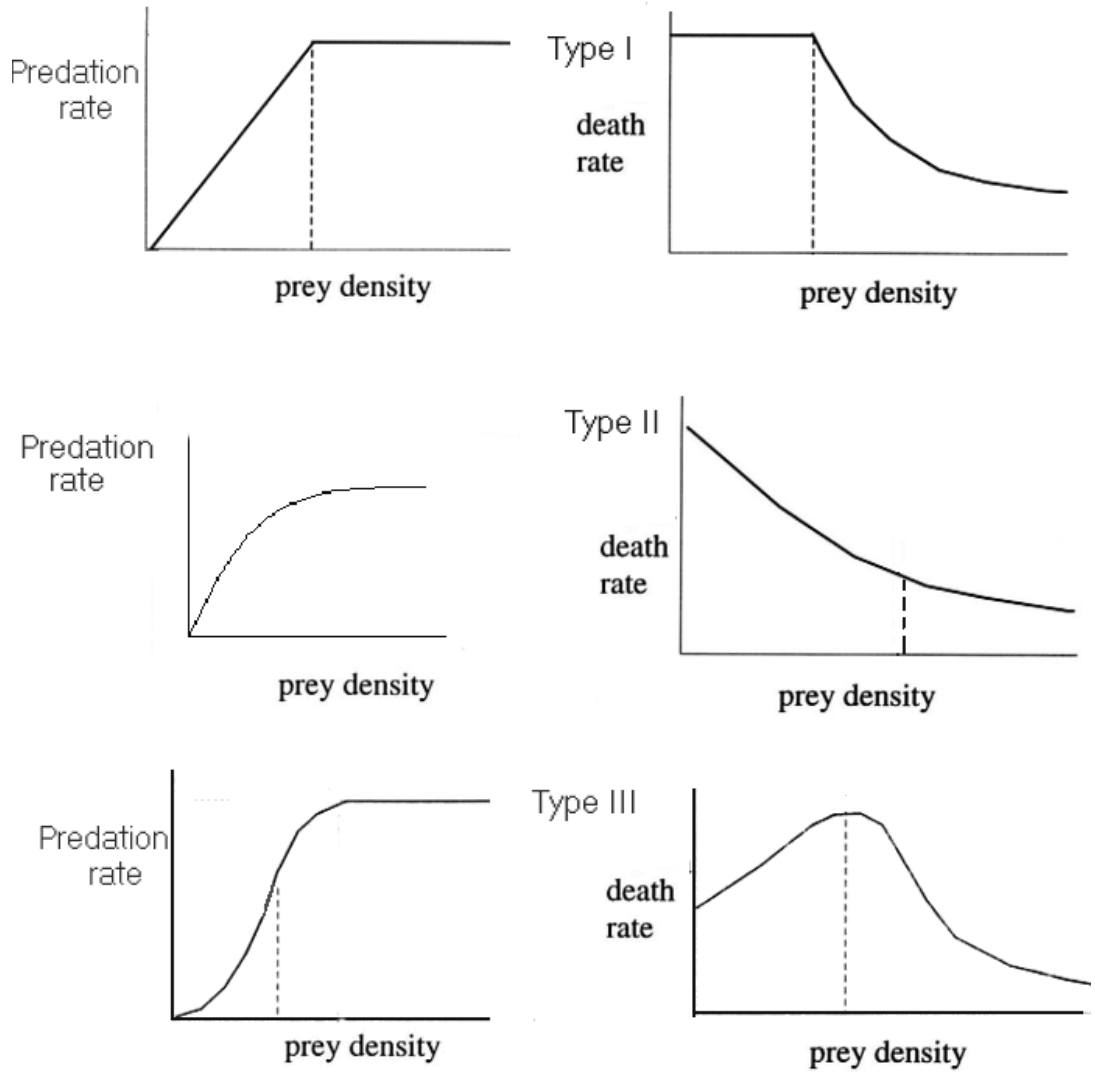


Figure 5 : Functional response type models and the prey mortality dynamic corresponding (HOLZER G., 2002).

The functional response is an important factor that can determine if a predator is able to regulate the density of its prey (SCHENK & BACHER, 2002). Indeed, the type III is the only one allowing controlling a population of prey when the predator density is constant. It is the only model where the mortality increases with the prey density. However, this happens only in the interval of the prey density where the mortality increases. When the density get higher than the upper limit, the mortality decreases and the predators are not controlling the prey any longer. That doesn't mean that the biocontrol agents have to present a type III functional response to be effective. In natural conditions, the predator density is increasing with the pest population. That effect is reported as the numerical response. The most they eat, the most they can spend energy to reproduction. The predator mortality also decreases when the prey density increases (SHAROV, 1996).

The aggregation response completes the previous dynamic model. That response shows how predators are attracted to prey aggregations. The biocontrol agents should have a strong aggregation response to be able to suppress the pests (SHAROV, 1996).

On an individual scale, the effectiveness of a predator varies with the time it spends to search the prey and the time it spends to handle the prey. According to the optimal foraging theory a predator has to maximize the ratio gain of energy on time unit. This theory can be applied to every predator even animal ones. The time spending to search a prey doesn't provide any energy. Once the prey is selected, and the handling begins, the predator gets energy. But as time goes by, and as the prey is consumed, the energy provided by time unit decreases as shown in the figure 6.

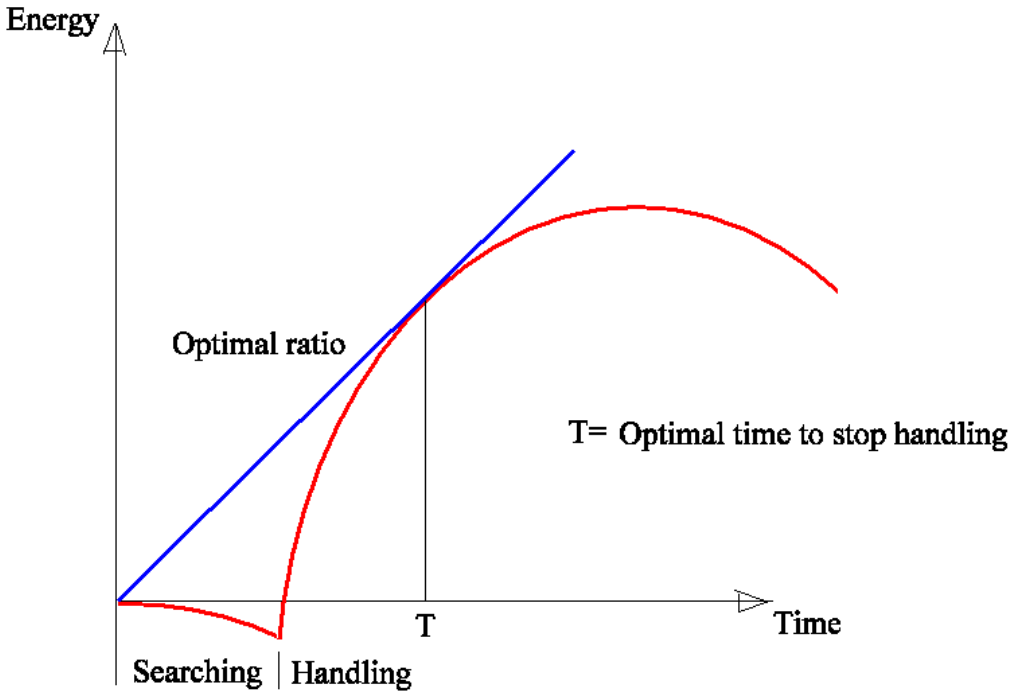


Figure 6: Optimal foraging theory. X = time; y = Energy. The red curve represents the evolution of the energy of a predator during the searching and the handling time. The blue line permits to find the optimal time to leave the prey.

This optimization of the ratio gain of energy on time sometimes forces the predator to leave a prey partially consumed.

A predator can also act differently when it is involved in a "one predator-two prey" system. For instance, *Amblyseius swirwkii* used against thrips, has not much impact on whitelfy populations. However, when the two preys, whiteflies and thrips, were present, the whitefly population decreased as fast as the thrip population. This is partly because the predator was increasing faster on a mixed diet (VAN MAANEN, 2012).

Parasitoids

The other biocontrol agent type is the parasitoids. They belong to the families Aphidiidae and Aphelinidae of the Hymenoptera (PERDIKIS, *et al.*, 2008). In contrast to predators, parasitoids eat their prey from the inside and only one in their whole life (MCPARTLAND, *et al.*, 2000). They are usually very small wasps and flies. The adult stage is free-living and may be predaceous (HOFFMANN & FRODSHAM, 1993). The parasitoid adult females lay their eggs in, on or near the host depending on whether it is an endoparasitoid or an ectoparasitoid. When the egg hatches in the abdomen host, the larva feeds on the host and develops in it until

its adult stage. If the egg is laid on or near the host, when it hatches the larva enters the host and feeds on it (HOFFMANN & FRODSHAM, 1993; MCPARTLAND, *et al*., 2000; BARBERCHACK, 2011). In both case, the parasitoids eat the fluids first, then the tisues beginning with non vital organs to keep the host alive the longest. The larva completes its development and pupates in its host. At that time, the host is killed, and its skin begins to harden transforming it into a mummy. The parasitoid cuts a hole in the underside of the host and attaches it to the leaf by silk (PERDIKIS, *et al.,* 2008). The parasitoid emerges from the mummy when it reaches its adult stage through a round hole (MCPARTLAND, *et al*., 2000). Most parasitoids attack a particular stage of its host. It can be egg, larva, pupa or adult. The host can be parasitized by only one larva parasitoid or several up to hundred (HOFFMANN & FRODSHAM, 1993). Sometimes a parasitoid can be parasitized by another parasitoid specie, this phenomenon is called hyperparasitism. *Encarsia tricolor* Förster and *Encarsia pergandiella* Howard are both hyper-parasitoids used to control whiteflies. The use of these two parasitoids increases the effectiveness of the biocontrol but it isn't the case with every parasitoids. The conditions for that kind of biocontrol to work are very stricts (PERDIKIS, *et al.,* 2008). The parasitoids are more efficient at finding prey than predators and hunt until the pests are nearly eradicated. They are also more specific to one pest specie sometime they are even prey and crop specific (MCPARTLAND, *et al*., 2000). However, the pests die more slowly. Some parasitized host can still lay eggs and feed and sometimes even at a higher rate. But usually the parasitoids complete their life cycle more quickly and increase their number much faster than the predators. The adults are also more susceptible to pesticides than the immature stages protected in the host and than the predators too (HOFFMANN & FRODSHAM, 1993). Examples of commercialized parasitoids are *Encarsia Formosa*, *Aphidius colemani* or *Trichogramma* wasps.

Figure 7 : Aphidius colemani attacking an aphid (DOURLOT S.)

One of the pests

The biological control is very useful against aphids in greenhouses. Indeed, some aphid species have developed resistance to several insecticides, most notably the pyrethroids (FOSTER *et al*., 2000; KUHAR *et al*., 2009). They remain among the main pests of the greenhouse crops. Twenty five percent of the plant species are infested with aphids (GORDON RAMEL, 2010). They belong to the Aphidoidae superfamily (INRA, 2010). More than 4000 aphid species are divided into 10 families, listed in the picture 5 and 250species are serious pests (GORDON RAMEL, 2010).

Table 1:4000 aphid species are divided into ten families belonging to the Aphidoidea superfamily (GORDON RAMEL, 2010)

Superfamily
Aphidoidea
Pemphigidae
Anoeciidae
Hormaphididae
Mindaridae
Thelaxidae
Drepanosiphidae
Phloeomyzidae
Greenideidae
Aphididae
Lachnidae

They damage the plants by sucking the sap. The plant is weaken, its development is reduced and infested young leaves may be deformed later (BIOBEST, 2012). By sucking, aphids can transmit over 100 different viruses (GIORDANENGO *et al.*, 2010), such as potato virus Y (INRA, 2008) or barley yellow dwarf virus (BYDV) (INRA, 2008). Furthermore, an excess of sugar is secreted as honeydew on the plant which, in turns, favors the infestation of fungi (BIOBEST, 2012). Most aphids found in the greenhouses are the cotton aphid (*Aphis gossypii*), the green peach aphid (*Myzus persicae*), the potato aphid (*Macrosiphum euphorbiae*), the glasshouse potato aphid (*Aulacorthum solani*) (MURPHY & FERGUSON, 2006; BIOBEST, 2012) and the tobacco peach aphid (*Myzus nicotianae*) (BIOBEST, 2012). *Myzus persicae* is the one used in the following experiments.

Figure 8: *Myzus persicae* (De Backer L.)

Myzus persicae Sulzer [Hemiptera: Aphididae], is a 1,2 to 2,6 mm green aphid. The colour varies a lot, from yellow-green to green, some are even red (BIOBEST, 2012). The winged adults are bigger than the wingless. They have a black thorax, a green abdomen with a black patch and four translucent wings (FRAVAL, 1997; CAPINERA, 2005). The life cycle, shown in figure 9, varies considerably depending on the temperatures. The population growth can be very fast (CAPINERA, 2005; BIOBEST, 2012), often 10 to 12 days for a complete generation and with more than 20 generations a year (CAPINERA, 2005). The green peach aphid usually crosses the winter as an egg (1 on figure 9) in its winter host, *Prunus* spp., or in the greenhouses (BIOBEST, 2012). The eggs are yellow or green and become black as they develop (CAPINERA, 2005; KUHAR *et al*., 2009). In the spring, the eggs hatch and the nymphs (2 on figure 9) feed on phloem from flowers, young foliage, and stems (CAPINERA, 2005). They are green just like the adult, pinkish sometimes (KUHAR *et al*., 2009), viviparous and reproduce by parthenogenesis (FRAVAL, 1997; CAPINERA, 2005). A wingless female can deposit up to 80eggs but the reproduction rate varies with the temperature with a threshold at about 4,3°C (CAPINERA, 2005). Three generations later (2, 3 and 4 on figure 9) (FRAVAL, 1997), winged adults (5 on figure 9) appeared and deposit eggs on the secondary host (CAPINERA, 2005). The primary host is free from aphids from the middle of May or early June depending on the temperatures (FRAVAL, 1997). Several generations of wingless and winged aphids occur in the secondary host. In Autumn, as days shorten and temperatures are getting colder, winged males (8 on figure 9) are produced by sexuparous females (7 on figure 9). At the same time, some winged sexuparae (7 on figure 9) return on the winter host and give birth to egg-laying apterous forms (8 on figure 9). Males, attracted by the pheromone, come back on the primary host where they mate with several oviparous females (CAPINERA, 2005). Pinkish eggs (1 on figure 9) (KUHAR *et al*., 2009) are deposited near the buds (FRAVAL, 1997; KUHAR *et al*., 2009). The adults can survive 3 month at 5°C but only 10 days at 25°C (FRAVAL, 1997).

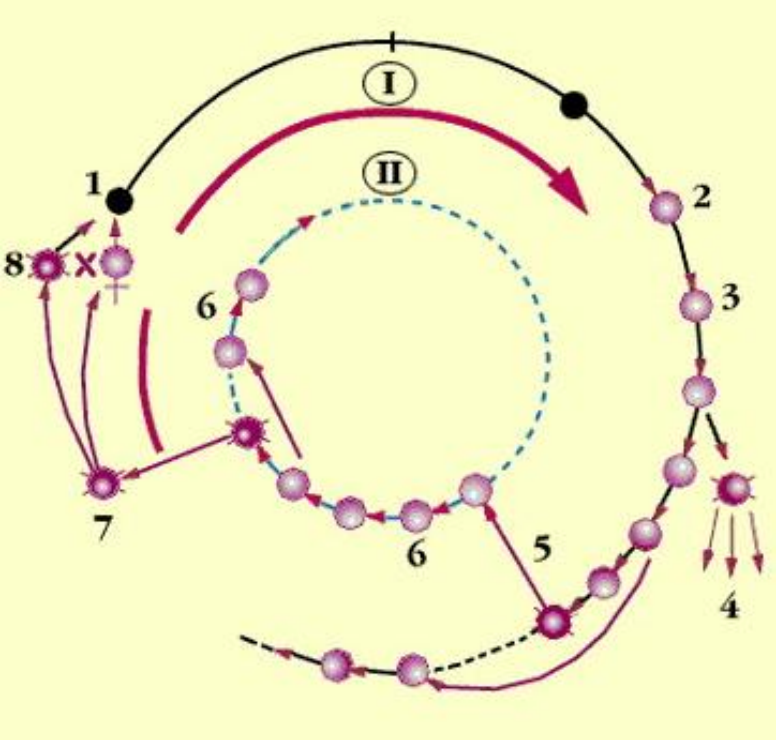


Figure 9:Life cycle diagram of Myzus persicae (FRAVAL, 1997). The aphid alternate between Prunus spp. (I) and secondary host (II), annual herbaceous plants. 1: diapausing egg; 2: viviparous, virginiparous, apterous fundatrix; 3: fundatrigeniae; 4: winged virgino parae, ensuring dissemination; 5: winged virginoparae migration to the secondary host; 6: winged and apterous virginoparae; 7: winged sexuparae; 8: sexual forms, winged males and oviparous apterous females.

The parthenogenesis allows them to increase at a high rate and confers them a shortened pre-reproductive time (GIORDANENGO *et al.*, 2010) making them very quickly uncontrollable by biocontrol agents in a curative way. Moreover, the winged adults colonize new hosts while the wingless invest their resources in reproduction (GIORDANENGO *et al.*, 2010). *M. persicae* is a phloem feeder. It uses the semiochemicals to select its hosts before landing. A first superficial stylet insertion helps it to decide if the host is suitable or if it has to search another one. If the host is accepted, the aphid initiates the feeding (CHAMBERLAIN *et al.*, 2001; GIORDANENGO *et al.*, 2010). It inserts its stylet between the cells of the plant tissues to reach the phloem (GORDON RAMEL, 2010). This sap is very rich in carbohydrates and very low in protein. To balance these supplies, the excess of carbohydrates is excreted as honeydew (VOECKEL *et al.*, 2004). *M. persicae* is found mainly on the upper leaves (ATHANASSIOU *et al.*, 2003) because the nitrogen content is higher in the young leaves. However, on a lettuce the aphids are mostly distributed over the oldest leaves (EENINK & DIELEMAN, 1977). It also prefers to settle on the abaxial leaf surface (CALABRESE & EDWARDS, 1976; EENINK & DIELEMAN, 1977). This behaviour is among others guided by light and gravity during daylight and by gravity only during darkness (CALABRESE & EDWARDS, 1976).

Several biological control agents are already commercially available including *Adalia bipunctata* [Coleoptera: Coccinelidae], *Aphidoletes aphidomyzae* [Hymenoptera: Aphididae],

Aphidius ervi [Hymenoptera: Aphididae], *Aphidius colemani* [Hymenoptera: Aphididae] and *Aphelinus abdominalis* [Hymenoptera: Aphididae] (BIOBEST, 2012). The lady beetle is a predator while the others are parasitoids.

The limits

The main bottleneck in the use of biological agent against aphids lies in the food. A predator has to find food to persist when the pests are getting scarce; and a parasitoid requires sugar sources, such as floral nectar (PORTILLO *et al*., 2012; BARBERCHACK, 2011). In the commercial crops, grown as monocultures, these sugar sources are often lacking, undermining the effectiveness of biocontrol. The dicyphine *Macrolophus pygmaeus* [Heteroptera: Miridae], already identified as aphid predator, offers a solution to that sugar source issue. Indeed, it can obtain carbohydrates and other nutrients by feeding on plant tissues directly. This ability makes it independent of nectar availability and of pests decreasing.

Macrolophus pygmaeus

Macrolophus pygmaeus [Heteroptera: Miridea] is a highly polyphagous predator originating in the Mediterranean. It is a 2 to 4mm green bug, with red eyes, piercing-sucking mouthparts and green antennae with a black base (BIOBEST, 2012). Its long legs allow it to move quickly even on hairy leaves (OMYA AG AGRO, 1998). The females are bigger than the male and have an ovipositor on the abdomen which helps to differentiate them easily. Three days after copulation the female deposits her eggs with her ovipositor. They are deeply buried in the tissue of the leaf, vein or stalk making them unavailable and invisible. At 25°C nymphs are born after 11 days. There are 5 nymphal stages. During the first stage nymphs are yellowish green, but older nymphs are green like the adults. The five stages last 19 days and the female adult live 40days. The males live a bit longer (BIOBEST, 2012). The development takes more time when the temperature decreases (MOHD RASDI *et al.*, 2009; BIOBEST, 2012). *M. pygmaeus* survives longer at 15°C but its fertility is higher at 20°C (PERDIKIS & LYKOURESSIS, 2004). The duration also varies with the amount of food available (BIOBEST, 2012). For instance, feeding on whiteflies, it can complete its development cycle in 22days (WHEELER A.G. JR, 2001).

The life of the adult females is divided into three periods: the pre-oviposition, from the emergence to the first egg laid; the oviposition and the post-oviposition, from the last egg laid to the death. The mating usually occurs about three days after the emergence and the first egg is laid three days after the mating (FAUVEL *et al.*, 1987). *Macrolophus pygmaeus* is monogame. Females accept to mate after several contacts. The males began to pursuit her actively and one of them is able to mate with her. The copula lasts 4,1 minutes on average (FRANCO *et al*., 2010). After that, females become reluctant to mate again (GEMENO *et al*., 2007) and run away from the males or reject them by moving their abdomen from side to side. The sperm stays in the ovarioles of the female making her able to lay eggs during her whole life (FRANCO *et al*., 2010). If the oviposition doesn't start within 4 to 5 days after the mating, generally, it doesn't occur at all. The fertility depends on the temperature, the host plant and the diet. In the investigation of FAUVEL *et al*.on *Macrolophus caliginosus,* 51 eggs were laid

in 30 days feeding on whiteflies on brinjal plants. BERENGERE *et al.* reported that macrolophus caliginosus lays 3eggs per day on tobacco plant (FAUVEL *et al.*, 1987).

The larvae and the adult are generalist predators (OMYA AG AGRO, 1998) feeding on several soft-bodied pest such as aphids, mites, trips and moth eggs (ALBAJES & ALOMAR, 2002; PERDIKIS, *et al.*, 2008). When there are various preys, it tends to eat different ones (MOHD RASDI *et al.*, 2009). Although, it clearly prefers whiteflies, *Trialeurodes vaporariorum* [Homoptera: Aleyrodidae] as well its eggs as its larvae or pupae (BIOBEST, 2012). An adult can eat on average 6 whitefly larvae in a day (MOHD RASDI *et al.*, 2009) and suck empty about 40 to 50 eggs (BIOBEST, 2012). The predator injects its stylet into the egg, larvae or pupae to suck it, leaving a small hole making the predate prey recognizable (PERDIKIS *et al.*, 2008; MOHD RASDI *et al.*, 2009; BIOBEST, 2012). The plant where the prey has been reared influences the predation rate; for instance brinjal produces better preys than tomatoes (MOHD RASDI Z. *et al.*, 2009).

An important issue of the information research in the literature about *Macrolophus pygmaeus* is the confusion of the two species *caliginosus* and *pygmaeus.* Many studies confuse the two species (WHEELER, 2001). *M. pygmaeus* and *M. caliginosus* are closely related, separated only by the different colour pattern on the first antennal segment. *M. caliginosus* presents a first antennal segment with a white central band and *M. pygmaeus* first antennal segment is entirely black (PERDIKIS *et al.*, 2003). PERDIKIS *et al* proved that *Macrolopus pygmaeus* is the predator usually commercialised and used for biocontrol. Although, the literature gives more information about the use of *Macrolophus caliginosus* as biocontrol agent. The "Macrolophus-system" produced by Biobest and used in this trial is made of *Marolophus pygmaeus* although it is described as *M. caliginosus*. Actually, *M. caliginosus* has become the synonym of *M. melanotoma* (WHEELER, 2001).Therefore, the conclusions established in the literature about *M. caliginosus* are supposed to be transposable to *M. pygmaeus*.

Figure 10 : Black first antennal segment of *Macrolophus pygmaeus* (De Backer L.)

As biocontrol agent, *Macrolophus pygmaeus*, has proven to be effective in controlling whiteflies *Trialeurodes vaporariorum* [Homoptera: Aleyrodidae] (MOHD RASDI *et al.*, 2009), *Bemisia tabaci* (INRA, 2010) and the leafminer *Tuta absoluta* [Lepidoptera: Gelechiidae] in greenhouses (BIOBEST, 2012). The predator is commercialised by Biobest and other companies to control the whiteflies. It is used by inoculation or inundative releases. Its effectiveness can be improved by providing him some alternative food such as *Ephestia kuehniella* eggs (PERDIKIS, *et al.*, 2008). In prevention, it is recommended to rear *Macrolophus pygmaeus* for two months before the critical period to build up a good population. It is thus recommended to introduce the predator twice around February and at minimum 0,5 *Mcrolophus*/m². In curative applications, 0,5 to 1 predators have to be released twice to four times on the crops. In the whiteflies hotspots, a release of 5 to 10 predators is also necessary (BIOBEST, 2012).

As mentioned earlier, the zoophytophagous predator *Macrolophus pygmaeus* offers the advantage of obtaining carbohydrates and other nutrients by feeding directly on plant tissues making it independent of nectar and prey availability (PORTILLO *et al*., 2012). *M. pygmaeus* can complete its development and reproduce at low levels when only feeding on stem and leaves (PORTILLO *et al*., 2012) from cultivated plants such as tomato, eggplant, cucumber or pepper (LYKOURESSIS *et al.* 2007). A study shows that it can survive 3,67 days without any food and 25,67 days feeding on plant only (MOHD RASDI *et al.*, 2009). The predator develops better while feeding on prey, and its performances depend on the plant he's feeding on. On some plants, females did not oviposit at all (PERDIKIS & LYKOURESSIS, 2004). For instance, *M. pygmaeus* can increase in number on *Solanum nigrum*, commonly named black nightshade, but performs poorly on *Dittrichia viscosa*, also known as false yellowhead or woody fleabane. These plants are non-cultivated but can be planted next to the crop to increase *M. pygmaeus* survival when preys are decreasing in the crop (LYKOURESSIS *et al.* 2007). Occasionally, it can cause crop damage when few or no prey are available or high population of *Macrolophus* occurs, like hundreds of individuals on the entire plant or 50 individuals in the top of the plant. Some crops and varieties are more sensitive such as cherry tomatoes and small-truss tomato (BIOBEST, 2012).

Macrolophus pygmaeus can complete its development while feeding only on certain aphid species. For instance, *Aphis Solanella*, *Aphis gossypii*, *Macrosiphum euphorbiae* and *Myzus persicae* allow it to finish its development in a similar period. However *Myzus persicae* allows it to achieve a better longevity and fertility (LYKOURESSIS *et al.* 2008) and the predation rate is higher on it than on *M. euphorbiae* (LYKOURESSIS *et al.* 2007). A study showed that *M. pygmaeus* performs very well when it feeds on *M. persicae* on pepper plant. At 15°C males survived 132,9 days and females 120,7days (PERDIKIS & LYKOURESSIS, 2004). In the field, females live 110 days on average at 15°C, and males a bit longer (BIOBEST, 2012). Nevertheless, fewer eggs are laid when only aphids are eaten (BIOBEST, 2012). The predator shows a type II functional response when feeding on *Myzus persicae*. The predation rate of the predator increases with increasing prey density. *Macrolophus pygmaeus* prefers the aphid first instars probably to maximize the net nutrionnal gain as suggested by the optimal foraging theory (FANTINOU, *et al.*, 2009).

Figure 11 : *Macrolophus pygmaeus* first instars (De Backer L.).

Little information is available regarding the effectiveness of *Macrolophus pygmaeus* to keep the aphids under the economic threshold (WHEELER, 2001). However, a study made on chrysanthemums infested by the chrysanthemum aphid, *Macrosiphoniella sanborni*, proved that *Macrolophus costalis* was able to reduce the aphids at predator-prey ratio of 1:2 or 1:3 and was more effective at higher prey densities (WHEELER, 2001). *M. costalis* has been proposed as biocontrol agent against *Myzus pesicae* by DIRIMANOV M. & DIMITROV A. (1975) but no information about this practice has been reported (FAUVEL *et al.*, 1987).

A few studies have been made to evaluate the sustainability *Macrolophus caliginosus* when feeding only on aphids. The confusion between the two taxa causes some doubts about the specie really used in the studies. FAUVEL G. *et al.* proved that *Macrolophus caliginosus* is unable to finish its development when feeding on *Aphis fabae, Aphis craccivora* or *Macrosyphum euphorbiae*. A 95% mortality of the first instars is observed in the first 24hours. However, *Myzus persicae* and *Aulacorthum circumflexum* make the development of *M. caliginosus* possible. While feeding on *Myzus persicae*, the predator larvae development lasts 27 days. That is longer than when it is feeding on whiteflies larvae (26 days) but faster than when feeding on whiteflies eggs (29,4 days). The fertility of the predator is better when it feeds on *M. persicae* (60eggs by female) than on *Aphis gossypii* (35 eggs by female) as shown on the graph 1. (FAUVEL *et al.*, 1987). In field conditions, female deposits 100 to 250 eggs on average (OMYA AG AGRO, 1998; BIOBEST, 2012).

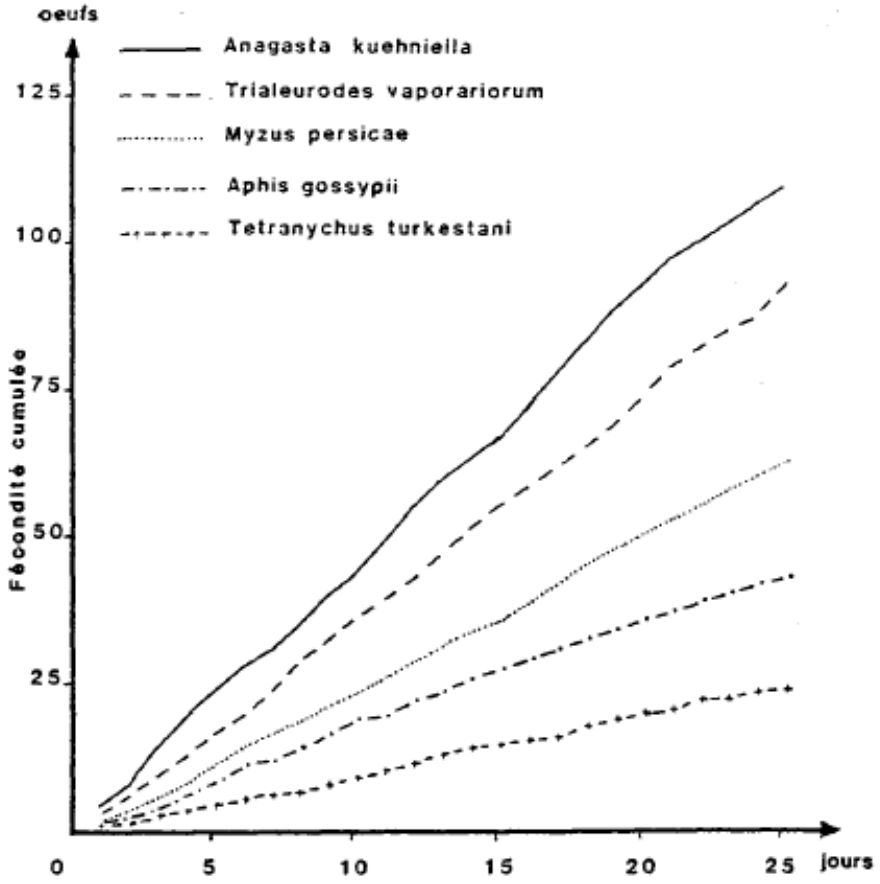


Figure 12: Effect of the prey specie on the fertility of *M. caliginosus* (FAUVEL *et al.*, 1987).

M. persicae has a good nutritional value making it a prey as suitable as *T. vaporariorum* (FAUVEL *et al.*, 1987). However, *Macrolophus caliginosus* presents a survival equal to 64,3% while feeding on *Aphis gossypii* and to 71,4 while feeding on *Macrosiphum euphorbiae* for 24hours. The fifth instar larvae was able to eat on average 10 *Aphis gossypii* and 13,2 *Macrosiphum euphoribae* while the adult can eat respectively 30,9 and 36,5 (ALVARADO *et al.*, 1997).

A biocontrol agent has to present several abilities to be effective. First, it must be able to detect and attack the prey. Olfactometric bioassays showed that the *Macrolophus pygmaeus* females are attracted to whitefly infested tomato, but that whiteflies alone do not attract the predator neither *Myzus persicae* (INGEGNO *et al.*, 2011). However, *Macrolophus pygmaeus* is identified as an aphid predator. The agent cannot interfere with the natural enemies of the aphids. A study about *M. caliginosus* proved that it seems to be more often the prey than the predator. The most dangerous aphid predators for *M. caliginosus* are the *Nabis ferus*, *Nabis mirmecoides* and *Coccinella septempunctata* with a predation rate reaching 80% (LUCAS & ALOMAR, 2000). The predator must stay unchanged after mass rearing (VERHEGGEN, 2011). As *Macrolophus pygmaeus* is already commercialized, it is given that the mass rearing is possible and let the predator efficient. Furthermore, the main inconveniences of the biocontrol agents are the transport and the mass rearing, the effectiveness, the agent specificity and the action delay (VERHEGGEN, 2011). For *Macrolophus pygmaeus*, the transport and the rearing

have already been studied and provide good results. It is not specific, making it more persistent than other predators. Its plant-feeding behaviour provides it carbohydrates and allows it to survive when preys are scarce (ATHANASSIOU *et al*., 2003). But its specificity may also make it less effective. The response for its effectiveness and action delay on aphid biocontrol will be given by the experiments.

Figure 13 :*Macrolophus pygmaeus* (De Backer L.)

Plant defences

The second part of this study is about induced plant defences. As the plants are sedentary, they have developed some strategies to defend themselves against the biotic attacks (KARBAN & BALDWIN, 1997). The defences can be classified depending on different characteristics. One of these classifications divided them in two groups; the constitutive and the inducible (GIORDANENGO *et al.*, 2010).

Constitutive defences

The constitutive defences are every device present all the time on the plant, like spines, trichomes, waxes, cuttins or suberins. These would spend too much energy to set during an attack (VERHEGGEN, 2011). For instance, caterpillars chewing the plant are bursting vacuoles allowing the release of a compound that will make contact with the appropriate enzyme. The

enzyme will degrade this substrate and make it toxic like isothyocyanate in brassicaceous plants (BRUCE & PICKETT, 2007).

Induced plant defences

The induced defences are set only during an attack of an insect, a fungus or an animal and usually are chemical defences (VERHEGGEN, 2011) like proteinase inhibitors, toxins (BRUCE & PICKETT, 2007) or volatile compounds repelling the pest (GIORDANENGO *et al.*, 2010; ANDERSON *et al.*, 2011) or attracting its predator (BRUCE & PICKETT, 2007). The organic volatile compounds varying quantatively and qualitatively depending on plant species and pest species (INGEGNO *et al.*, 2011). The plant will react differently if attacked by a leaf-chewer, a phloem feeder or a single-cell feeder (MAFFEI, 2010) or even by more than one organism (BRUCE & PICKETT, 2007). These compounds present intraspecific variations too (RAGHAVA *et al*., 2010). The response occurs at the damaged site but also at some distance from the site (KARBAN & BALDWIN, 1997). A below-ground attack can thus stimulate an above-ground response by a systemic induction (BRUCE & PICKETT, 2007). The volatile organic compounds are issued from the secondary metabolism.

It takes several main steps to induce the defences. The first step is the recognition of the attacker by some physical and chemical signals. Then a signal is emitted inducing the expression of several specific genes responsible for the defences (BENHAMOU & PICARD, 1999; ARIMURA *et al.*, 2005).

Elicitors are extracellular signals allowing the recognition of the attacker. They are present in the oral secretions, saliva or regurgitates of the organism. For instance, the volicitin (N-(17-hydroxylinolenoyl)-L-glutamine), a fatty acid-amino acid conjugate found in the oral secretions of *Spodoptera exigua,* is the main elicitor of the herbivore-induced plant volatiles (HIPVs) in maize plants (ARIMURA *et al.,* 2005; GIORDANENGO *et al.*, 2010) and the β-glucosidase is the elicitor from *Pieris brassicae* (GIORDANENGO *et al.*, 2010). The wounding is also important and can elicit a response alone. Indeed the herbivore bite causes a depolarization of the membrane potential and an intracellular calcium influx. However, when the leaf is mechanically wounded, a depolarization occurs but without the calcium influx. The response elicited will be different already at this early step (ARIMURA *et al.,* 2005). The plant can also recognise its own molecules altered by a bioagressor as an elicitor. For instance, *Spodoptera frugiperda* caterpillars infesting *Vigna unguiculata* ingest chloroplastic ATP synthase γ-subunit proteins and regurgitate them in the form of peptides eliciting the defence response (GIORDANENGO *et al.,* 2010). However, there is still much to be learned about the mechanisms used to differentiate the attacker (BRUCE & PICKETT, 2007).

The initial signal eliciting the defences is highly specific to the attacker (BRUCE & PICKETT, 2007). Nevertheless the early responses to different biotic attacks share common events such as protein phosphorylation, membrane depolarization, calcium influx and release of reactive oxygen species (such as H_2O_2) (GIORDANENGO *et al.*, 2010). The subsequent signalling, resulting in a cascade of reactions (ARIMURA *et al.*, 2005), and the gene expression response present similarities too (BRUCE & PICKETT, 2007). The salicylic acid (SA), the jasmonic acid

(JA) the ethylene (ET) and abscissic acid (ABA) pathway are differently involved in the responses. The involving can be different by the pathways used but also by the proportions of the compounds produced by each pathway. For instance, chewing insect attacks mainly involve the JA-pathway while spider mites involve the JA-pathway as well as the SA-pathway (BRUCE & PICKETT, 2007). Some enzymes and proteins intervening in the cascades have been identified such as the mitogen-activated protein kinase (MAPK) starting the MAPK cascade. Its transcripts begin to accumulate in the leaves one minute only after the wounding. This protein kinase (WIPK) is considered as essential for the JA induced responses. WIPK has also been reported to elicit the transcription of a gene for a fatty acid desaturase catalysing the conversion of linoleic acid into linolenic acid which is a precursor of JA (CHAMBERLAIN *et al*., 2001).

These different mechanisms and pathways end up to the activation of different genes and to the production of the herbivore-induced plant volatiles (HIPV's). They belong to the group of terpenoids (homo-, mono-, di-, sesquiterpenoids), fatty acids, phenylpropanoid aromatic compounds (like methyl salicylate, MeSA (CHAMBERLAIN *et al*., 2001), and indole), as well as certain alkanes, alkenes, alcohols, esters, aldehydes, and ketones (MAFFEI, 2010). These compounds are emitted as a complex blend specific to the attacker species. The emissions are repelling the herbivores but they also can attract their natural enemies (DICKE, 2009).

The phloem feeders with piercing and sucking mouthparts like aphids do less structural damage to the plant (BRUCE & PICKETT, 2007). They insert their flexible stylet between the cells up to the sieve tubes. However, they cause alterations to their host such as morphological changes, modified resource allocation and local as well as systemic symptoms (GIORDANENGO *et al.*, 2010). The responses present more similarities with the pathogen elicited responses than with the chewing insect elicited responses (BRUCE & PICKETT, 2007). In aphids, the oligogalacturonides released after being hydrolysed by the salivary secretions during the stylet penetration are known to induce the plant defences but no elicitors have been formerly identified. They are known to activate the SA pathway and reduce the expression of the JA pathway (GIORDANENGO *et al.*, 2010). DE VOS *et al.* (2006) compared the genes expression responses of *Arabidopsis thaliana* elicited by five attacker types; a pathogenic bacterium (*Pseudomonas syringae)*, a pathogenic fungus (*Alternaria Brassicola*), a chewing lepidopteran larva (*Pieris rapae*), a cell feeder thrips *(Frankliniella occidentalis*) and *Myzus persicae*. It showed that the aphids induced the largest number of changes in gene expression but didn't induce the largest production levels of SA, JA or ET. Another trial has been led to investigate the impact of *Myzus persicae* on the direct and indirect defences of the cotton plant *Gossypium arboreum*. It turned out that the aphids decreased the extrafloral nectar secretion (URBANUS, VAN RIJN & WÄCKERS). This is in contrast with the strong induction of foliar nectar secretion in response to caterpillar feeding to attract their predators (ARIMURA *et al.*, 2005). The foliar terpenoid levels were also increasing. The plant can also react to a phloem feeder attack by producing some semiochemicals attracting its natural enemies. A study led on *Vicia faba*, shows that the parasitoid *Aphidius ervi* was attracted to the aphid previously infested plants up to 24h after the aphids had been removed. It can even

discriminate two different aphid species by the semiochemicals induced by aphid feeding (CHAMBERLAIN *et al*., 2001).

The plants responding to an attack may do less well than plants that do not respond. Indeed one of the best documented physiological responses to leaf damage is the increased rate of photosynthesis in the undamaged leaves of a damaged plant. This increase is frequently associated with an increasing of photosynthetic pigments and ribulose-1,5-biphosphate carboxylase-oxygenase. Unfortunately, RuBPCase increases the nutritional quality of the leaf for herbivores (KARBAN & BALDWIN, 1997).

Herbivore adaptations

On the other hand, the plant defence response can be suppressed by specialised attacking organisms. These organisms can produce some metabolites such as detoxifying enzymes or their own toxins that interfere with the plant metabolism. They can also produce some signals to disrupt the signalling pathways of the plant (BRUCE & PICKETT, 2007). The aphids are part of these organisms. As they are phloem feeders, they need time to access the sieve tubes and ingest sufficient amount of nutrients. To get that time they have to avoid the plant defences.

Figure 14 : *Myzus persicae* feeding on a plant (De Backer L.)

During their feeding they continuously inject gelling saliva to lubricate the stylet and seal the punctured cells. Watery saliva is injected in the plant cell cytosol by the punctures, mixes with the cytosol and is quickly reingested. This mechanism allows the aphids to locate the stylet in the plant tissues. Watery saliva is also injected in the sieve tubes before the phloem ingestion. The saliva is probably responsible for the defence counteracting. A common pattern in the gelling saliva composition can be found between different aphid species whereas the

watery saliva composition strongly differs between species. Proteins; such as phenoloxidases, peroxidises, pectinases and β glucosidase ; phospholipids and conjugate carbohydrates composed the gelling saliva. The watery saliva composition is more complex and includes among other components enzymes such as pectinase, pectinmethylesteras and polygalacturonase. The saliva presents some antagonist effects to the plant defences. The toxic phenolics released by the damaged tissues are absorbed with the saliva, and converted by oxidases in less toxic components. The glucose-oxydase found in the saliva of *Myzus persicae* can reroute the plant defences and lead to a weak JA pathway response. Several ways to do that are reported. The glucose oxidase can oxidize D-glucose and release H_2O_2 stimulating SA accumulation. SA and JA pathways are reported to be antagonists. They are also reported to have none interactions therefore the plant defences would be increasing. The oxidase also interferes with the early step of the response to a wound leading the biosynthesis of anti-insect secondary metabolism. It is reported as a potent inhibitor of lypoxygenase activity inhibiting the JA production. Normally when a sieve tube is injured, the plant immediately occludes it to prevent sap loss. This mechanism is activated by a sudden influx of Ca^{2+} ions inducing protein coagulation. Stylet penetration does not modify the sap flow. Indeed, at least two proteins of the watery saliva have calcium-binding properties, reducing the availability of the Ca^{2+} ions. As the gelling saliva attends to seal the punctures, it could seal a sieve tube. When the stylet reaches the phloem, an intense secretion of watery saliva begins to compete with the gelling saliva (GIORDANENGO *et al.*, 2010).

The aphids are able to manipulate the phloem to improve the plant suitability (VOECKEL *et al.*, 2004).The salivary secretions are also responsible for this change. The reason for this manipulation is that the phloem does not contain enough amount of nitrogen to fill aphid needs. The lack of nitrogen is partly compensated by an endosymbiotic bacterium *Buchnera aphidicola* but also by the alteration of the plant to adapt the phloem composition to the aphid requirement. On potato plant, *Myzus persicae* strongly increases glutamine synthase and glutamate dehydrogenase activities even in distant leaves. These enzymes are involved in the amino acid transport and ammonium from protein catabolism releasing. The aphids are taking advantage of this increasing translocation of nitrogen. (GIORDANENGO *et al.*, 2010).

As the aphids are making the plant more suitable, their reproduction rate may increase. Indeed, a trial investigates the behaviour of a second aphid infestation on a peach cultivar. It turns out that the female aphid larviposition was slightly enhanced by a 48h pre-infestation of *M. persicae*. That behaviour has been monitored for 8h. On preinfested plants, the aphids produced less sieve element salivation and more continuous sap ingestion than on uninfested plant (SAUGE *et al.*, 2002).

Herbivore predator inducing defences

Usually for the biocontrol, the plant defences are used to attract the natural enemies of the pest. In this case, the use of the predator *Macrolophus pygmaeus* as an inducer of the plant defences to act negatively on aphids will be tested. As *M. pygmaeus* can feed on leaf and stem, it could induce the same reactions as a plant pest. This type of relation, between the predator and the plant, must be investigated and may improve the biocontrol (PORTILLO *et al.*,

2012). Indeed, if some compounds are produced after *M. pygmaeus* fed on the plant they could repel the predator but also the aphids. For instance, among the responses, methyl salicylate is directly related to the SA-pathway, which is activated by phloem feeder attacks. It is also proven that aphids are repelled by this compound (CHAMBERLAIN *et al*., 2001). The plant could thus be protected by two effects: a direct one, whereby the predator feeds on aphids, and an indirect one: whereby its plant feeding can induce the production of some repellent compounds. These two mechanisms are being investigated in this study.

Objectives

The global aim of this study is to evaluate *Macrolophus pygmaeus* as biocontrol agent against aphids.

The first specific objective is to investigate the predation behaviour of *Macrolophus pygmaeus* on aphids. A first experience will evaluate the effectiveness of *Macrolophus pygmaeus* on controlling aphids when both are present on the plant and also when an alternative diet is present. Through a second trial, we will evaluate the effectiveness of the control depending on the density of *Macrolophus pygmaeus.*

The second specific objective is to investigate the ability of *Macrolophus pygmaeus* to stimulate the natural defence of the aphid host plant. This ability may improve the results of the biocontrol.

Figure 15: *Macrolophus pygmaeus* (De Backer L.).

Section I: "*Macrolophus pygmaeus* as an efficient aphid predator?"

Macrolophus pygmaeus is not used as a biocontrol agent against aphids even though it is often considered as an aphid predator. Indeed, *M. pygmaeus* seems to be effective under strict conditions that remain unknown. For a producer, it is important to know how many predators are needed to control an aphid infestation. Moreover, they need to know if their predator populations obtained after laboratory rearing are sufficient to control aphids or if another biocontrol agent has to be introduced.

The following experiments aim to evaluate the ability of *Macrolophus pygmaeus* to efficiently control an aphid population. To do so, three experiments have been conducted :

(1) **Preventive application of *M. pygmaeus* and second diet influence**: The first experiment has been conducted to demonstrate the ability of *M. pygmaeus* to reduce the fitness of an aphid colony. This qualitative experiment allowed us to follow the aphid population dynamic in presence or absence of *M. pygmaeus*. We also have evaluated the impact of the presence of an alternative diet on aphid predation by *M. pygmaeus*.

(2) **Curative application of *M. pygmaeus* on small aphid colonies**: In a second set of experiment, we have determined the predator density needed to control a small aphid colony, mimicking an accidental introduction of aphids in a greenhouse.

(3) **Curative application of *M. pygmaeus* on large aphid colonies:** The aim of this last experiment of this section is to evaluate the effectiveness of *M. pygmaeus* on higher aphid densities.

Figure 16: *Macrolophus pygmaeus* (De Baker L.)

Material and method

All three experiments have been conducted under daylight in large cages (2,5m; 1,5m; 2m) spread into two different greenhouses. Each large cage contained 4 bell pepper plants (*Capsicum anuum*) (Figure 17). All plants were 60 cm high and had 25 true leaves on average. Temperature and humidity have been recorded by a data logger (Lascar Electronics, EL-USB 2). The temperature in the greenhouses was 24,79°C ± 3,25°C and 25,40°C ± 3,44°C. The humidity was 41,11 % rh ± 7,68 % and 37,86% rh ± 7,49% rh .

Figure 17 : Big cages used and disposition of the bell peppers.

Preventive application of *M. pygmaeus* and second diet influence

This experiment aimed to demonstrate the ability of *M. pygmaeus* to reduce the fitness of an aphid colony when applied in a prevention way. It took place from February 13rd to March 22nd

The predators *M. pygmaeus* were reared for 4 weeks in the large cages used for the trial (Figure 17). Then, herbivore pests (the peach aphid, *Myzus persicae*) were added in the cages. To evaluate the impact of alternative diet on the efficiency of *M. pygmaeus* as an aphid predator, additional replicates were conducted where either a second herbivorous insect pest, *Trialeurodes vaporariorum*, or a second source of food, *Ephestia kuehniella* eggs, were added.

A control has been realized where bell peppers were infested by aphids and were left in absence of predators. In total, four different treatments are applied. They are summarized on table 2, along with their respective codenames, that will be referred to in the following text.

Table 2: Codenames of the different treatments.

Treatments	Cages
Myzus persicae	Mzp
M. pygmaeus + M. persicae	Mp + Mzp
M. pygmaeus + M. persicae + Ephestia eggs	Mp + Mzp + Ek
M. pygmaeus + M. persicae + Trialeurodes vaporariorum	Mp + Mzp + Tv

Each treatment was repeated four times. A total of 16 cages were used with four plants in each, for a total of 64 plants, spread in 2 greenhouses, with eight cages in each. The 4 treatments are arranged as shown on the table 3.

Table 3: Arrangement of the cages in the greenhouses.

4 Mp + Mzp	4 Mp + Mzp + Ek	4 Mp + Mzp + Tv	4 Mzp
3 Mp + Mzp	3 Mp + Mzp + Ek	3 Mp + Mzp + Tv	3 Mzp
2 Mp + Mzp	2 Mp + Mzp + Ek	2 Mp + Mzp + Tv	2 Mzp
1 Mp + Mzp	1 Mp + Mzp + Ek	1 Mp + Mzp + Tv	1 Mzp
Greenhouse 17		Greenhouse 15	

Adult *Macrolophus pygmaeus* predators were provided by Biobest "Macrolophus-System". They are reared on tobacco plants (*Nicotiana tabacum*) before being packaged. They were sexed based on the physical difference between males and females. The females have an ovipositor making the abdomen looking bigger. Five females and five males are released in the cages. The predators are fed every week with a food mix called "Nutrimac" made of pyralid *Ephestia kuehniella* (Zeller) eggs and *Artemia* sp. cysts, produced by Biobest. The *Artemia* sp.cysts are used to avoid the agglutination of *Ephestia* eggs. Water was sprayed over the plants and the eggs were poured on the top of the plants. The water makes the eggs stayed on the leaves.

Five winged and five wingless adult *Myzus persicae* aphids were added 4 weeks after the predator introduction in the cages. In the "Mp + Mzp + Ek" cages, a few *Ephestia* eggs are poured on the top of the leaves twice a week. Ten unsexed adult whiteflies were added in each "Mp + Mzp + Tv" cage. Twice a week, aphids, whiteflies and *M. pygmaeus* were counted in each cage.

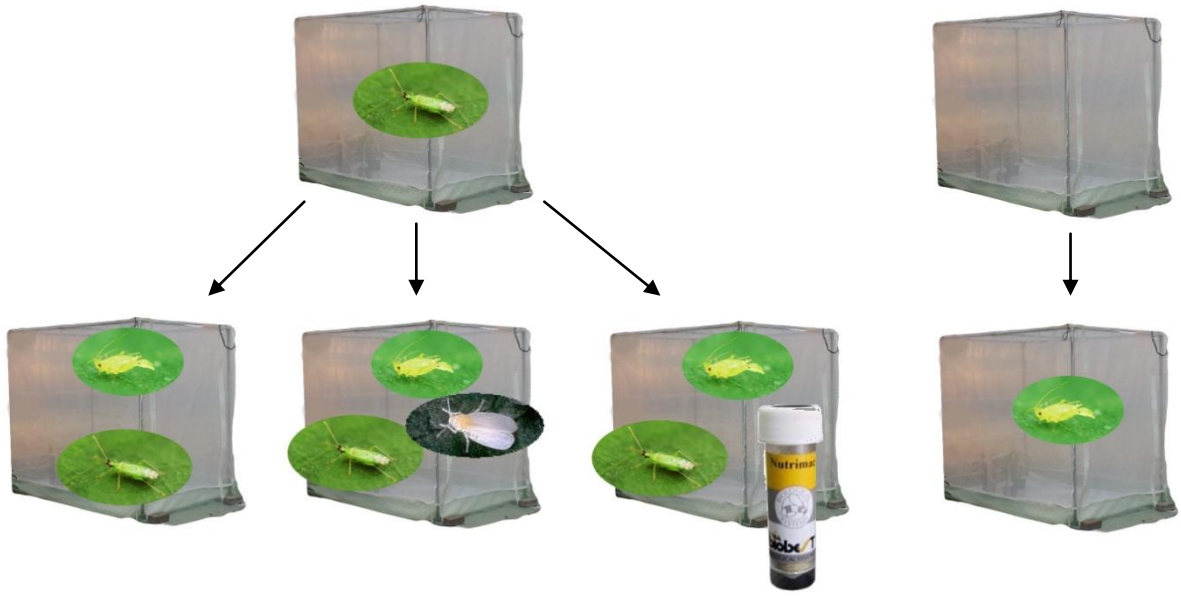


Figure 18: Experiment procedure.

Curative application of *M. pygmaeus* on small aphid colonies

This experiment tests the effectiveness of different predator densities on determined aphid density mimicking a natural introduction of aphids in a greenhouse. This time, the predators will be applied in a curative way.

It took place from April 25th to May 15th. Sixteen big cages containing four pepper plants (*Capsicum anuum*) were used.

Three different densities of *M. pygmaeus* were tested and compared to a control, thus four different densities were applied as shown in the table 4.

Table 4: Different densities of *Macrolophus pygmaeus* tested and number of predators and aphid per cage corresponding.

Macrolophus pygmaeus density	Macrolophus pygmaeus per cage	Myzus persicae per cage
0 / plant	0	10
4 / plant	16	
8 / plant	32	
12 / plant	48	

Four repetitions were made. *M. pygmaeus* predators were collected with a sucking device (Figure 19) from a laboratory rearing. *M. persicae* aphids were collected with a paintbrush from a laboratory rearing on bell pepper. Five winglets and five apterous were put in the same tube. In total 160 aphids. Ten aphid and 0, 16, 32 or 48 predators are released in every cage.

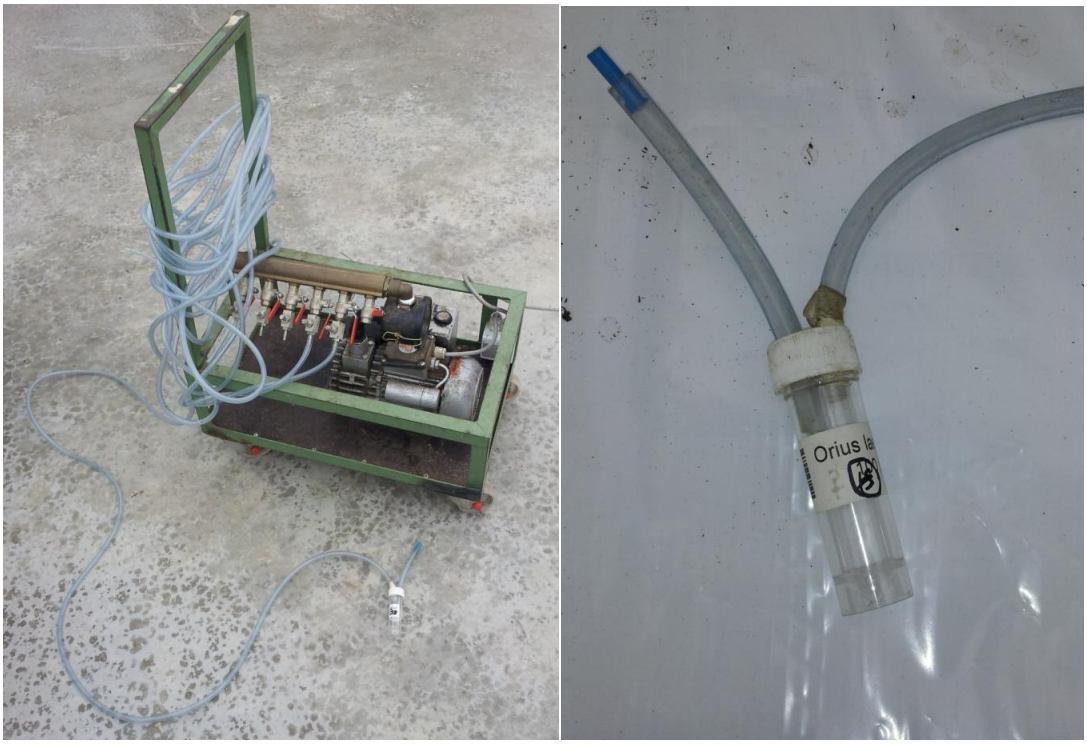


Figure 19 : Sucking device.

Aphids and aphid predators were counted every two days until no aphids were present or until new born predator nymphs appeared and modified the density tested.

The initial density of *Macrolophus pygmaeus* has been maintained by adding the missing number of predators after each counting.The results analysis will reveal if there is a difference in the control effectiveness depending on the density.

Curative application of *M. pygmaeus* on large aphid colonies

Usually aphid colonies are noticed when they are much more than ten. The predator effectiveness must also be tested on high aphid populations.

This experiment has been conducted from June 13th until June 29th.

The efficiency of four *Macrolophus pygmaeus* per plant has been evaluated on two aphid densities: 50 and 200 aphids per cage. Four repetitions were made for the two densities and the control cages.

Table 5 : The three different treatments applied and the code names corresponding.

Myzus persicae densities	Macrolophus pygmaeus density	Code name
50	4 / plant = 16 / cage	50 + M
200		200 + M
200	0 / plant	Control

Predators were collected from the rearing with a sucking device. In total, 128 *M. pygmaeus* are spread into 8 tubes. Four tubes are filled with 50 aphids and eight tubes are filled with 200 aphids collected with a paintbrush. Sixteen predators and fifty or 200 aphids are released on the same time in eight cages out of the twelve. The aphid tubes were emptied on the highest formed leaf of one out of the four plants per cage. The last four tubes containing 200 aphids are emptied in the four last cages.

Aphids and aphid predators were counted every two days until nymphs appear on the plants. *M. pygmaeus* were added to maintain a density of four predators per plant.

Results and discussion

Preventive application of *M. pygmaeus* and second diet influence

The plants used were grown in a big greenhouse with other plants and were full of *Aphidius colemani*. This was no problem though, because they do die after 3 days without aphids and nectar (F. WACKËRS, personal communication), and there were no aphids yet on the plants. The flowers were removed to preclude nectar feeding.

A week later, 3 dead *Macrolophus* were found in each of the cages 3Mp + Mzp + Ek, and 2 and 3Mp + Mzp + Tv. At least one predator was seen in the cages 2 and 4 Mp + Mzp, 2 and 4 Mp + Mzp + Ek, and all the control cages. The open flowers were removed. Some *Aphidius colemanii* were seen in the cages 3Mp + Mzp, 2Mp + Mzp + Ek and 1Mp + Mzp + Tv.

During the first two weeks, few *Macrolophus pygmaeus* were seen on the plant. Three weeks later, nymphs appeared in each of the cages.

Twenty eight days after the trial started, the predators were counted in every cage. On average 43,3 were recorded in the control cages. The maximum number of *M. pygmaeus* (66) was found in the 3 Mp + Mzp cage. The minimum (16) was found in the 1 Mp + Mzp cage.

Thirty one days into the experiment, thus three days after the pest introduction, the results show that the *M. pygmaeus* were increasing. Only one whitefly was seen in the 1st cage of that treatment. The adults were probably already dead. Aphid numbers showed a slight decrease in the cages treated only with aphids and *M. pygmaeus*. Decreases were more pronounced in the cages where there are whiteflies too. In contrast, the aphids were increasing in the cages with *Ephestia* eggs but at a rate that was less than half the rate seen in the control cages.

The aphids keep increasing in those cages but still only at half the rate as in the control cages. The aphids in the cages with the whiteflies keep on decreasing. Finally, the aphids in the cages with only *M. pygmaeus* are increasing. This increase is probably the consequence of the decreasing number of predators in the cages.

At day thirty eight, the aphids are gone in almost all of the cages and the aphid are not counted any longer. The results are shown in the figure 20.

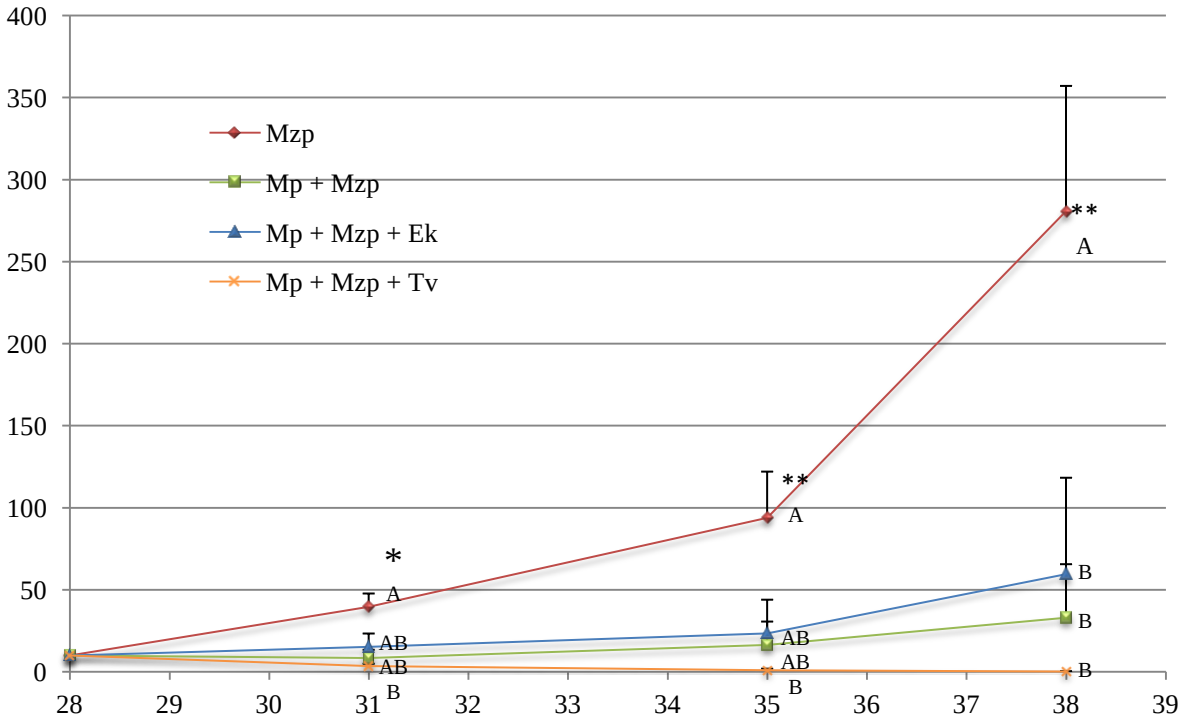


Figure 20: Mean aphid number for different treatments. X= time in days after the beginning of the trial, the aphids were added after 28 days; y= aphid number; n = 4; * = P<0.05; ** P<0.01. The results sharing the same letter are not significantly different.

The variance is analysed with "Minitab 16". First, the normality of the populations and the equality of the variances must be checked with a normality test and a Ryan-Joiner test. The variances of the aphid number for each counting are equals. The populations of the aphid for each treatment and each counting are normal except for the populations of the treatments with only *M. pygmaeus* and *M. pygmaeus* and *Ephestia* eggs for the counting after 35 and 38days. This is caused by one cage in each treatment presenting higher results. The results are transformed. The logarithms ten of the results are calculated.

All the populations are now normal and the variances are still equals. The analysis will be made on the logarithms of the results. The null hypothesis is that all the means of the aphid number per counting are equals. The variance is analysed for each counting with a One-way ANOVA. The null hypothesis is rejected for every counting. The p-values corresponding to the different time counting are shown in the table 6.

Table 6: P-Values of the analysis of the variance of the logarithms of the results depending on the treatments, for the different counting days.

Days	31	35	38
P-value	0,015	0,008	0,006

A Tukey mean structuration of the results is made to compare every mean to each other and not only compared to the control. After 31 and 35 days, the means are spread into two groups. The means into the same group cannot be considered as different. The first group includes the control cages, the Mp + Mzp treatment and the Mp + Mzp + Ek treatment. The second group includes the Mp + Mzp treatment, the Mp + Mzp + Ek treatment and the Mp + Mzp + Tv treatment. After 38 days, the aphid number from the control cages is significantly different from the three other treatments. *M. pygmaeus* helps to reduce the aphid number. No difference is shown between the predation when an alternative diet is present or not.

A regression of the results is made for every treatment following an exponential model.

With :

- A = aphid number
- A_i = initial aphid number
- T = Time
- X = coefficient estimated

Figure 21 shows the regressions and the equation corresponding.

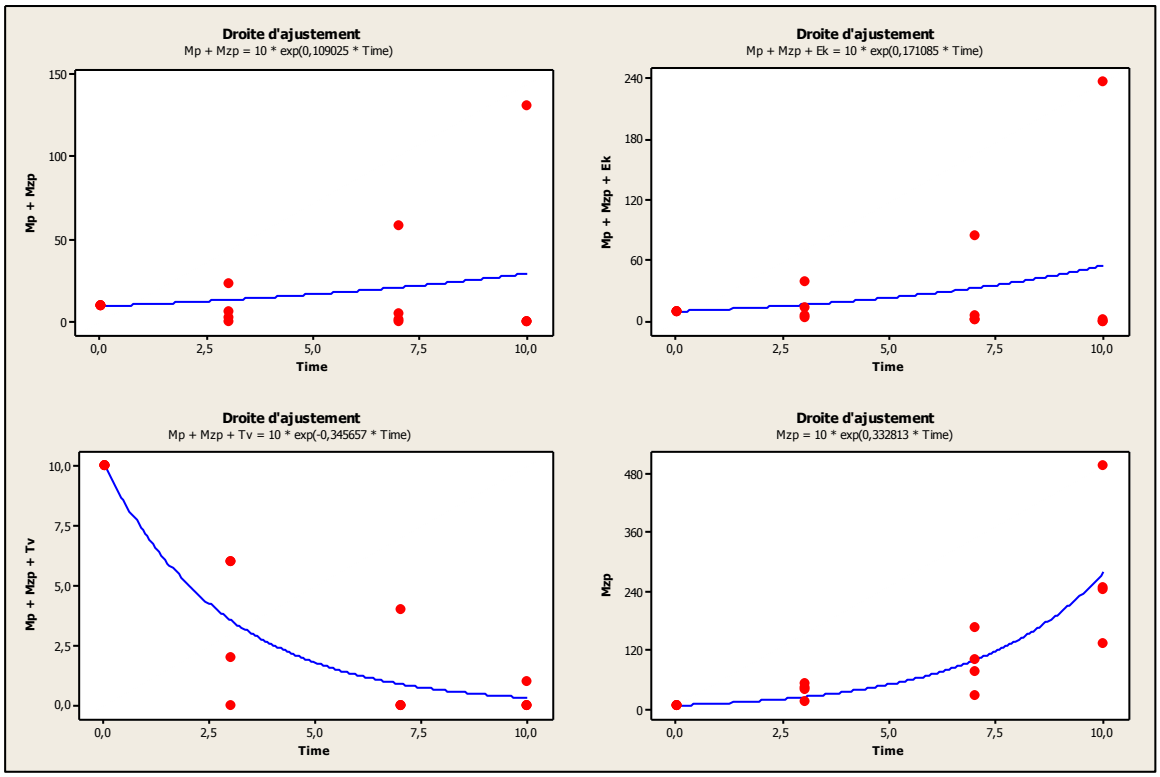


Figure 21 : regression of the results depending on the predator densities. x = time; y = aphid number; n = 4.

The figure 21 shows that the aphid population keep increasing in the cages with *M. pygmaeus* alone and with *Ephestia* eggs. Actually it's only because the aphids are increasing in two cages as shown in the figure 22 presenting the aphid number in each cage on the last counting. The aphids are gone in every cage except the 1Mp + Mzp and the 4 Mp + Mzp + Ek. In these two, the *M. pygmaeus* were probably too few to control the aphids. In the cage 1Mp + Mzp there was 16 *M. pygmaeus* when the aphids were added, and there were 30 predators in the 4 Mp + Mzp + Ek cage. For these cages, a rearing of one month has not been sufficient. On the other hand, the aphids expand faster in the 4 Mp + Mzp + Ek cage than in the 1Mp + Mzp even if there were nearly twice as much predator. That might suggest a preference of the predator for the *Ephestia* eggs. But this suggestion is only based on one observation; more repetitions are needed to confirm it. On the other hand, the aphid numbers in these two cages are equal to two control cages. So, the aphid colony in the cages with predators develops as slowly as in the two control cages and the predators are not efficient at all; or the aphid colony develops as fast as the colony in the fourth control cage and the predators are efficient but not enough comparing to the development rate.

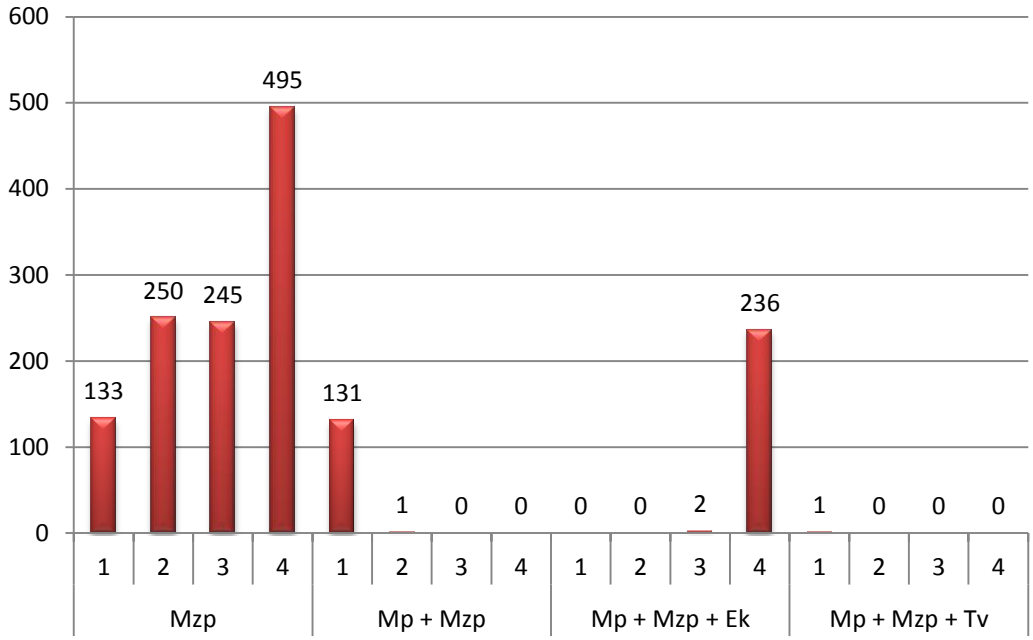


Figure 22 : Aphid number in each cage 10 days after their introduction. X=each cage; y = aphid number.

The 1Mp + Mzp and the 4 Mp + Mzp + Ek cages correspond to the smallest numbers of predators present at the moment of the introduction of the aphids. The table 7 resumes the predator numbers after 28days of rearing.

Table 7: Number of *Macrolophus pygmaeus* in every cage when the pests were added, after 28 days of rearing.

Treatment	Cages	Number of Macrolophus pygmaeus
Mp + Mzp	1	16
Mp + Mzp	2	45
Mp + Mzp	3	66
Mp + Mzp	4	60
Mp + Mzp + Ek	1	44
Mp + Mzp + Ek	2	37
Mp + Mzp + Ek	3	39
Mp + Mzp + Ek	4	30
Mp + Mzp + Tv	1	44
Mp + Mzp + Tv	2	51
Mp + Mzp + Tv	3	46
Mp + Mzp + Tv	4	42
Mzp	1	0
Mzp	2	0
Mzp	3	0
Mzp	4	0

A One-Way Anova analysis showed that aphid number at the end of the experiment was strongly related to the predator number at the end of the rearing.

The graphic is corrected by removing the two cages from the results. The figure 23 shows a decreasing of the aphid number for every treatment except the control.

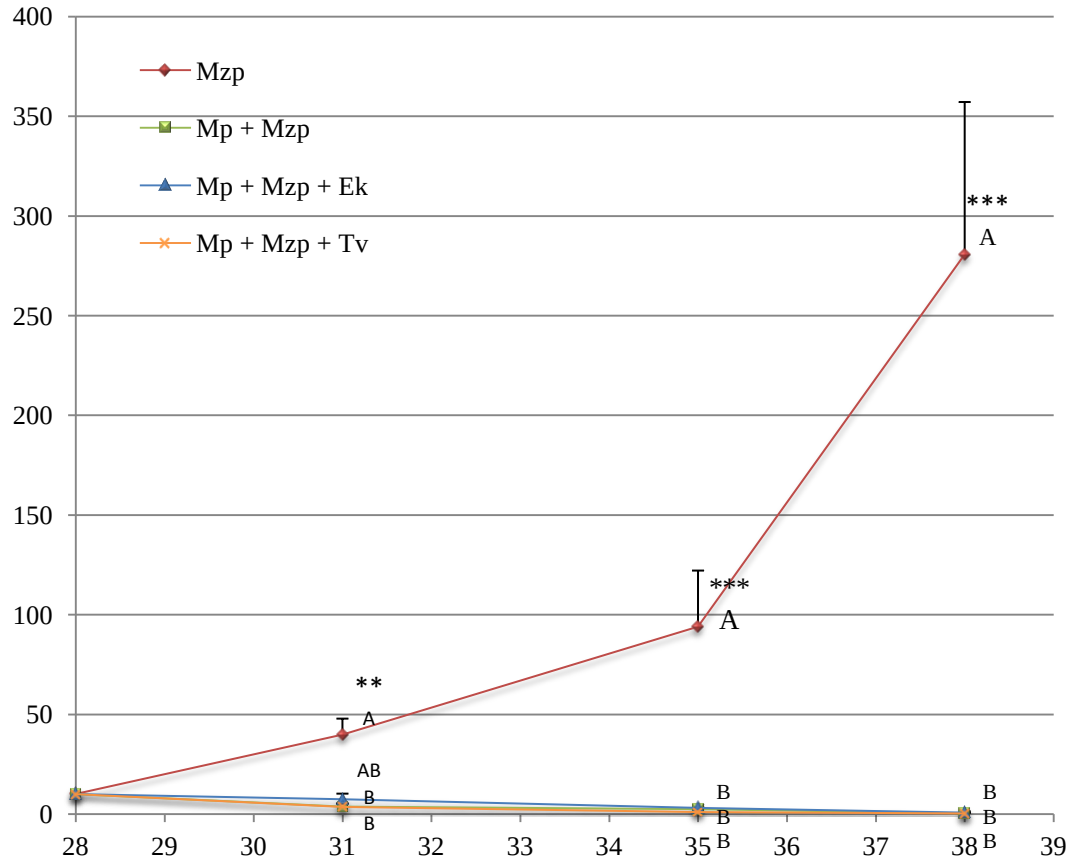


Figure 23 : Corrected mean aphid number for different treatments. X= time in days after the beginning of the trial, the aphids were added after 28 days; y= aphid number; n = 3 for the treatments Mp+ Mzp and Mp + Mzp + Ek and n = 4 for the treatments Mp + Mzp + Tv and Mzp; ** = p<0,01; *** = p<0,001. The results sharing the same letter are not significantly different.

An analysis with minitab of the aphid number logarithm is made again without the 1Mp + Mzp and the 4Mp + Mzp + Ek cages. The populations are normal and the variances are equals. The One-way Anova analysis of the logarithm of the aphid number still shows a difference between the control cages and the other treatments. The treatment are very significantly different 3 days after the introduction of the aphids, and highly significantly different after 7 days. The p-values are shown in the table 8. In every cage treated with *Macrolophus pygmaeus*, the predator are reducing aphid growth comparing to the control cages.

Table 8 : : P-Value of the analysis of the variance of the logarithms of the results without the cage 1 Mp+ Mzp and 4 Mp + Mzp + Ek depending on the treatments, for the different countings.

Days	**31**	**35**	**38**
P-value	0,003	< 0,001	< 0,001

The means are structured with a Tukey test. After 31 days, the control cages and the Mp + Mzp + Ek treatment cannot be considered as different, and the three treatments with predators cannot be considered as different neither. After 35 days, the aphid number in the control cages is highly significantly different from the other treatment.

The population dynamic of the predator is also interesting. The predator number means for each treatment are shown in the figure 24.

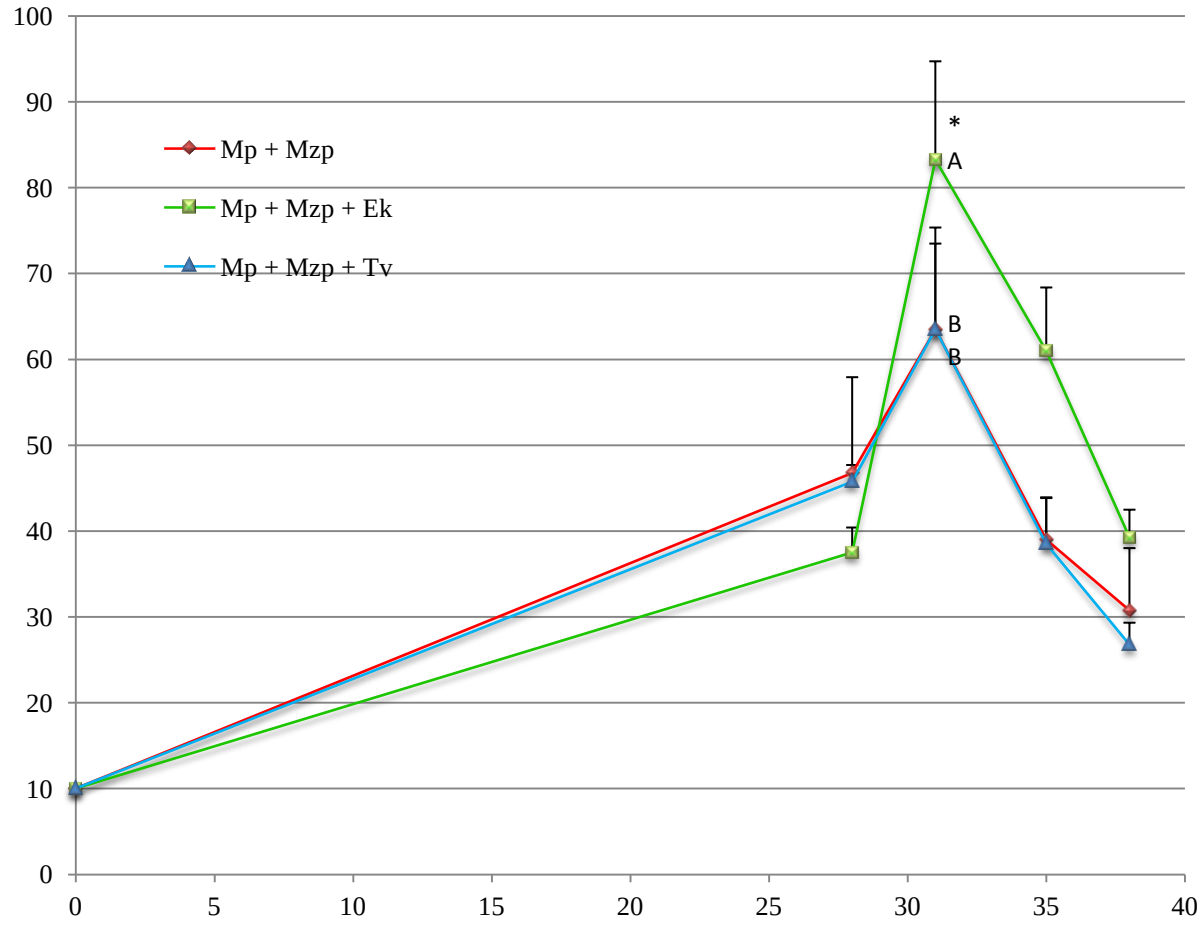


Figure 24: *Macrolophus pygmaeus* number depending on the diet. x = time in days since the beginning of the trial; y = number of predators; n = 4; * = significantly different. The results sharing the same letter are not different.

A GLM analysis of the results depending on the time and the treatments is made. The 12 populations of *Macrolophus pygmaeus*, three treatments and four counting, are normal. The variances are also equal. The predator numbers are highly significantly different depending on the time but not on the treatments and there isn't an interaction between the two factors. The results are analysed by counting day. A One-way ANOVA in made for each counting with a null hypothesis that all the means are equal. The null hypothesis is rejected for the counting after 35 days. The p-values of each counting are shown in the table 9. An addition of *Ephestia kuehniella* eggs with the aphids increases the predator population

Table 9: P-values corresponding of the One Way ANOVA of the *Macrolophus pygmaeus* number after 28, 31, 35 and 38 days of rearing.

Day	**28**	**31**	**35**	**38**
P-value	0,587	0,389	0,041	0,227

A structuration of Fisher for the means of the 35th day shows that the *M. pygmaeus* are significantly more prevalent in the cages with aphids and *Ephestia* eggs. The structuration with Tukey doesn't show any difference. A little rearing made on the side of *Macrolophus pygmaeus* in individual cages fed on *Myzus persicae* only and on *Myzus persicae* and *Ephestia* eggs showed that there is no difference of reproduction between the two diets.

At thirty five days, predator numbers were decreasing. This may be because the prey scarcity. Understanding that the plant does not provide the same quality of food as the prey. However, a decrease was also observed in the cages where they have aphids and *Ephestia* eggs. The decreasing of the *M. pygmaeus* after 31 days, March the 15th, could be caused by a climate change. Indeed, the temperatures started increasing around the13th of February and reach their highest point around the 16th. The fertility is the best at 20°C and decreases with the temperature (PERDIKIS D.C. & LYKOURESSIS D.P., 2004). The mortality increases very fast with the temperature and reaches 40% at 30°C and 100% at 40°C when feeding on *Ephestia kuehniella* eggs (FAUVEL G. *et al.*, 1987). Unfortunately, the data loggers indicate that the temperatures are kept very constant in the greenhouses. The humidity reached a low value of 21,5% but increased again in the next days. The cannibalism might be a cause of the decrease in predator numbers.

This experiment showed that *M. pygmaeus* presence decreased the development rate of the aphid colonies and that a second diet didn't influence *M. pygmaeus* predation on aphid. In 10 cases out of the 12, the predator was efficient to control and eliminate aphids. The two cases where aphids were not under control were related to a low predator number after the rearing. However, predators keep increasing and reach a number of 50 individuals in the cage 4 Mp + Mzp + Ek. This density of predator should have be able to control the aphid. Unless if the aphids are yet too numerous to be controlled. The failure in the control can also be explain by a different development rate of the aphid colonies. A low predator density would be very influenced by that development rate whereas a higher density would get over any development rate.

In these two cages, the aphid number is higher in the cage with *Ephestia* eggs. This could mean that *M. pygmaeus* prefers *Ephestia* eggs than aphids. It's probably easier to feed on eggs than on defending aphids. However, this presumption is only based on one observation.

The predator developed very well on bell peppers making a month of rearing sufficient to build up a population. The predator decreasing after 31 days is probably due to cannibalism encouraged by high predator densities .

Curative application of *M. pygmaeus* on small aphid colonies

After 5 days, the aphids are gone from one of the cages started with 8 predators per plant. After 7 days, two cages out of the four started with 12 predators per plant are free from aphids. There are one and three aphids left in the other two cages, and they are gone after 9 days. After 13 days, two cages started with 4 predators per plant are also under control.

Table 10 : Time needed to eliminate the aphids depending on the density of *M. pygmaeus.*

Time from the introduction	Cage number	Density (predators/plant)
5 Days	4	8
7 Days	2	12
7 Days	3	12
9 Days	1	12
9 Days	4	12
13 Days	1	4
13 Days	4	4
20 Days	2	8

The variance of the results is analysed with Minitab. The conditions to apply a Balanced Anova model are verified. Some populations do not have a normal distribution, for the 0/plant density the results after 7,9,13 and 15 days, for the 4/plant density after 9,13,15 and 20 days and for the 8/plant density after 9,13,15 and 20 days. The test of the equality of the variances shows that the variances of the different densities after 5, 7, 13, 15 and 20 days are not equal. The results have to be transformed. The neperien logarithm of the results is calculated. The variances are now equal. The populations are normal except the 0/ plant density after 7 days. The variance of the neperien logarithm of the results is analysed with a Balanced Anova Model with two factors, the time and the densities. The results are highly significantly different depending on the densities and the interaction of the time and the densities. The variance of the neperien logarithm of the results is analysed with a One-way Anova model with one factor, the densities, for each counting. The mean are structured with a Tukey test. This test permits to see the difference between the densities and the control but between the different densities too. After 5 days, the results are significantly different. The mean are spread into two groups. The first group includes the densities 0/plant, 4/plant and 8/plant and the second group includes the densities 4/plant, 8/plant and 12/plant. Only the densities 0/plant and 12/plant are different. After 7days, the results are very significantly different. The structure of the mean is the same as after 5 days. The results stay very significantly different until the end of the experiment. But after 13 days the structure changed. The density 0/plant stay only in the first group and the three other densities are together in a second group.

Table 11 : P-values of the analysis of the variances of the different densities of predator for each counting.

Days	**2**	**5**	**7**	**9**	**13**	**15**	**20**
P-value	0,587	0,05	0,014	0,004	0,002	0,002	0,002

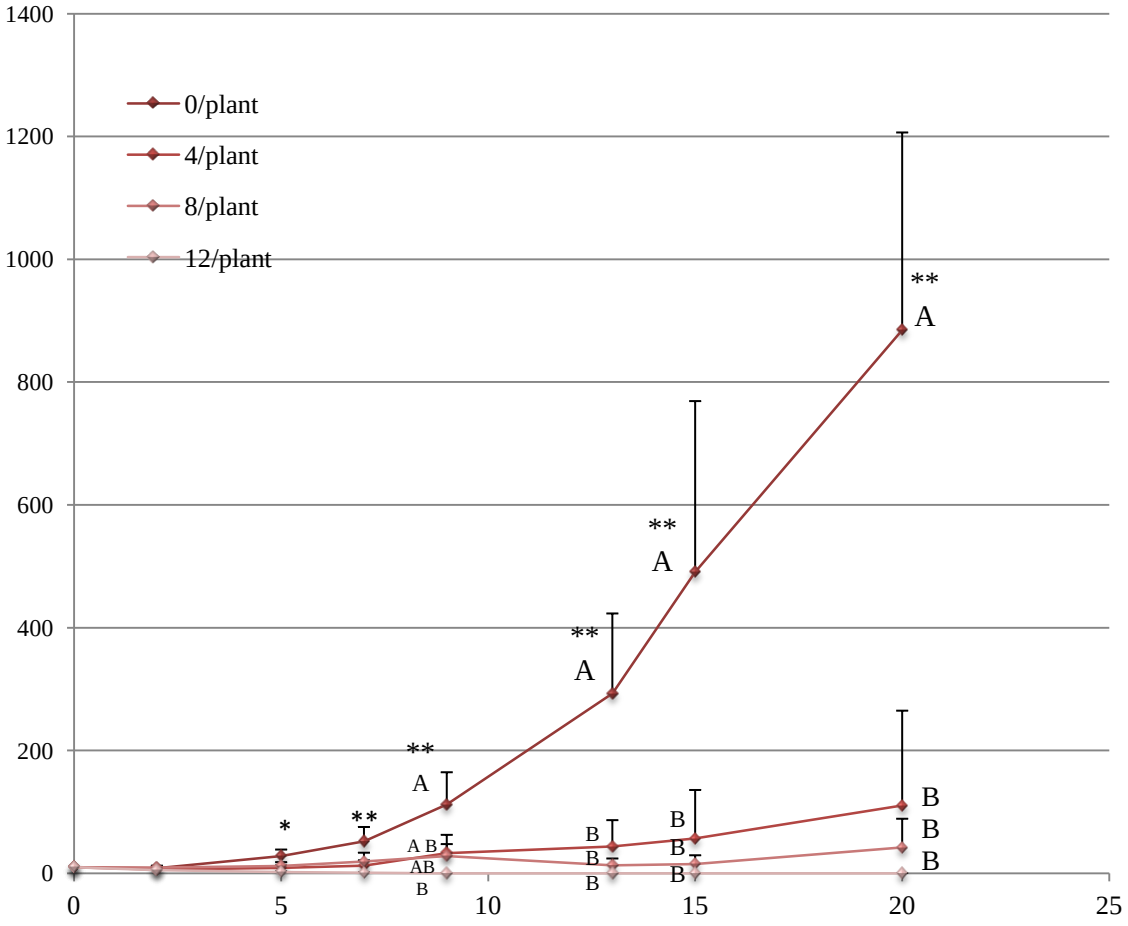


Figure 25 : Mean aphid number evolution depending on the density of *M. pygmaeus*. X = time in days; Y = aphids; n = 4; * = p < 0,05; ** = p< 0,01. The densities sharing the same letter can't be considered as different.

The analysis showed that there was not any apparent difference in aphid control between the different densities of *M. pygmaeus.* However, the mean of the aphids in the cages with a density of 4 predators per plant reached 221 aphids after only 20 days. This would be too high for a greenhouse crop. But, actually, the results of each cage showed that after 13 days two cages with 4 predators per plant were free from aphids and one out of the two left stayed at 2 aphids after 15 and 20 days. 439 aphids were found in the last cage. The cages with 8 predators per plant followed the same schema. After 20 days, 2 cages were free from aphids, 4aphids were left in one cage and 164 were found in the last one. In the cages with 12 predators per plant are very soon free from aphids. The details of each cage are showed in figure 26.

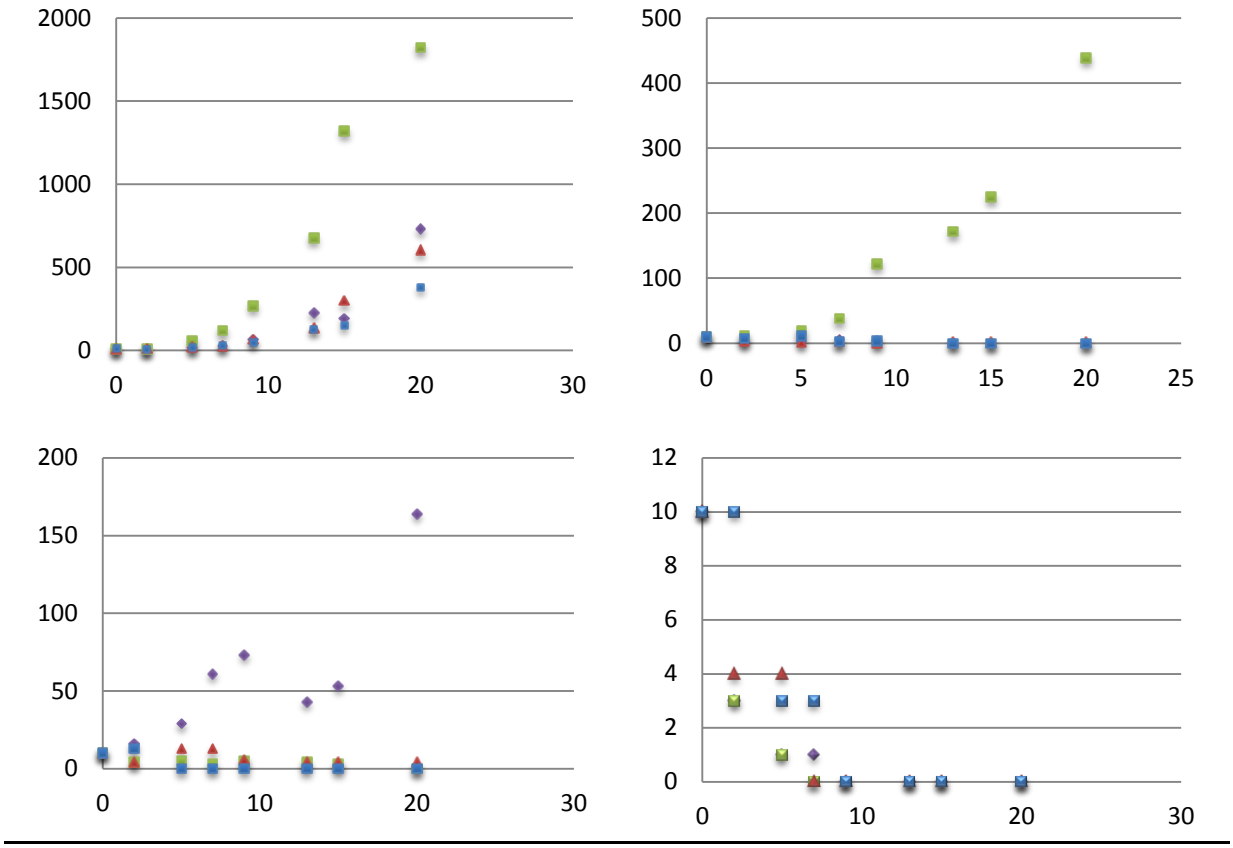


Figure 26 : Graphics of the aphid number in each four cages for different predator densities. From left to right and up to down: 0 *M. pygmaeus* / plant; 4 *M. pygmaeus* / plant; 8 *M. pygmaeus* / plant and 12 *M. pygmaeus* / plant. X = time in days; Y = aphid; n = 4.

The fact that one cage out of the four is not under control with the two lowest densities of predators could mean that one colony out of four develops faster than the others. Indeed, the detailed results showed that in every treatment one colony developed faster than the other. Even, with the highest density, one colony seemed to be controlled with more difficulty.

The mortality of the predators is very high in every cage. The mean mortality after the first predator introduction was equal to 67,19%, 54,69% and 59,90% in the cages with a density with 4, 8 and 12 *Macrolophus pygmaeus* per plant. After 5 days, the mean mortality reached a normal rate, given the temperature, for the 4 predators per plant density but stayed high in the other cages. After that, it kept increasing, with the highest number for the highest density. The mean mortalities after each counting are shown in the table 12.

Table 12 : Mortality of the predators depending on the densities

Density (predators / plant)	2Days	5 Days	7Days	9Days	13Days	15 Days	20 Days
4	67,19	20,31	35,94	39,06	43,75	37,50	-90,63
8	54,69	39,06	44,53	46,88	54,17	43,75	-54,17
12	59,90	41,67	55,73	60,42	*	*	*

These results suggest that the predators may engage in cannibalistic behaviour when the density is too high. Such cannibalism by mirid predators had already been reported in other studies (WHEELER, 2001; MORENO-RIPOLL, 2012).

After 15 days, two young nymphs are born in one cage out of the 12 cages containing *Macrolophus pygmaeus*, both in a cage with a density of 8 predators per plant. After 20 days, small nymphs are seen in every cage. In all cages the number of predators is higher at this point than the initial density. The results of the counting of the predators after 20 days are removed from the analysis.

The variance of the mortalities are analysed with a One-Way Anova model counting day by counting day. The conditions to apply the analysis, the normality of the population and the equality of the variances are verified. The mortalities are not significantly different.

Table 13 : P-values of the variance analysis of the mortalities of *M. pygmaeus* depending on the densities for each counting.

Days	**2**	**5**	**7**	**9**	**13**	**15**
P-values	0,153	0,095	0,355	0,324	0,375	0,385

Once again, one aphid colony out of four developed faster than the others keeping *M. pygmaeus* from eliminating every aphids. Still, *M. pygmaeus* did reduce their development. Only the highest density of 12 predators per plant was sufficient to eliminate the aphid even when developing faster. Although, the analysis didn't detect any difference between the treatments. Anyway, such a high density of predator seems to be the only way to surely eliminate the aphid whereas the introduction of so many predators per plant in a crop is unfeasible. After the experiment, *M. pygmaeus* may be considered on one case out of four as effective as biocontrol agent on a small aphid population of 10 individuals even at a density of 4predators per plant. On crop situation, the *M. pygmaeus* should move from the plant without prey to the infested plant. This way, their density should increase and achieve a higher level of elimination.

M. pygmaeus present a high mortality without prey. As it can survive and develop only feeding on plant, there is no doubt about their cannibal behaviour.

Curative application of *M. pygmaeus* on large aphid colonies

After 12 days, a few nymphs are seen in four different cages.

The aphids mostly stay on the plant they were put on. They seem to spread on other plant only when the predators are present.

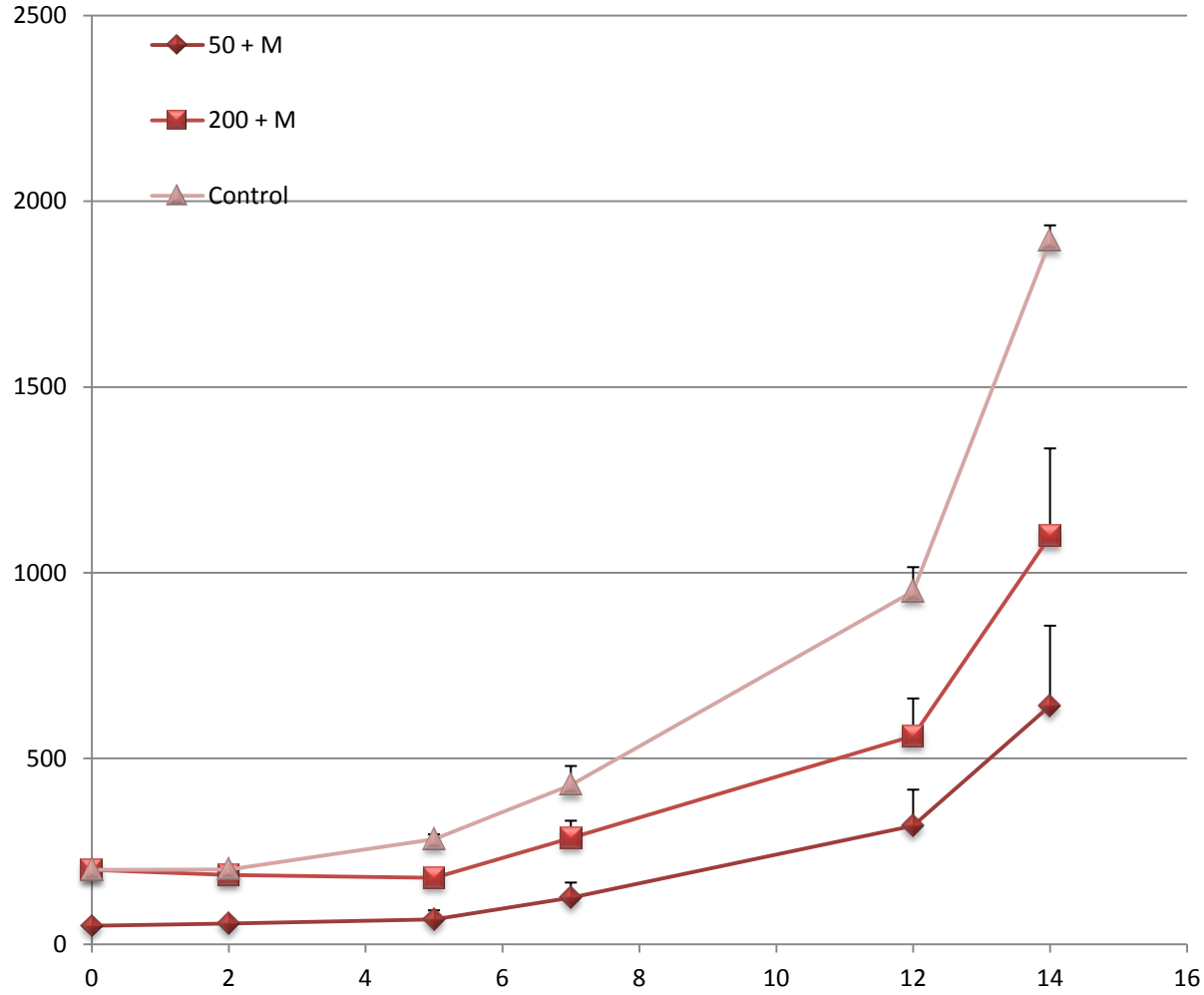


Figure 27 : Aphid population evolution depending of their initial density and the presence or absence of predation. X = time in days; Y= aphid number; n = 4; 50 + M = initial density of 50 aphids and presence of predation; 200 + M = initial density of 200 aphids and presence of predation; Control = initial density of 200 aphid and absence of predation.

Given that the initial aphid densities are different, the results must be transformed to be analysed. Indeed, the aphid number mean after 14 days is surely lower for an initial aphid density of 50 aphids than 200 aphids. The gradient must be analysed not the mean. The general equation of the aphid number depending on the time for the different density and the absence or presence of predation is:

It can be transformed as:

The analysis of the variance of "ln A – ln A_i" is made with a model of two factor, treatment and time and with the time as covariable. A Levene test shows that the variances of the

different treatment for each count are equals. Although, 5 populations out of 15 are not normal, because one out of the four results is lower than the other. The results are let untransformed. The GLM analysis shows that the results are not different depending on the treatments.

By analysing with a One-Way Anova model the variance between the control and the "200 + M" treatment, which can be compared, as the initial aphid density is the same, it's found that the means are highly significantly different with a "time" and "treatment" model. When the results are analysed for each count, the results are very significantly different after 5 days, not significantly different after 7 days, than significantly different after 12 days. The p-values corresponding to each count are displayed in table 14.

Table 14: p-values of the GLM analysis of the aphid number for each count

Days	**2**	**5**	**7**	**12**	**14**
P-Values	0,202	0,005	0,085	0,018	0,016

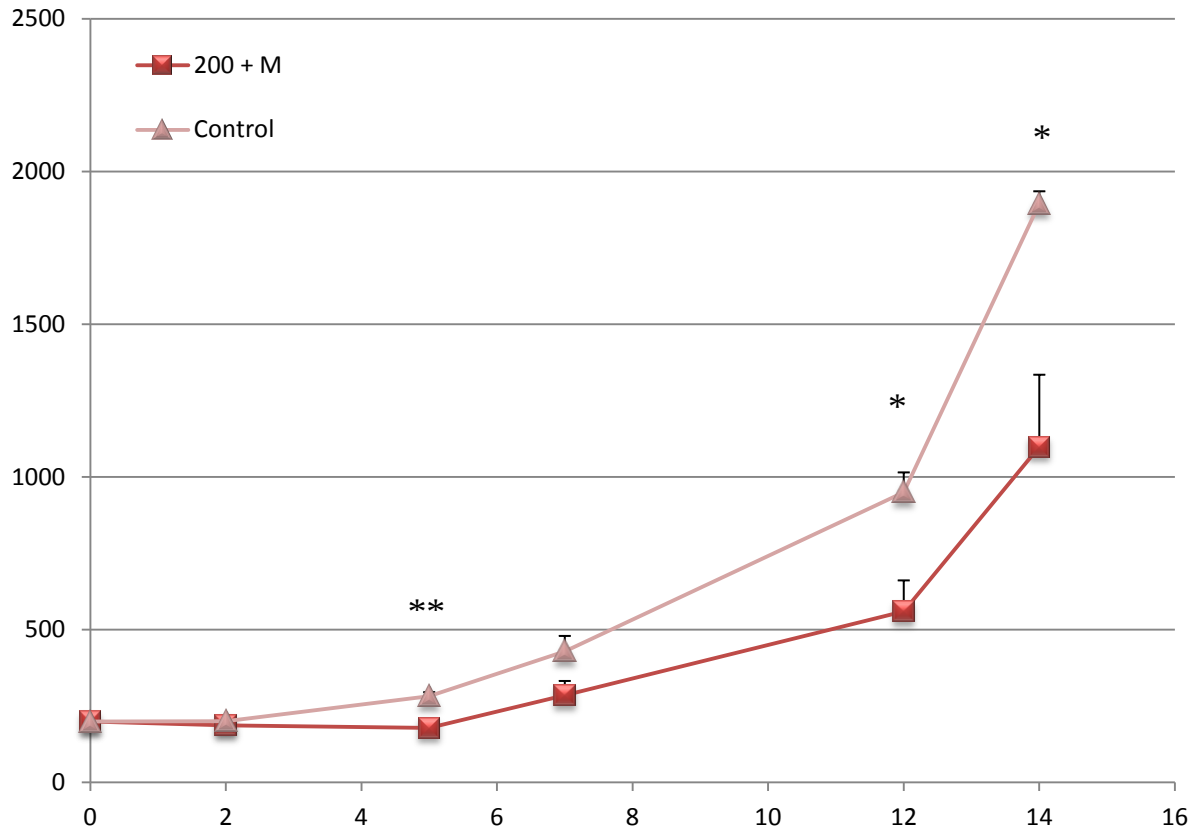


Figure 28 : Aphid population evolution depending of their initial density and the presence or absence of predation. X = time in days; Y= aphid number; n = 4; 200 + M = initial density of 200 aphids and presence of predation; Control = initial density of 200 aphid and absence of predation; * = p < 0,05; ** = p < 0,01.

The analysis of the development rate did not manage to detect any difference depending on the aphid initial density and the absence or presence of predation. However, after 2weeks, the aphid numbers are significantly different when *M. pygmaeus* is present and when it's not. It can be told that *M. pygmaeus* helps to reduce the development of a large aphid colony of 200 individuals. Unfortunately, no conclusion can be made about the colony of 50 individuals, as the analysis did not detect any difference of growth rate compared to the control but neither compared to the larger colony with predation.

It can be supposed that *M. pygmaeus* had also an impact on colonies of 50 individuals but that the analysis cannot detect a difference in the development rate as soon as 2weeks. The difference might get harder with the time.

Section II: plant defences induction

This section investigates the plant defences induction by *Macrolophus pygmaeus* and potential impacts on aphid populations. Under *M. pygmaeus* pre-exposition, three parameters were monitored: aphid behaviour on the plant, aphid reproduction rate and aphid suitability as a prey for *Macrolophus pygmaeus*.

The reproduction rate and the suitability to predation are two parameters important to investigate because they could influence the potential of *M. pygmaeus* as a biocontrol agent against aphids. The aphid behaviour may provide indications if some compounds are emitted or not by the plant. Indeed, if the plant emits some repellent compounds under *M. pygmaeus* presence, these chemicals could be translocated to the top of the plants (F. WÄCKERS, personnal communication). The higher concentrations would thus be found in the top of the plants (RAGHAVA T. *et al*., 2010) forcing the aphids to move down of the plant.

Understanding the results and the circumstances of the experiment, it has been made again and split into two experiments: one experiment testing the aphid pre-exposition and especially the aphid suitability when fed on plant pre-exposed to aphids; and a second one, testing the impact of a pre-exposition to *Macrolophus pygmaeus.*

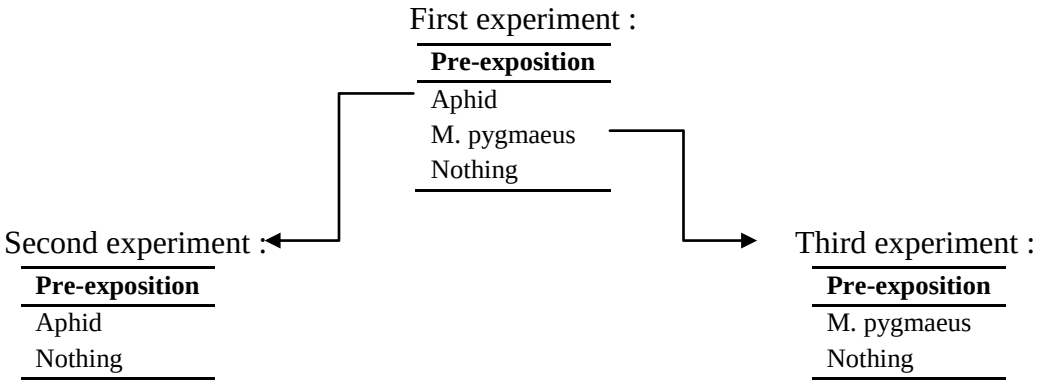


Figure 29 : Experiments made to test the induction of the plant defences.

Figure 30 : Female *Macrolophus pygmaeus* (De Backer L.).

Material and method

The three above-mentioned experiments were conducted on bell peppers placed individually in net cages (Figure 32). The temperature and the humidity were recorded every five minutes by a data logger. The temperature and the humidity were respectively 25,4°C ± 3,44°C and 37,85% rh ± 7,48% rh.

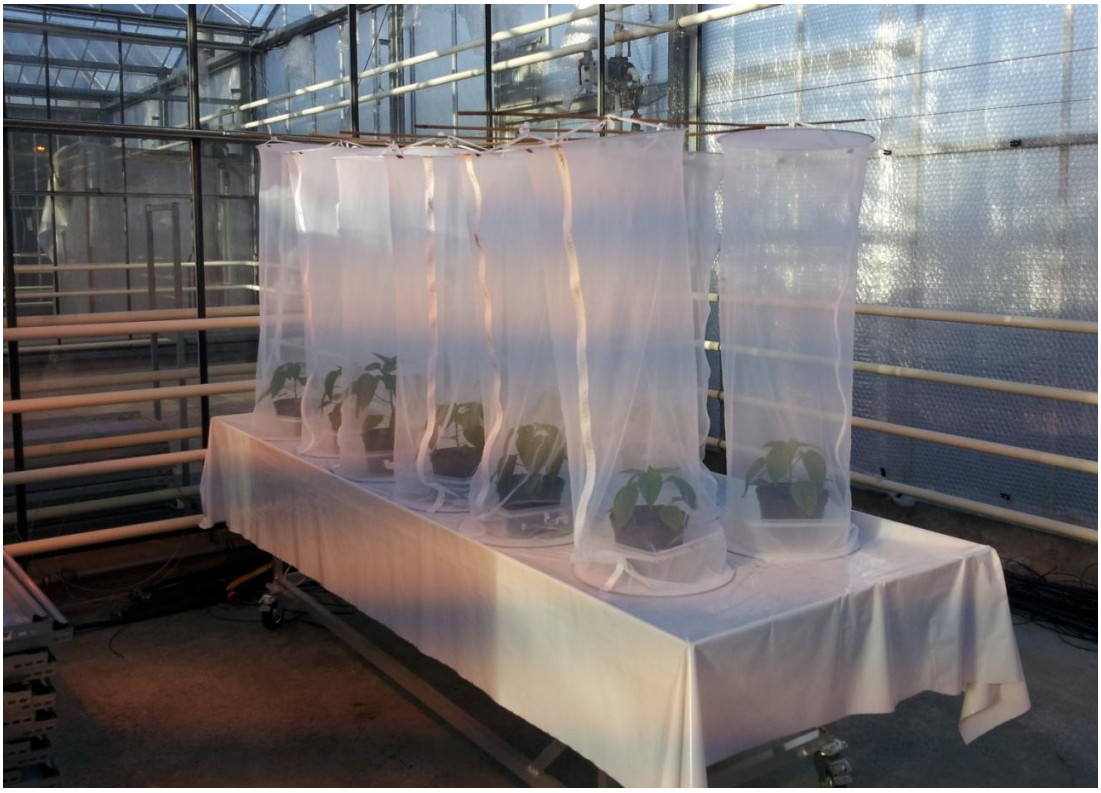


Figure 31 : Net cages containing a single bell pepper plant used to evaluate the induction of chemical defence under infestation by *Macrolophus pygmaeus.*

Macrolophus pygmaeus and aphid pre-exposition impact

This experiment took place from February 14th until March 12th.

The impact of a pre-exposition to *M. pygmaeus* was compared to a control corresponding to plants that were not subjected to any pre-expositions. It is also compared to plants pre-exposed to aphids which are known to interact with the plants. Thus three different pre-exposition were applied on bell peppers: 50 *Myzus persicae*, 5 *Macrolophus pygmaeus* (3 females and 2 males) and nothing. The figure 33 shows the procedure of the experiment.

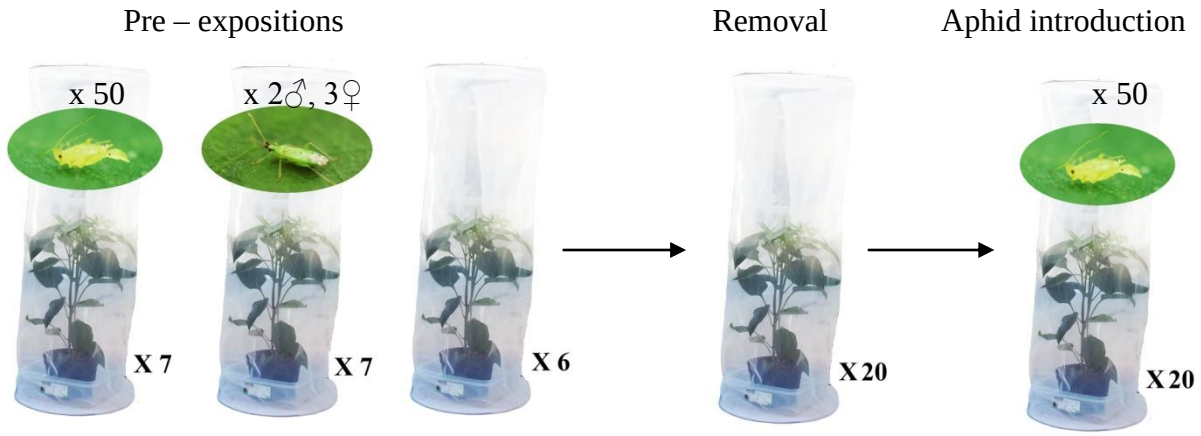


Figure 32 : Experiment testing the plant defence induction.

The plants were 25cm high and had 14 to 18 leaves. Young plants are likely to be express more induced defences under sucking-insect infestations (F. WACKËRS, personal communication). Seven repetitions were conducted for the aphid and the predator pre-exposition and six repetitions for the control. A total of twenty cages were disposed on two tables as shown in figure 34.

M. pygmaeus	Control	*M. persicae*	*M. pygmaeus*	*M. pygmaeus*	*M. persicae*
M. persicae	Control	*M. persicae*	Control	Control	*M. pygmaeus*

Control	*M. pygmaeus*	Control	Control	*M. pygmaeus*
	M. persicae	*M. persicae*	*M. pygmaeus*	*M. persicae*

Figure 33 : Disposition of the individual cages.

The 350 aphids were collected with a paintbrush from a laboratory rearing and spread into 7 tubes. Fifty aphids were released on seven out of the twenty plants. Twenty one predator females and forteen males were collected from the commercialised product "Macrolophus-System" (Biobest). Three females and two males were released on seven out of the thirteen plants left.

After 3 days, aphids, *M. pygmaeus* adults and nymphs were easily removed but predator eggs were too deep in the plant tissues (BIOBEST, 2012) to be removed. The plants have been watched for 5days and eventual appeared nymphs were removed.

Five days after removing predators and pests, a thousand aphids were collected with a paintbrush from a pepper plant and spread into twenty tubes. Fifty aphids are added on each plant in the net cage on the highest formed leaf. The tube was carefully emptied with a paintbrush.

The leaves were numbered from the top. If the stem branched out, the leaf at that point was numbered as the fifth. All the plants did not have as many leaves as each other. For this experiment it was important that all the top leaves and all the bottom leaves had the same number to be able to see the distribution of the aphids. In that aim, each leaf on plants

counting exactly 14 leaves was numbered. For the plants with more leaves than 14, the leaves below the fifth were counted two by two to reach the same number of 14 leaves.

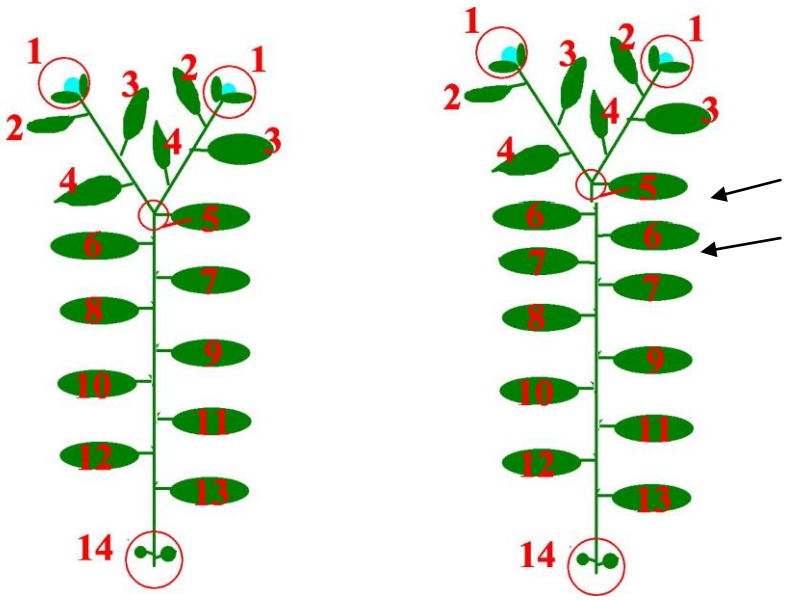


Figure 34: Numbers attribution for a plant with 14 leaves and a plant with 16 leaves.

Aphids were counted on every leaf after 2 hours, 24 hours and 6 days to evaluate their reproduction rate and their distribution on the plant.

To test the suitability of the aphids as prey for *M. pygmaeus*, the predatory rate has been compared to the aphid growth without predation. The figure 36 shows the procedure applied. Twenty one days after the beginning of the experiment, the 10^{th} and 11^{th} leaves were cut from each plant. All aphids but 25 individuals were evacuated from each leaf. The leaves were put on a wet piece of paper in a plastic box closed with a rubber band (Figure 37). Forty leaves were collected thus forty boxes were needed in total. Two *M. pygmaeus* nymphs were added in one of the two boxes containing the leaves from the same plant. The second box was used as a control to monitor the aphid population growth. The boxes were closed.

Living aphids were counted after 24 hours, 48 hours and 6days.

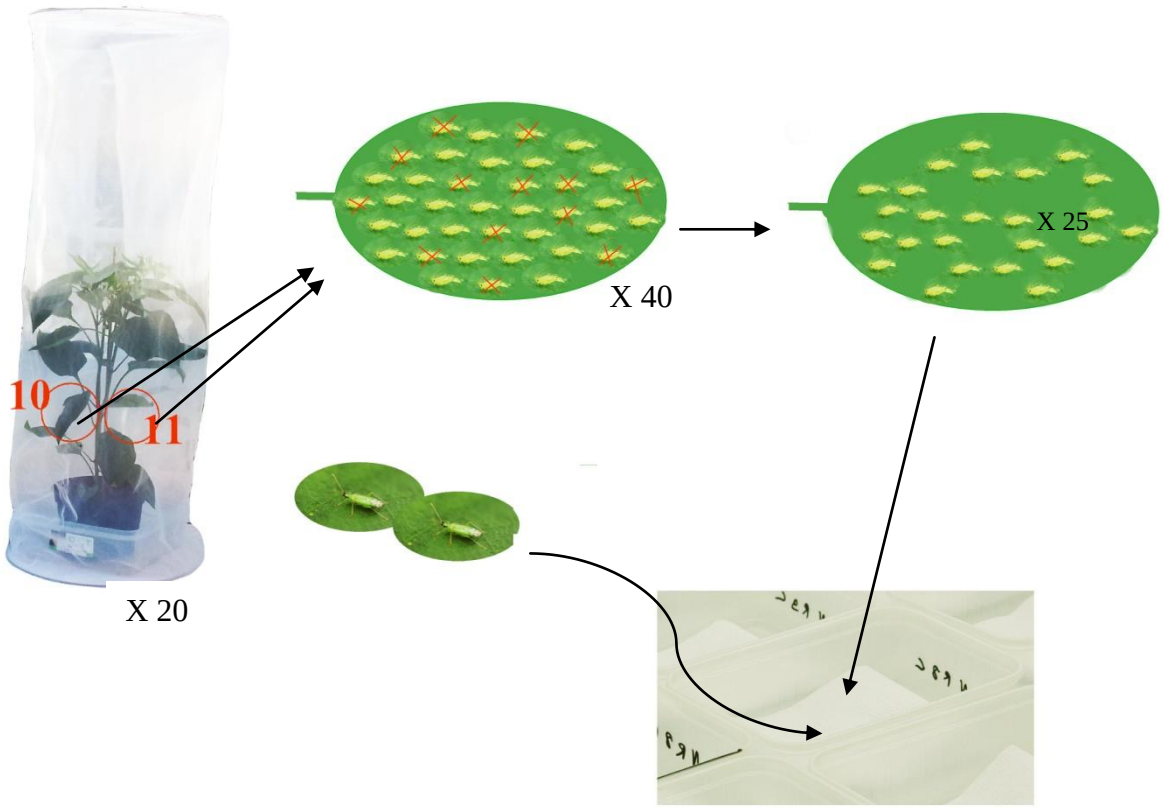


Figure 35 : Preparation of the plastic boxes for the evaluation of the aphid suitability.

Figure 36: Plastic boxes used to evaluate the suitability of the aphids feeding on plant pre-exposed to *M. pygmaeus*, aphids and not pre-exposed..

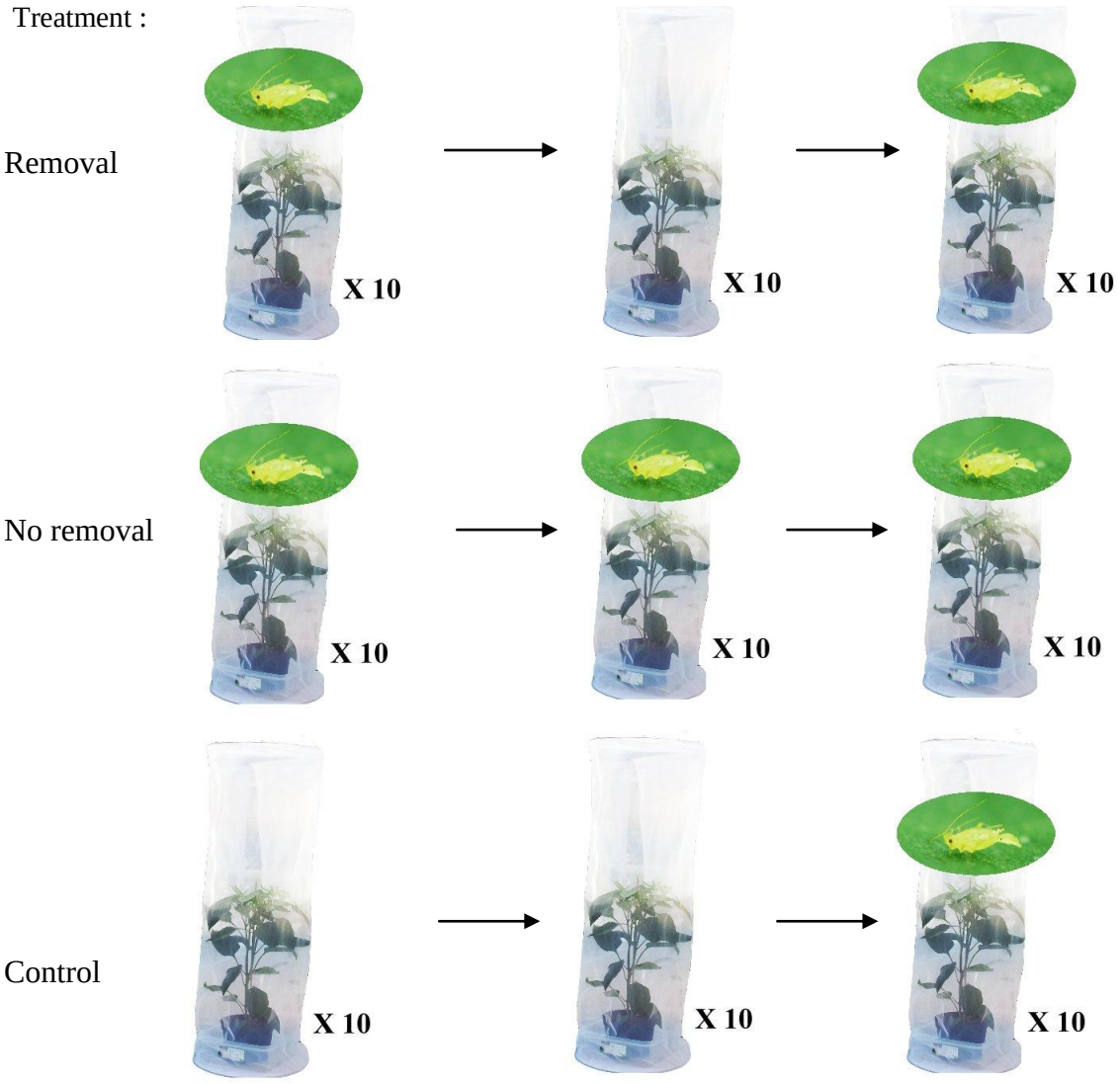
Aphid pre-exposition impact on the aphid suitability

This experiment aimed to confirm previous results about the influence of an aphid pre-exposition of the plant on the suitability of the second infestation aphids for *Macrolophus pygmaeus*. It began on March 19th and ended on April 13th.

Thirty bell peppers (*Capsicum anuum*) in individual cages were used. The plants were about 35cm high and the leaves were numbered as in the first trial from 1 to 14.

The protocol was nearly the same as the previous experiment but without the predator pre-exposition. This time, the importance of the aphid removal was also tested.

The impact of a pre-exposition to aphids was compared to a long infestation, where the aphids were not removed, and to a control. The long infestation permits to know if the effect observed was caused by the pre-exposition, or if it just needs time to occur and become visible. Ten repetitions of these three treatments were made.



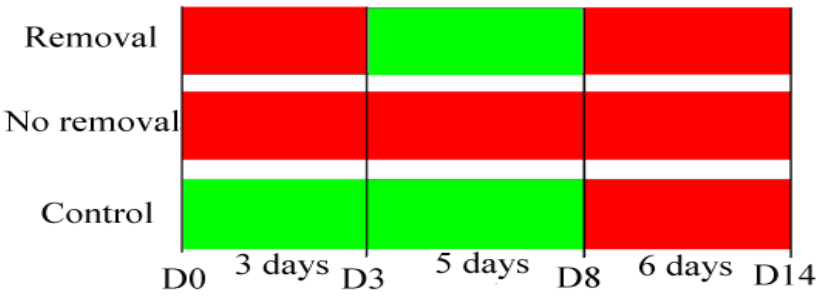


Figure 37 : Aphid presence and absence in the cages depending on the treatment and time repartition of the expositions to aphids depending on the treatment; in red; and the removal or the aphid absence, in green.

One thousand aphids were collected from a bell pepper with a paintbrush and spread into twenty tubes. Fifty aphids were introduced in twenty out of the thirty plants. The tubes were carefully emptied with a paintbrush on the highest formed leaf numbered 2 of each plant.

After 3 days, the aphids from half of the exposed plants were removed. The plants were checked two hours after the removal to be sure there was no aphid left. The aphids on the other half were left. Five days later, fifty new aphids were added on the cleaned pre-exposed plants and on the control plants. They were added on the highest formed leaf. Excess aphids on the "no removal" treated plants were removed until 50 were left on the highest formed leaf. This allows comparing the aphid population growth on the different treated plants. The aphids were counted 6 days after the second infestation.

Thirteen days after, the10^{th} and 11^{th} leaves were cut from each plant, the aphids were removed until 25 were left and the leaves were put on a wet paper in a box. The box was closed by a cloth held by a rubber band. Two *Macrolophus pygmaeus* were added in one of the two boxes. The other box was the control.

The aphids were counted each day for three days. The results analysis will reveal if there was a difference of predation depending on the pre-exposition of the plant.

Plant defences induction by *Macrolophus pygmaeus*

This experiment investigates the negative impact of the plant defences induction by *Macrolophus pygmaeus* and whether these defences had any effect on a newly established aphid population. In this aim, plants were exposed to predators before being infested by aphids. Two parameters were monitored: the aphid behaviour on the plant and their reproduction rate.

The experiment was conducted from June 15^{th} until July 5^{th}.

The leaves of forty bell peppers were numbered from the top of the plant as in the previous experiments. Leaf number 1 was the top of the plant and includes non-formed leaves and buds. The cotyledons were both numbered as the 13^{th} leaf.

The plants were exposed to *M. pygmaeus* males and unmated females separately to avoid the eggs laying and the predation of the nymphs emerged after the introduction of aphids. The impact of these expositions was compared to a negative control (plants unexposed to anything), and to a positive control (plants exposed to a defence inducer). The defence inducer was methyl jasmonate (MeJA), as several studies prove its ability to induce the plant defences (FRANCESCHI *et al.*, 2002; HUDGINS *et al.*, 2003) such as trypsin inhibitor (TI), a proteinase inhibitor increasing mortality of the attacking insect and polyphenol oxidase (PPO), an oxidative enzymes destroying or modifying dietary amino acids and fatty acids (TAN *et al.*, 2011). In total, 4 different treatments were applied on ten repetitions.

- Nothing
- *M. pygmaeus* females
- *M. pygmaeus* males
- Plant defence inducer : methyl jasmonate

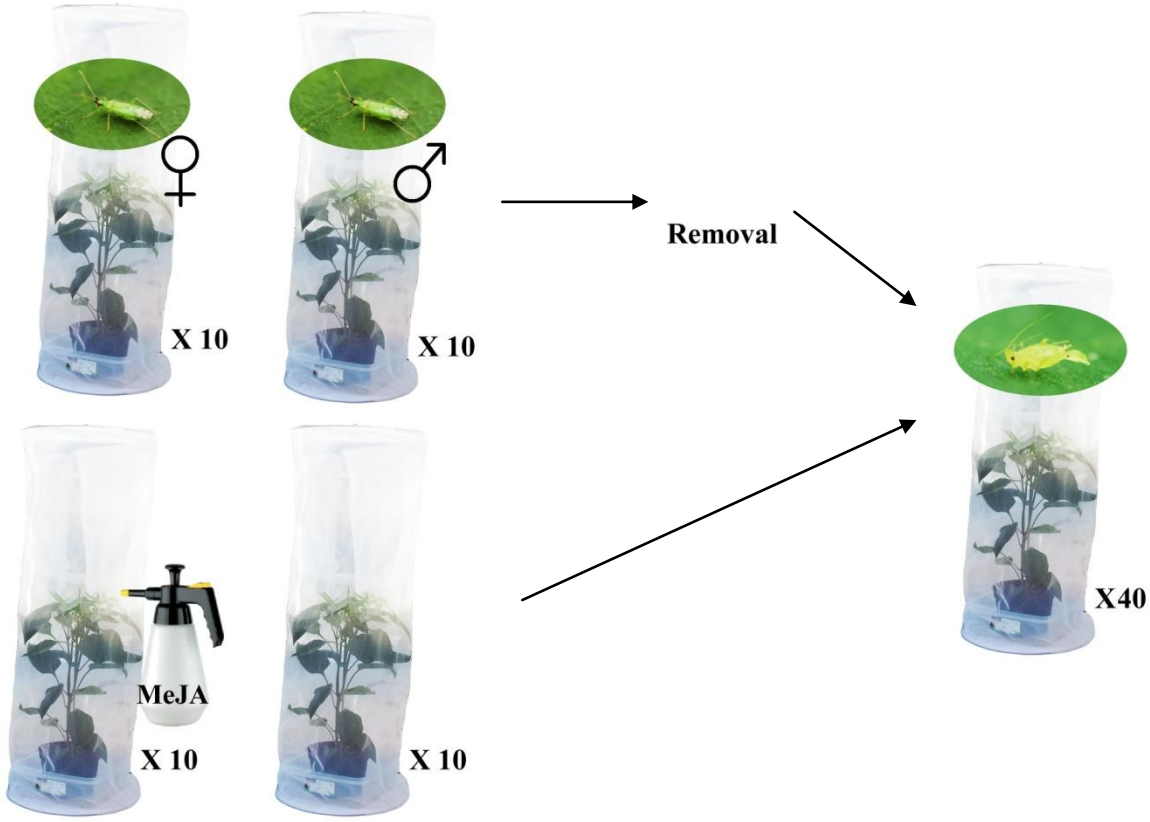


Figure 38 : Schema of the experimental procedure.

Because previous tests showed that *Macrolophus caliginosus* could survive 25,67 ± 2,33 days only feeding on brinjal leaf (MODH RASDI, 2009), additional food was not needed.

One week before the experiment started, one hundred 3rd, 4th and 5th nymphs were collected from a laboratory rearing on tobacco plants with a sucking device. They were put in a plastic box for conservation with *Ephestia* eggs and three small tobacco plants (Figure 40). Three days later, males and females were separated and stored in two plastic boxes (Figure 40).

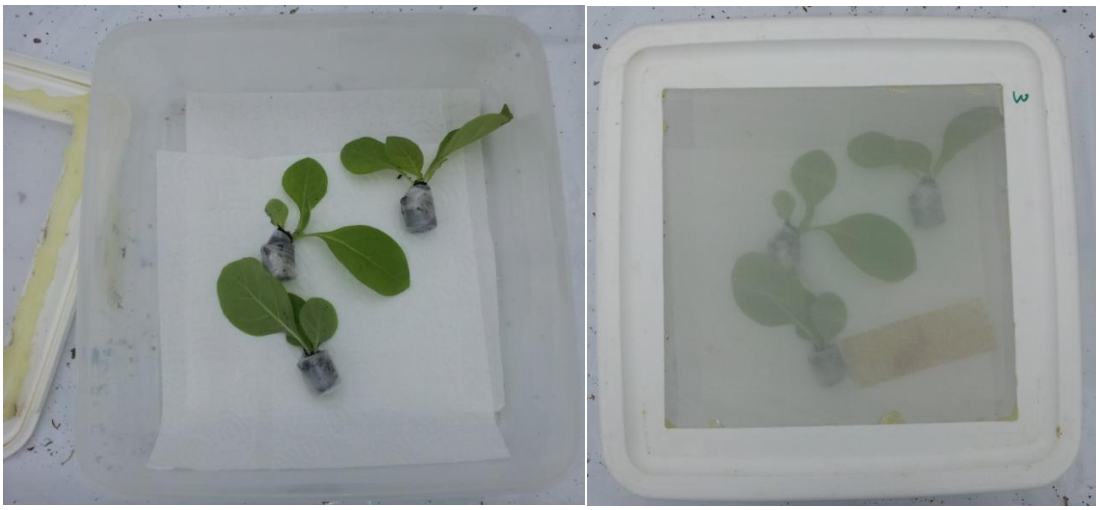


Figure 39 : plastic box for conservation with *Ephestia* eggs and small tobacco plants.

Two days later, forty unmated females were collected from the plastic box and spread into ten tubes. Forty males were collected in the same way. The four predators contained in one tube were released on a plant and the cage was closed. In total, twenty plants were treated with predators, ten with females and ten with males.

Five days after the predator introduction, ten plants were treated with methyl jasmonate (Sigma-Aldrich Chemie Gmbh, Germany) a concentration of 1,5mM (TAN *et al.*, 2011). It was first dissolved in 96% alcohol (1:10). As the molar mass of MeJA is 224,3g/mol, the volumic mass is 1,03g/ml and the product was 95% concentrated, 0,345µl were removed with a micropipette and added to 3,43ml of alcohol. Water was added to reach 1l. Surfactant (Trend®90, Dupond, Belgium) was added (1:10000) to the solution. The solution was sprayed above the plants until it dripped from the leaves.

M. pygmaeus were removed one week after their introduction. On the same day, 50 aphids were added on the highest formed leaf (numbered 2nd) of every forty plants.The aphids were counted on every leaf of every plant after 3 and 6 days.

Results and discussion

Macrolophus pygmaeus and aphid pre-exposition impact

The screen cages were in the same compartment than the big cages containing *M. pygmaeus*, aphids and *Ephestia* eggs.

Three days after the beginning of the experiment, *Macrolophus* were removed, most of them were found dead. The aphids were removed from the plant pre-exposed to aphids with a paintbrush; they were over than 70 in each cage. The plants were watched for five days. Two nymphs appeared and were removed. Some aphids left involuntarily on the plant were also removed, 19 in total.

Five days later, aphids were added. Some may accidentally die because of the manipulation; indeed, if their stylets are damaged they will die (SHARP J.C. & ANDRADE M., 1994).

Reproduction rate

After only 24hours, the results show a big increasing of the aphid population on the plants pre-exposed to aphids. On average after 6days, the total of the aphids on a plant was equal to 767,71 and reached about four times the total on the plants previously exposed to *Macrolophus pygmaeus* (184,14) and the control plants (156,00). The total numbers of aphids per plants depending on the pre-exposition are shown in the figure 41.

The mean daily growth rates calculated over the results after one and six days of the aphids on plant pre-exposed to *M. pygmaeus* and not pre-exposed were respectively 3,14 and 2,59. The aphids on the plants pre-exposed to aphids reached a daily growth rate of 11,86.

The results were analysed with minitab counting by counting. Every population was normal, and the variances of the first counting were equals but not any longer after 1 day. The logarithm was calculated for the two last countings. The variances were equals and the populations were normal. A one-way ANOVA was made for the three countings. The numbers of aphids were very significantly different after 2 hours and became highly significantly different after 1 day.

Table 15: P-values of the One way ANOVA of the total number of aphids depending on the pre-exposition of the plant.

Time after the introduction	2Hours	1Day	6 Days
P-values	0,004	< 0,001	< 0,001

The mean are structured with a Tukey test. After 2 hours, the total number of aphids on the plants pre-exposed to aphids and *M. pyugmaeus* was considered as the same. After 1 day, the number of aphids on the plants pre-exposed to *M. pygmaeus* was the same as the control plants. It stayed unchanged on the 6^{th} day.

The aphids were reproducing faster on the plants previously exposed to aphids. The same phenomenon was already been reported (SAUGE M-H. *et al*, 2002). The aphids are able to manipulate the phloem to make the plant more adapted to their needs. As the plant is more suitable, they can reproduce faster.

A pre-exposition to *Macrolophus pygmaeus* did not have the same effect on the aphids. The reproduction rate stayed unchanged with a pre-exposition to *M. pygmaeus* compared to no pre-exposition. It means that when *M. pygmaeus* is feeding on the plant, the aphid new arrival will grow at a normal rate. However, aphids arriving on a plant previously exposed to aphids will grow much faster than the normal rate and compromise the biocontrol.

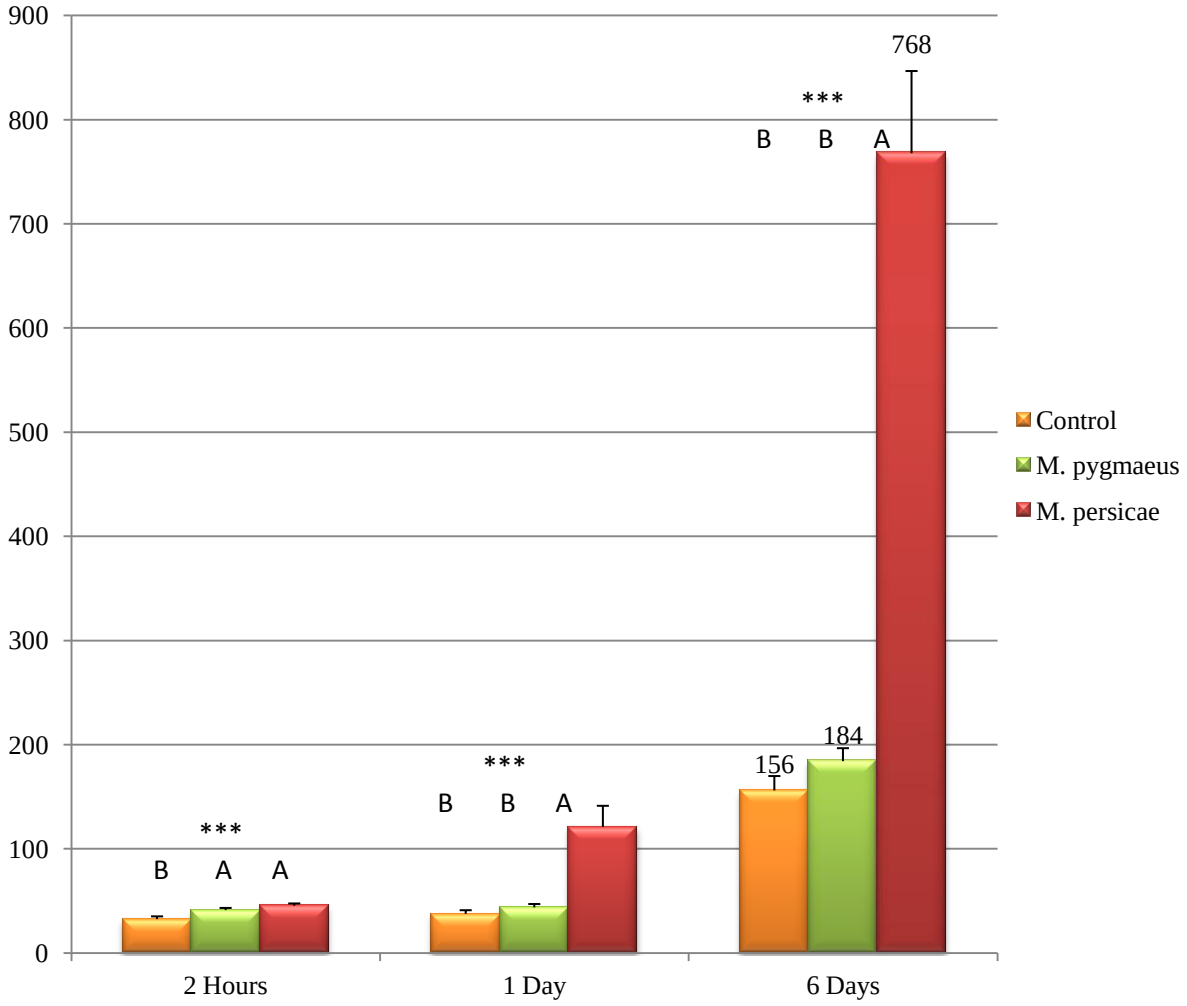


Figure 40 : Mean aphid number per plant previously exposed to *Macrolophus pygmaeus*, *Myzus persicae* and not previously exposed. X = time; y = aphid number; n = 7 for the aphids and *M. pygmaeus* pre-exposition and 6 for the control; *** = p < 0,001. The results sharing the same letter cannot be considered as different.

Aphid behaviour

The mean proportions of the aphids on each leaf of a plant depending on the pre-exposition of the plant after 6 days are shown in the figure 42.

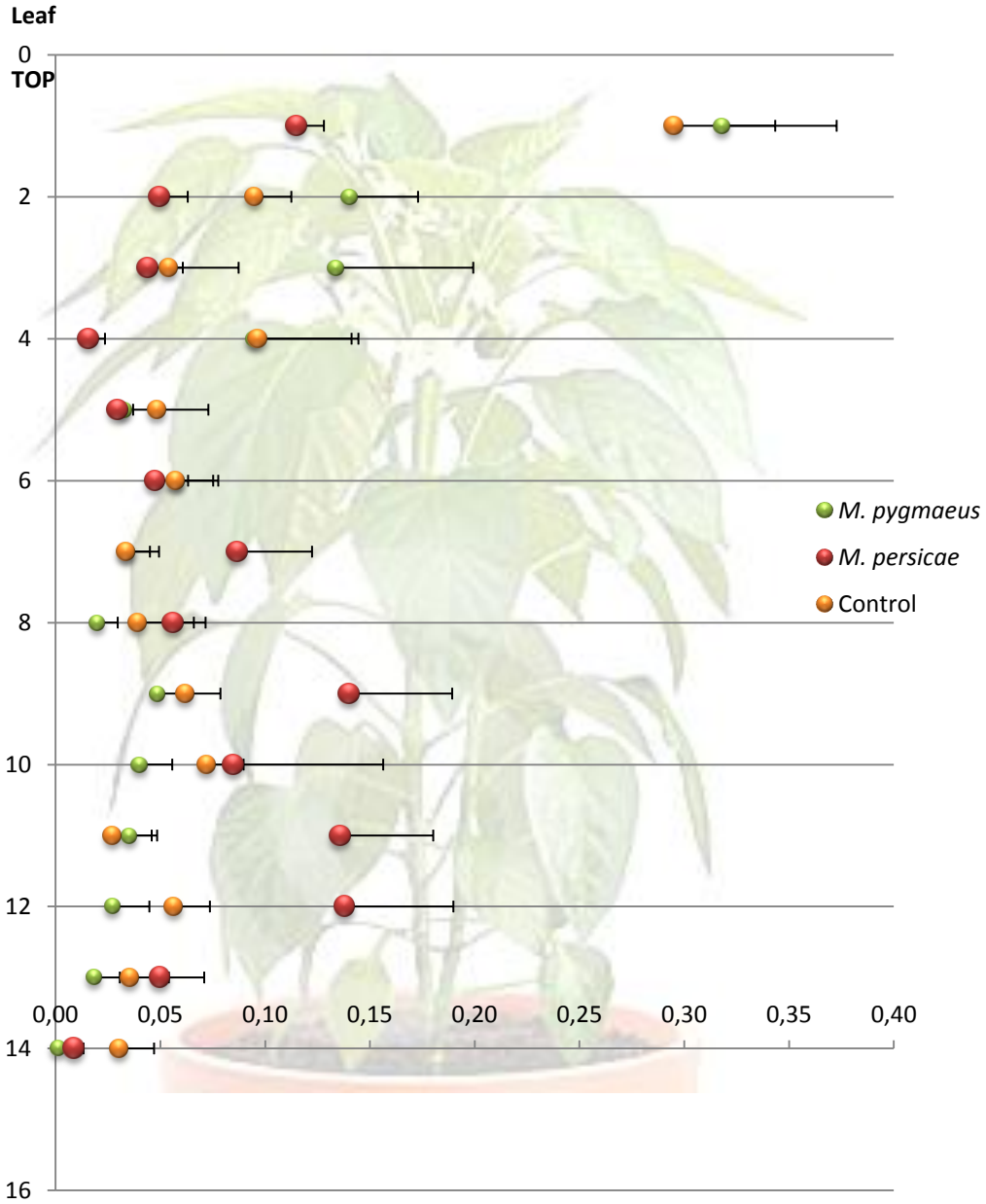


Figure 41 : Mean aphid probabilities on each leaf depending on the pre-exposition of the plant after 6 days. X = the probability; y = number of each leaf with 1 being the top and 14 the cotyledons; n = 7 for the aphids and *M. pygmaeus* pre-exposition and n = 6 for the control plants.

The aphid distribution was analysed with a Chi square test made with minitab. For each counting, the number of aphids on each leaf was summed by pre-exposition. A table per counting of the total number of aphids by leaf depending on the pre-exposition was obtained.

After 2 hours, the aphids were mostly stayed on the leaf they have been released on. The Chi square test shows that the distributions of the aphids on the plants differently treated were not different. Chi square equal to 2,567 was below the critical value 5,99 found in the table with a degree of freedom equal to 2 and $\alpha = 0,05$.

After 1 day, a Chi square test was not possible because many estimated values were below 5. The results were grouped by two leaves except for the first two leaves. The distribution of the aphids on the plants pre-exposed to aphids was highly significantly different compared to the other pre-exposition. Chi square was equal to 360,655 and was higher than 23,69 found in the table with a degree of freedom equal to 14 and $\alpha = 0,05$

After 6 days, the distributions were highly significantly different. The Chi square, equal to 1524,801 was higher than the critical value 38,89 (DF = 26; $\alpha = 0,05$). The p-value of the test was below 0,001.

The values used in the tests are displayed in the table 16.

Table 16: Values used in the Chi square tests of the aphid distribution on the plant depending on the pre-exposition of the plant.

Time	F.D	χ^2	χ^2_{th}	P-value
2 Hours	2	2,567	5,99	0,277
1 Day	14	360,655	23,69	< 0,001
6 Days	26	1524,801	38,89	< 0,001

The Chi square tests were not made based on the same columns number table, so the χ^2 values cannot be compared.

By that time, the aphids on the healthy plants and on the *M. pygmaeus* pre-exposed plants were mostly on the top. The probability for the aphids to be on the top on a plant previously exposed to *Macrolophus pygmaeus* was 0,335, and 0,278 on a control plant. These were the highest probabilities of the aphid position on a plant for these treatments. When the aphids were released on a plant previously exposed to aphids, the highest probability was 0,145 followed by 0,132 associated to the 10^{th} and 14^{th} leaves. The mean proportions of the aphids on the plant differently pre-exposed are shown in the table 17.

Table 17 : mean proportion of the aphids on each leaf on the plant depending on the pre-exposition

	Control	M. pygmaeus	M.persicae
1	0,278	0,335	0,114
2	0,089	0,140	0,049
3	0,048	0,136	0,043
4	0,093	0,085	0,014
5	0,053	0,030	0,031
6	0,052	0,056	0,045
7	0,037	0,033	0,094
8	0,044	0,018	0,063
9	0,064	0,048	0,145
10	0,074	0,039	0,068
11	0,032	0,032	0,132
12	0,062	0,031	0,136
13	0,040	0,017	0,058
14	0,034	0,001	0,009

The aphids are naturally settling on the top of the plant because the young leaves contain more nitrogen (F. WÄCKERS, personnal communication). It was the case on the plants pre-exposed to the predator and on the control plants. On the same time, the plant could defending itself by producing some systemically compounds (DICKE M., 1993) translocated to the top of the plant. The highest concentrations of compounds should be found in the top of the plant because of the accumulation and because the flowers and stems displayed a highly compromised response as compared to leaves (RAGHAVA T. *et al*., 2010). This increasing amount of repellent should make the aphids move down on the plant.

Presumably, as the aphids are suppressing the defences, they stay on the top. One of the possible explanations of the aphid distribution on the plants pre-exposed to aphids is that the plants recovered during the removal of the aphids, and the defences became effective. The aphids from the second infestation were thus moving down. If the distributions were not yet different after 2 hours, it was probably just because the aphids need time to be disturbed by the defences and to move down.

As the aphids did not move down on the plants pre-exposed to *M. pygmaeus*, it would mean that the predator did not induce the defences effective against aphids. The mechanisms and interactions between aphids and plants are largely unknown. Only suppositions can be made to interpret the results.

The combination of the distribution and the high reproduction rate is shown in the figure 43.

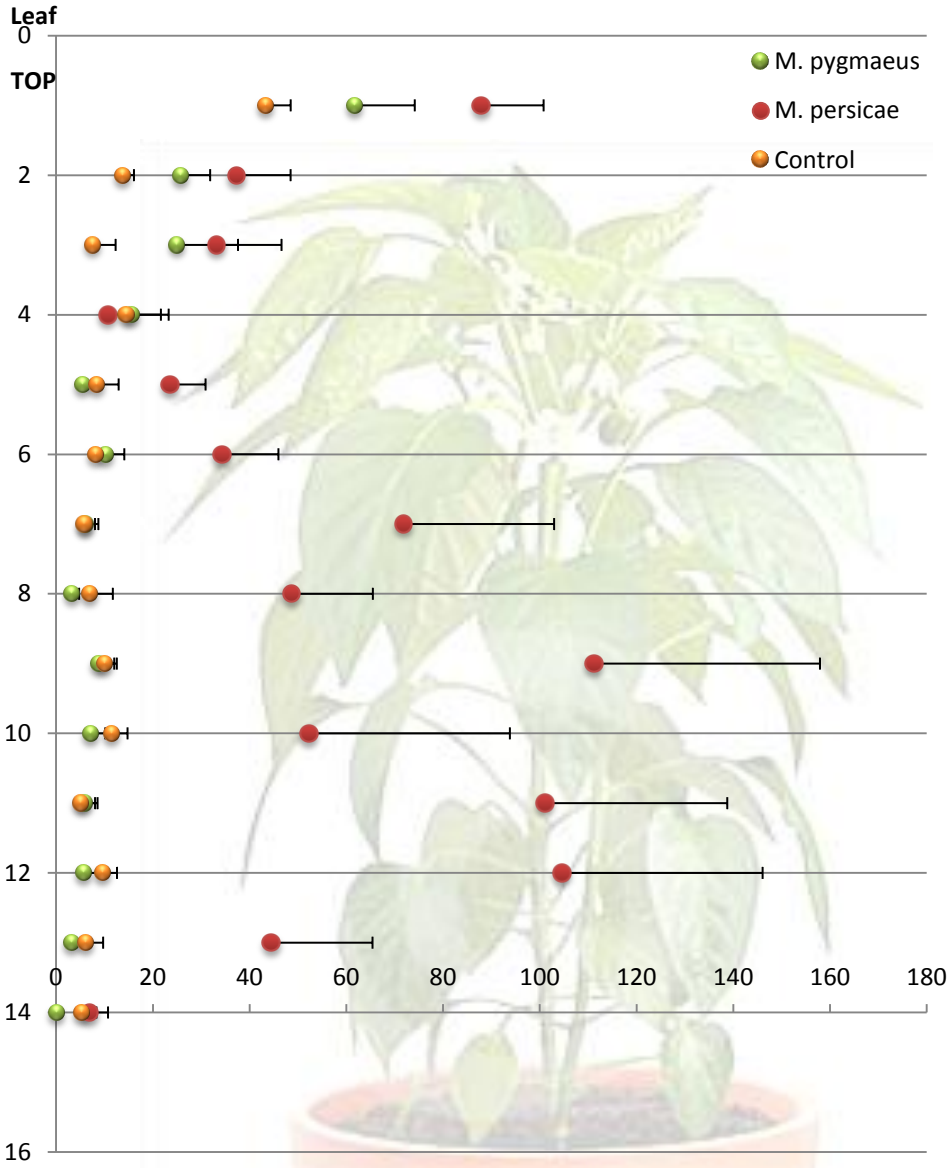


Figure 42 : Mean distribution of the aphids on each leaf depending on the pre-exposition of the plant after 6 days. X = aphid number; y = number of each leaf with 1 being the top and 14 the cotyledons; n = 7 for the aphids and *M. pygmaeus* pre-exposition and n = 6

Suitability

After 6 days, certain leaves became very dried and the results were no longer significant.

The mean living aphid numbers in the plastic boxes are shown in the figure 44.

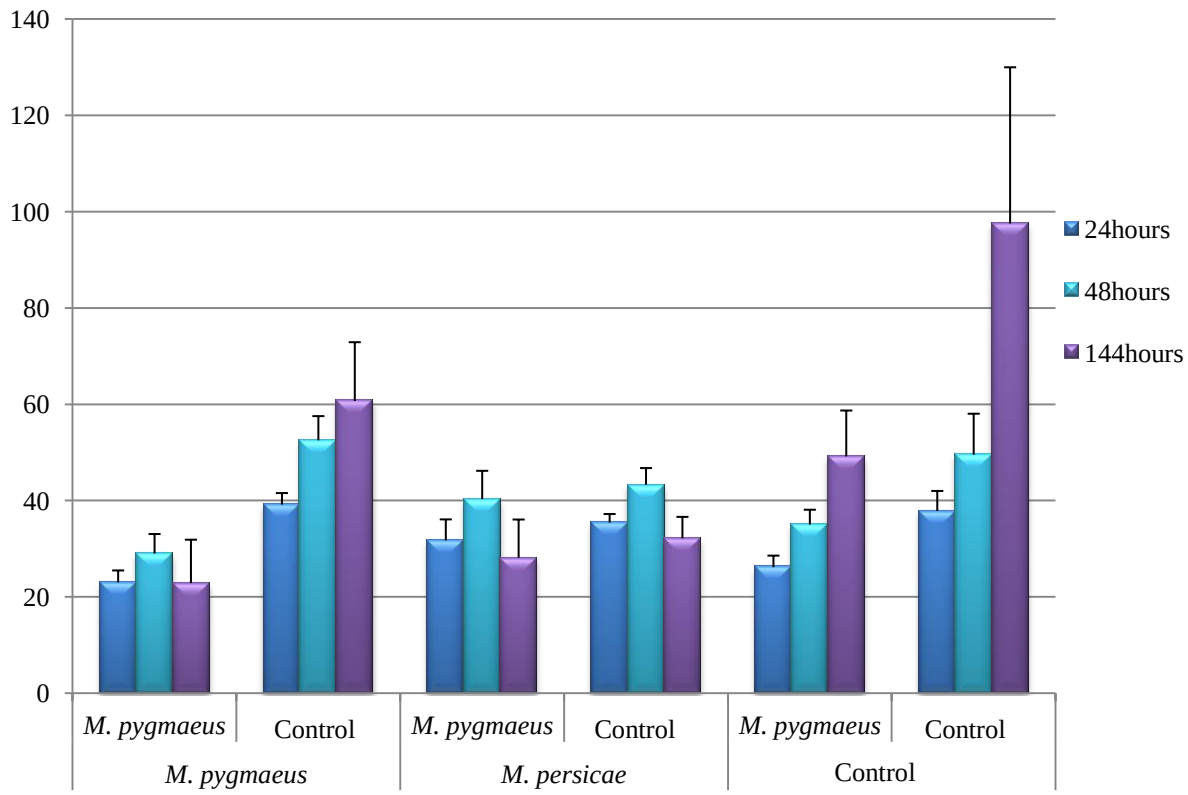


Figure 43: Mean aphid number left in the boxes depending of the pre-exposition of the plants and the predation. X = the predation and the pre-exposition; y = aphid number; n = 7 for the pre-exposition to aphids an *M. pygmaeus* and n= 6 for the control plants.

The number of aphids after 24 hours and 48 hours was lower in the boxes with *M. pygmaeus* than in the control boxes. That is a positive result for the biocontrol use of the predator.

The results were analysed with minitab in a GLM model with two factors: the pre-exposition and the predation. A normality test of Ryan and Joiner was made for every population, 6 in total, 3 pre-expositions and 2 treatments. All the populations were normal. The variances were also equals. As the application conditions were verified the results can be analyzed.

The aphid number in the control boxes and in the boxes with *M. pygmaeus* was highly significantly different after 1 day. After 2 days, the difference became very significative. The aphid number was not different depending on the pre-expositions after 1 day or 2 days. There was not any interaction between the predation and the pre-exposition. The p-values are shown in the table 18.

Table 18: P-values of the GLM analysis of the aphid number depending on the pre-exposition of the plant and the predation.

	1Day	2 Days
Pre-exposition	0,719	0,960
Predation	< 0,001	0,004
Pre-exposition*Predation	0,140	0,160

However, in the graphic (Figure 44), the difference between the boxes with and without predation on aphids from plants pre-exposed to aphids seemed to be very weak compared to the other pre-exposition. That could mean that the aphids of the first infestation suppress the plant defences, manipulate the phloem to make the plant more suitable and they also make the aphids feed on that phloem less suitable for the predators. It is interesting to notice that the interaction between the predation and the pre-exposition was significantly different for 14 %.

The difference between the boxes with predation and the control ones was the biggest with the aphids from the *M. pygmaeus* pre-exposed plants. In other words, the biocontrol was the best when the plants have been pre-exposed to *Macrolophus pygmaeus*. That may be because the predator injects some saliva into the phloem while feeding on the plant. That saliva doesn't influence the aphid behaviour on the plant but could circulate in the aphids feeding on the same phloem and make them more suitable or faster recognisable. Once again, these results were seen on a graph but not significant.

In this experiment, it seemed that *M. pygmaeus* did not have an indirect impact on the aphid population. But, it is important to remember that most of the predators were found dead at the end of the pre-exposition time. It is possible that the predators are inducing some defences but without having an effect on aphid reproduction and behaviour. It is also possible that they died quickly after their introduction before they fed on the plant or before they fed enough on the plant to induce some defences. Indeed, the *M. pygmaeus* contained in the product "Macrolophus-System" are stored in cold room before being sold, this could have weakened them. Also, as the plant doesn't provide the same quality of food than prey, they could have been too weak to survive only feeding on the plant. This experiment has to be done again with *M. pygmaeus* from laboratory rearing.

Doubts are set about the susceptibility of the aphids to the predation when fed on plant pre-exposed to aphids. The previous results were not significant but the aphids seemed to be protected from the predator by an earlier infestation. The experiment has to be done again to ensure the results and definitely banish this new problem about aphids or to begin to study this ability.

Aphid pre-exposition impact on the aphid suitability

As this experiment were made to clarify the results about *M. pygmaeus* predation on aphids from plant pre-exposed to aphids, the results about aphid reproduction and behaviour are displayed but only briefly analyzed.

The mean aphid total numbers on plant pre-exposed to aphids with and without removal after 6 days were the same. They also were equals to the mean aphid total number on the plants pre-exposed to aphids from the first trial.

The total of aphids on a aphid pre-exposed plant with removal was equal to 897,5 and to 718,2 without removal. The total was equal to 767,7 aphids on the aphid pre-exposed plant in

the first trial. On the control plants, the aphids reached a number of 156 per plant in the first trial and 120,6 in the second trial. The results are shown in the figure 45.

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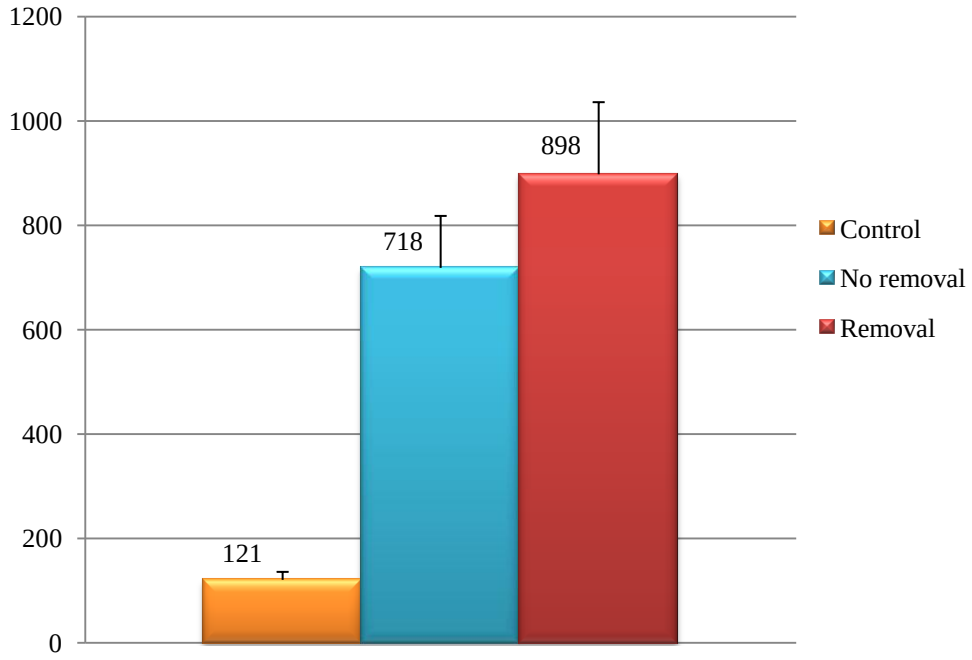

Figure 44 : Mean aphid number per plant depending on the treatment after 6 days and standard error. Y = aphid number; n = 10.

The results of the distribution of the aphids on the plants (Figure 46) were not as strong as in the first trial. But the probability to find aphid on the top of the aphid pre-exposed plant with removal was still below the probability of the other plants. The aphid distribution on the aphid pre-exposed plant without removal was quite the same as the control plants.

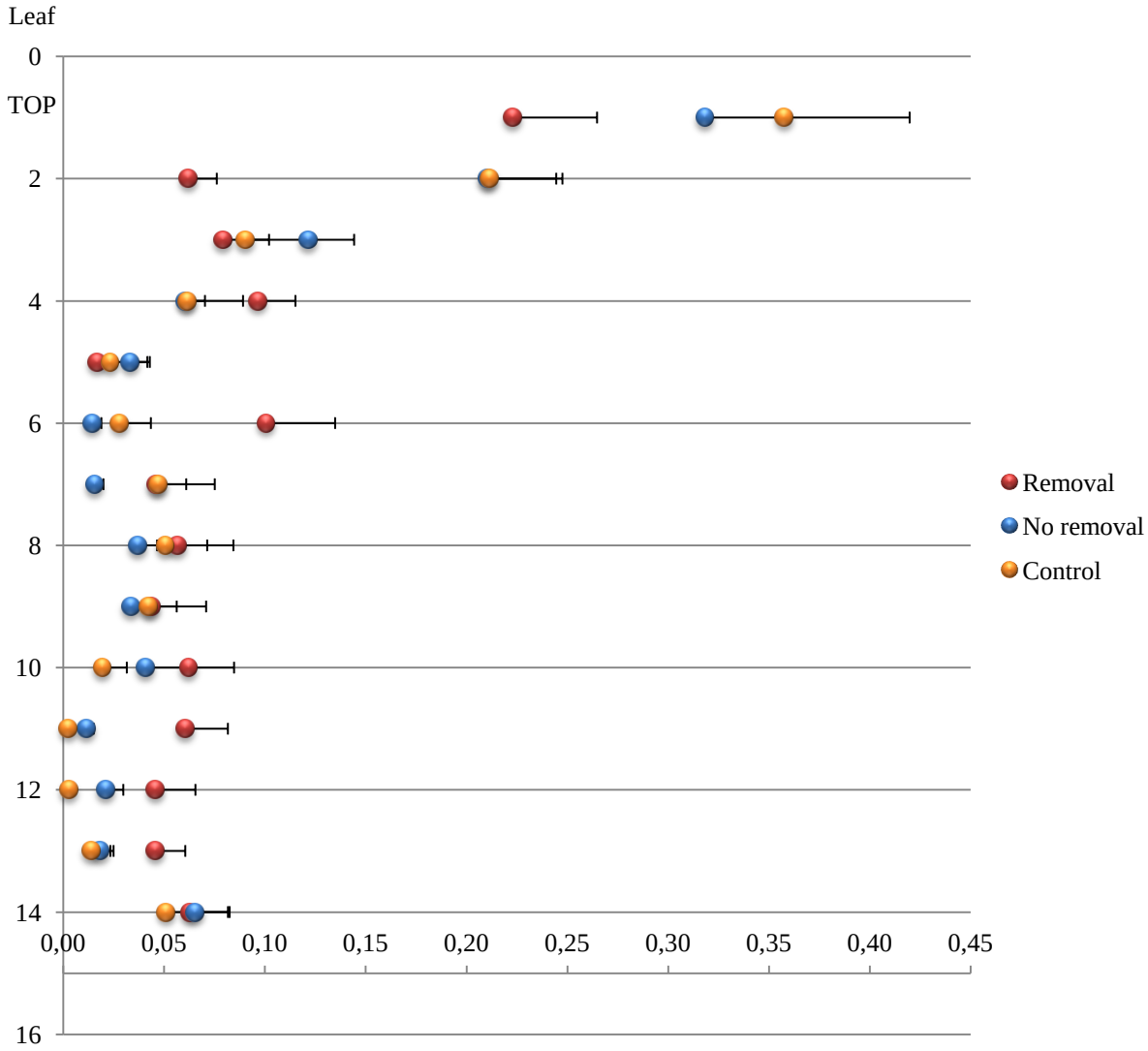

Figure 45 : Mean aphid probabilities on each leaf of plants pre-exposed to aphids with and without removal and control plants after 6days and standard error. X = probabilities; y = leaf number; n = 10.

A Chi square test confirms that the distributions were highly significantly different. The values are displayed in the table below.

Table 19: Values used in the Chi square test of the aphid distribution on the plants depending on their pre-exposition.

Time	F.D	χ^2	χ^2_{th}	P-value
6 Days	26	2496,917	38,89	< 0,001

The χ^2 value can not be compared to the value obtained in the first trial of the second section. The replication numbers were not the same, making the aphid number sum much higher in this trial.

The results for the suitability are shown in the figure 47. The results were analysed with minitab. The normality of the populations was verified. One of the populations was not

normal. When the results were transformed in logarithm or cosinus, more populations were not normal. The results were kept without transformation. The variances were equals. The variance was analysed by a Balanced Anova model with two factors, predation and pre-exposition. There were no significant difference between the pre-exposition neither between the presence and the absence of predation.

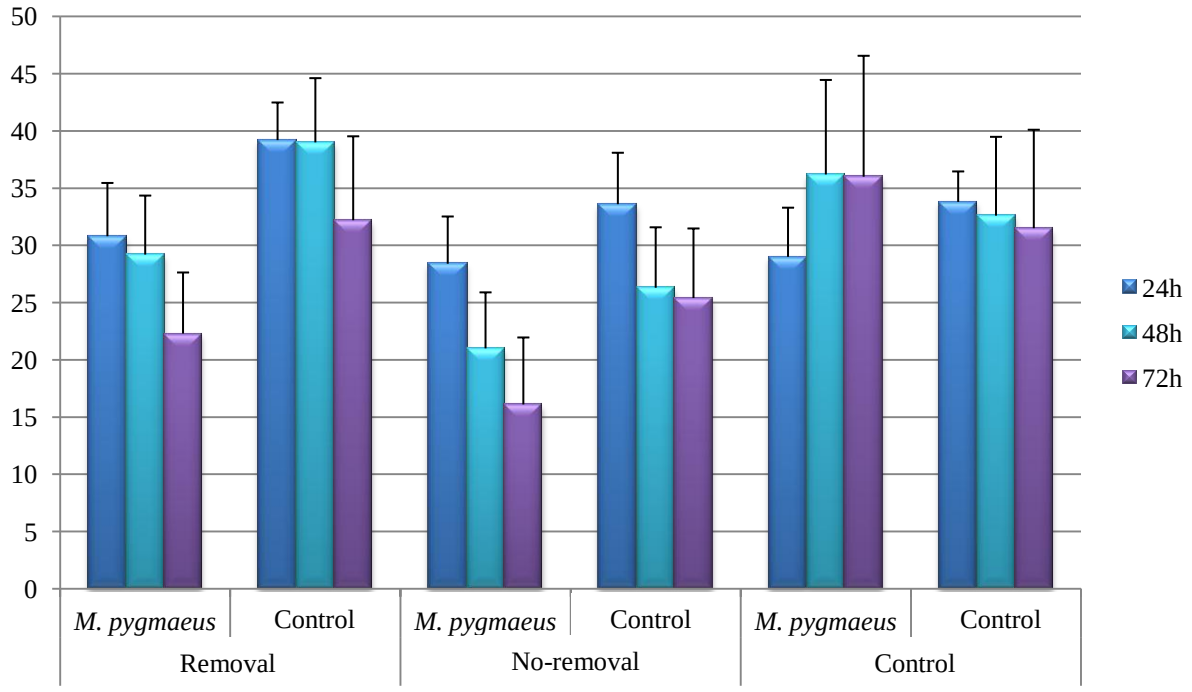

Figure 46 : Mean aphid number with and without predation depending on the pre-exposition of the plants and standard error. X = predation and pre-exposition; y = aphid number; n = 10.

However, the mean aphid number decreased in every pre-exposition with and even without predation except for the boxes containing aphids from control plants with predation. These results were not logical. Something must have gone wrong with the experiment.

The fact that the aphid distribution was the same for the control plants and for the aphid pre-exposed plants without removal could mean that the aphids were suppressing the defences of the plant. These two treatments only differ by the time the aphids spent on the plant; 6 days for the control plants, 14 for the "no-removal" treated plants. As the aphids were found on the top of the plant, it presumably means that the defences were not expressed. However, the aphids from the second infestation presented a different distribution on the plant in both trials. This suggests that they were subject to the plant defences and that the plant was probably recovering during the removal, and expressed its defences while the aphids are introduced the second time. The same aphid total number per plant for all the aphid pre-exposed plants supposes that aphids are making the plant more suitable and that the plant can't undo it during the removal. The aphid manipulation of the phloem probably takes longer to be undone thant 3 days.

Plant defences induction by *Macrolophus pygmaeus*

At the time of the predator removal, 9 cages were found empty out of twenty. On average, 1,5 ± 0,43 female predator and 1,1 ± 0,46 male predator were found alive on the plant. The detailed results are displayed in the annexes.

With regard to aphid development, firstly the aphid total number per plant was analysed to test for differences in their overall development. An analysis was made for each count. Every population was normal but the variances were not equals. The logarithm of the results was thus calculated and equalised the variances. A One-Way Anova was made. Results of both July 2nd (three days after aphid inoculation) and July 5th (6 days after inoculation) were very significantly different. A Tukey structuration of the means divided them in two groups. A first one with the control plants and the plants pre-exposed to males and a second with the control plants and the plants pre-exposed to methyl jasmonate and females. An analysis with repeated measures Anova does not change anything with only to countings.

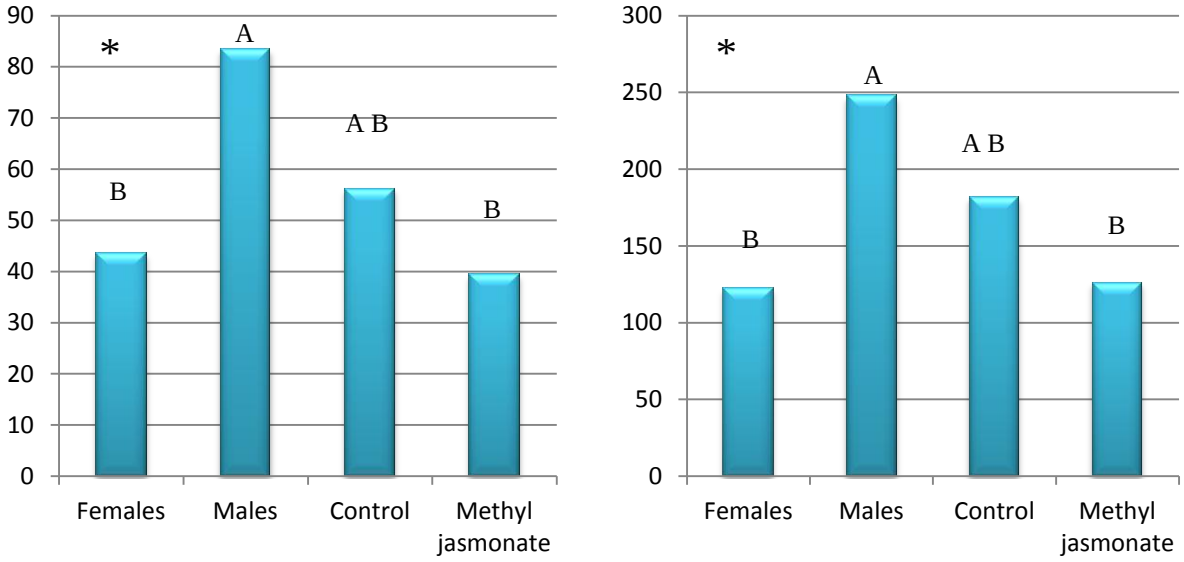

Figure 47: Total aphid number depending on the treatment for the count of July 2nd (left) and July 5th (right). X = aphid number; Y = plant pre-expositions. The results sharing the same letter cannot be considered as different.

The p-values of the analysis are displayed in table 20.

Table 20: P-values of the one-way Anova model analysis of the aphid total number on the plant depending on the pre-exposition.

Time	P-values
July 2nd (3 days)	0,012
July 5th (6 days)	0,021

The mean aphid distribution on a plant depending of the pre-exposition is shown in figure 49.

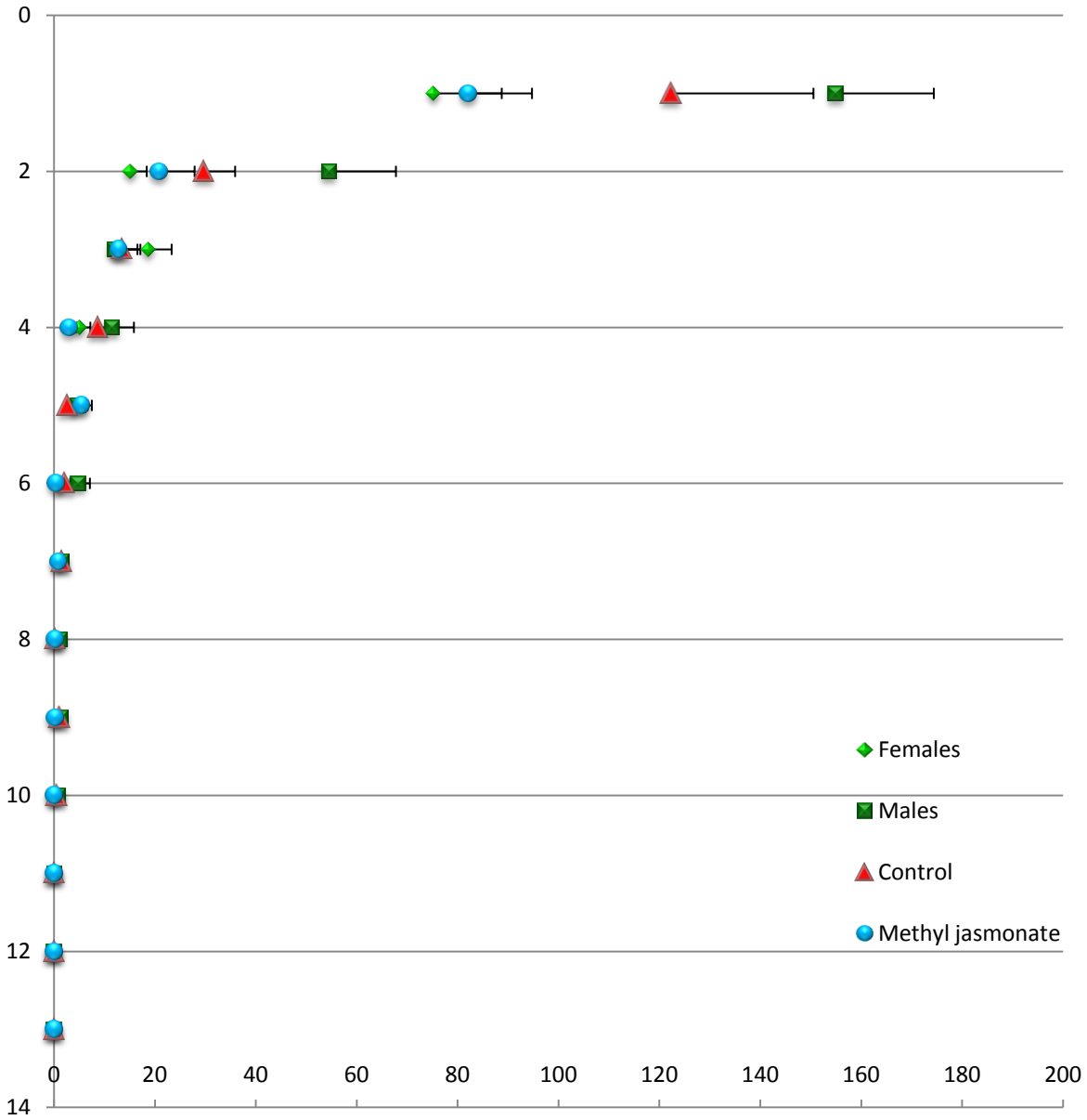

Figure 48 : Mean distribution of the aphid on a plant depending on the pre-exposition of the plant after 6 days. x = mean aphid number; y = leaf number, 1 being the top and 13 the cotyledons; n =10; black line = standard error.

Figure 49 represents both aphid proportions on each leaf and aphid abundance, as it shows the aphid mean number. Figure 50 represents only the aphid proportions on every leaf.

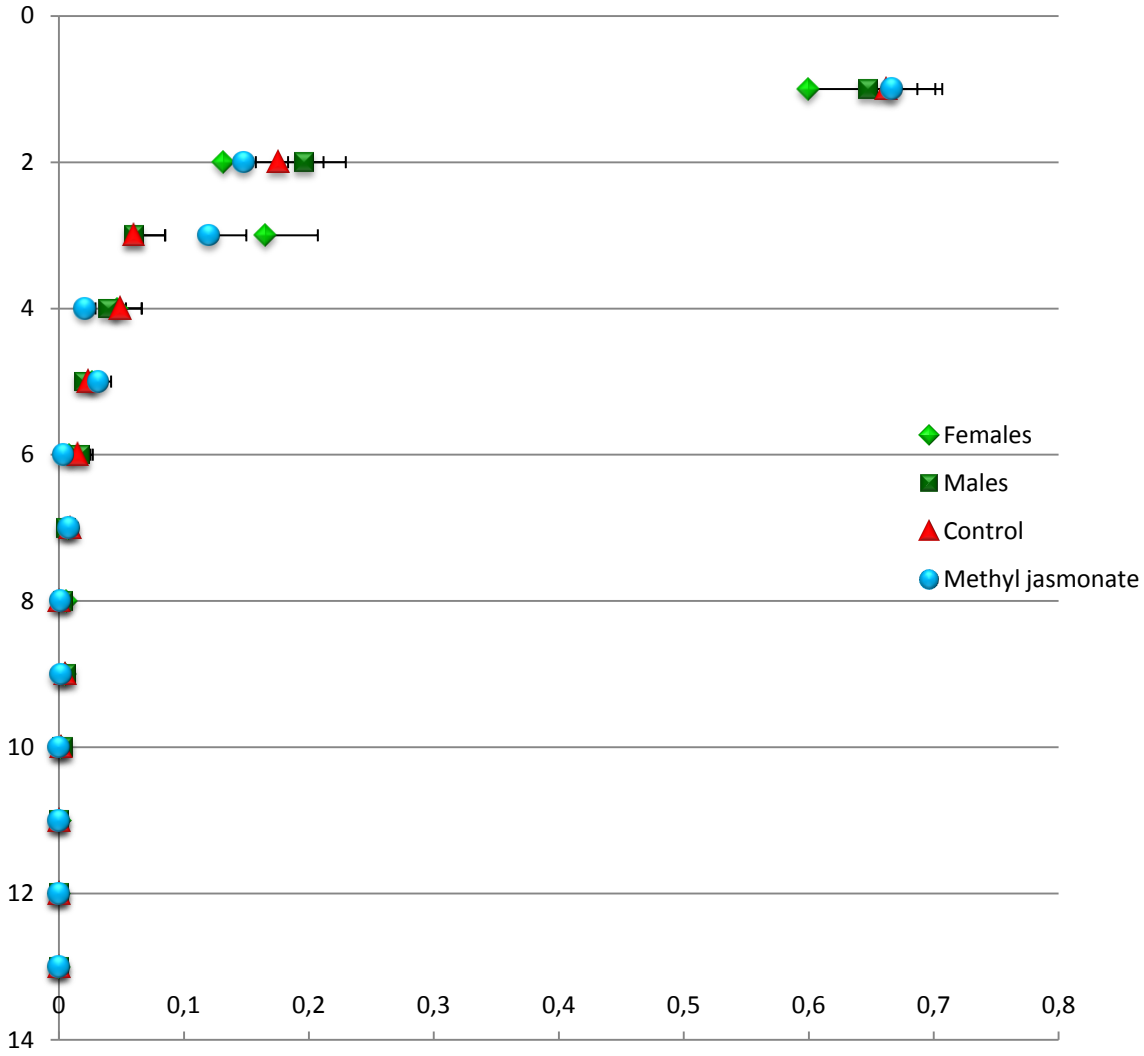

Figure 49: mean probabilities or aphid proportion on each leaf depending on the pre-exposition of the plants after 6 days. X = probability; Y = leaf number, 1 being the top of the plant and 13 the cotyledons; n = 10, black lines representing the standard errors.

A Chi square test was made on that table to see if the distributions were different depending on the pre-exposition. The total of aphids per leaf for all the plant was made. As few aphids were found on the lasts leaves, for the count of July the 2nd the aphids on the leaves 9, 10, 11, 12 and 13 were summed. For the count of July the 5th, the results of the leaves 10,11,12,13 were also counted together. Both Chi square tests indicate a difference highly significative depending on the pre-exposition. The χ^2 calculated were both above the theoretical values. The table below displays the values of the tests.

Table 21 : Values used for the Chi square tests.

Date	F.D	χ^2	χ^2_{th}	P-value
July 2nd	24	165,422	36,42	< 0,001
July 5th	27	249,303	40,11	< 0,001

An Anova analysis could not be made because the aphid development was different depending on the treatment and would influence the results of the distribution analysis. An Anova analysis could be made on the results transformed into probabilities. But this transformation makes the analysis loses its robustness.

To find out where were the differences in the distributions, several Chi square tests must have been done by removing one leaf after another until the test showed the same distributions for every pre-exposition. The table 22 helped to know in which order to remove the leaves. The standard deviation was calculated to know which leaves presented the most different probabilities.

Table 22: Mean probabilities of the aphid position on the plant depending on the pre-exposition.

Leaf	Females	Males	Control	Methyl jasmonate	Standard deviation
1	0,599	0,648	0,662	0,666	0,0307
2	0,132	0,196	0,175	0,148	0,0286
3	0,165	0,060	0,060	0,120	0,0512
4	0,047	0,040	0,049	0,021	0,0128
5	0,027	0,020	0,023	0,031	0,0049
6	0,008	0,017	0,015	0,004	0,0062
7	0,007	0,006	0,009	0,008	0,0012
8	0,006	0,004	0,000	0,001	0,0026
9	0,005	0,006	0,005	0,001	0,0020
10	0,002	0,003	0,002	0	0,0013
11	0,001	0,0003	0	0	0,0006
12	0	0	0	0	0
13	0	0	0	0	0
Total	1	1	1	1	0

The leaves were removed from the test by the order of the probabilities. After, several combinations are tested to ensure the results. the combinations tested and the values used in the different tests are displayed in table 23.

Table 23: Leaf combinations tested with a Chi sqaure tes and the values used in the different tests.

Leaves removed	F.D	χ^2	χ^2_{th}	P-value	Leaves left
3	24	129,986	36,42	< 0,001	1;2;4;5;6;7;8;9;10;11;12
3 ;1	21	102,711	32,67	< 0,001	2;4;5;6;7;8;9;10;11;12
3; 1; 2	18	82,933	28,87	< 0,001	4;5;6;7;8;9;10;11;12
3; 1; 2; 4	15	61,210	25	< 0,001	5;6;7;8;9;10;11;12
3; 1; 2; 6	15	63,673	25	< 0,001	4;5;7;8;9;10;11;12
3; 1; 2; 5	15	26,695	25	0,031	4;6;7;8;9;10;11;12
3; 1; 2; 4; 6	12	34,452	21,03	0,001	5;7;8;9;10;11;12
3; 1; 2; 4; 5	12	22,488	21,03	0,032	6;7;8;9;10;11;12
3; 1; 2; 5; 6	12	17,655	21,03	0,127	4;7;8;9;10;11;12
3; 1; 2; 4; 6; 5	9	13,470	16,92	0,142	7;8;9;10;11;12
3; 1; 2; 4; 6; 5; 7	6	8,456	12,59	0,207	8;9;10;11;12
3; 1; 2; 4; 6; 5; 8	6	5,578	12,59	0,472	7;9;10;11;12

These tests showed that the aphid distribution can be considered as the same on the leaves under the 7th and including the 4th. Whereas the standard deviation between the mean probabilities of the 4th leaf was higher than of the 5th and 6th leaves, the 4th seemed to play a minor role in the difference between the 4distributions. Probably because of the standard deviation calculated over every replication.

A pre-exposition of the plants to male predators favoured the aphid development compared to a pre-exposition to female predators or methyl jasmonate. However no difference was detected between a female pre-exposition and the control plant. It cannot be tell that females permit to decrease the aphid development rate, only that they decrease it comparing to a pre-exposition to male. The distribution of the aphids on the plant was different depending on the treatment. This difference placed from the plant top until the 6th leaf except for the 4th. According to the Chi square tests and the graphics of the mean aphid distribution and the mean probabilities, it seemed that a pre-exposition to females makes the aphid leave the top of the plant. Indeed, in those plants, the aphids preferred to settle on the third leaf than on the buds and youngest leaves as they usually do.

This impact on aphid was probably caused by a difference of secretions transmit or let on the plant between male and unmated female *M. pygmaeus*. Pheromones would have been volatised faster than 6 days ant the impact on aphid would have be decreasing and not increasing like it was the case. On the other hand, when feeding, *M. pygmaeus* does not reach the phloem whereas the aphid does. The secretions stay at the same place and might be swallowed by the aphid trying to reach the phloem by injecting and taking back its saliva. But that means that to have an impact the aphid have to feed on the exact same spot that the predator did. It is more likely a secretion let on the plant leaves.

Conclusion

The experiments conducted for this study improve the knowledge about the ability of *Macrolophus pygmaeus* to control aphid on bell peppers and about the strategy to adopt to achieve a good level of biocontrol in greenhouses.

M. pygmaeus is efficient on small aphid introduction on one case out of 4. Indeed, the results of the two first experiments matched. On the first, in 3 cases out of 4 in two different treatments, the aphids were nearly eliminated after 10 days, only one or two were left in some cages. In the last treatment, the aphids were gone in every cage. Thus, no matter the treatments, which turned out didn't influence the predation on aphid, in total in two cases out of 12 the aphid were not under control. In these two cases, the densities of predator were 4 per plant and 7,5 per plant. The second experiment proved that also in 1 case out of 4 with these densities, the control didn't work. Probably because these colonies expanded faster. Indeed, the results from the control cages from the first and the second experiment showed that both time one out of the four colonies developed faster. Only a density of 12 predators per plant managed to control every colony. Obviously, none producer will use twelve predators per plant in a crop to ensure the elimination of the aphids.

Fortunately, *Macrolophus pygmaeus* is efficient on small introduction of ten aphids on 75% with a density of only 4 individual per plant. For the 25% left, the aphid development was slowed by *M. pygmaeus* presence. Moreover, in crop situation, the predators from the healthy plants around the infested plants will probably be attracted by the prey, increase their density by moving and, this way, improve the control of the aphid.

The attraction of *M. pygmaeus* by the aphids should be investigated.

On higher populations of aphids (50 and 200 individuals), *M. pygmaeus* reduced slightly their development. Unfortunately, the development reduction is not strong enough to stay below an acceptable economic threshold.

A second diet made of *T. vaporariorum* or *Ephestia* eggs didn't influence *M. pygmaeus* predation on aphids.

The best strategy to use *Macrolophus pygmaeus* in greenhouse is the prevention. The population is built up quickly and easily. New born nymphs appear after two to three weeks, so a rearing of a month is sufficient to obtain enough individuals to control an introduction of aphids. The *Ephestia* egs are used to feed the predators during the rearing. When an aphid establishment is noticed, the food providing should be stopped for economical reasons. In high densities, the predators could damage the plant. But because of the cannibalism, the predators maintain riskless densities. When the predator number gets below the threshold of 4 individual per plant, food should be providing again.

The *Ephestia* eggs remain very expensive but some studies are being made to evaluate meat-based diet (CASTAÑÉ & ZAPATA, 2005) or the use of pollen as supplementary food to reduce

the *Ephesta* eggs amount (VANDEKERKHOVE & DE CLERCQ, 2010) to rear *M. pygmaeus*. A study evaluated the *Ephestia* eggs amount needed per *M. pygmaeus* per 3 days equal to 40 eggs (VANDEKERKHOVE & DE CLERCQ, 2010). Knowing this, the exact amount can be provided without waste. The *Ephestia* eggs must be applied uniformly on the crop to achieve a better dispersal of the predators (PUT *et al.*, 2010) and a higher probability to detect earlier an aphid introduction.

In a curative way, 4 *M. pygmaeus* per plant should be enough to control a small introduction of aphids, given the fact that they should move to the infested plants. Unfortunately, the predators seemed to be quickly overwhelmed when the aphid number increases. Further researches should continue the investigation about higher aphid population, and the establishment of a relationship between the aphid number and the predator density needed to control them.

The use of *Macrolophus pygmaeus* as biocontrol agent should be incorporated in an integrated pest management strategy such as physical methods to prevent the aphid introduction.

The temperatures are important to be monitored and controlled, the mortality of the predator increases very fast with the temperature. However, the predators seemed to be very effective in maintaining themselves without any food or water.

A pre-exposition of the plant to *Macrolophus pygmaeus* had an influence on the aphid development and distribution on the plant. The aphids on plants pre-exposed to males developed faster whereas the aphids on plants previously exposed to females developed at a lower rate compared to each other. The statistics did not allow considering these pre-exposition impacts different from the control plants. The aphids also seemed to be bother by a pre-exposition to unmated females. Indeed, they prefer to settle on the third leaf than on the top of the plant as they usually do, still compared to a pre-exposition to males.

Naturally, females and males occur in the same time cancelling both effects. Indeed, *M. pygmaeus* reproduce only by mating. A population made of males or females only couldn't maintain itself. Moreover, this study investigated a pre-exposition to unmated female which may induce a different effect on aphid than mated females. In natural situation, females stay virgin for maximum 3 days.

Anyway, it would be interesting to find out the difference in, probably, the secretions between males and unmated females. The compounds responsible for that effect could be applied on plants to reduce the aphid development.

Even if this trial was not about aphid, the second part of the investigation gives information about their relation with the plant. Indeed, a pre-exposition of the plant to aphid allows a second infestation, arriving 5days after the removal of the first infestation, to develop faster. This fact is likely due to their manipulation of the phloem. The aphids are able to suppress the plant defences, allowing them to establish on the top of the plant. It also seemed that during

the time of the removal, the plant recovered and manage to express some defences forcing the aphid of the second infestation to move down of the plant. Further researches should evaluate the time needed between to infestations to avoid these effects and also investigate the way to counteract the phloem manipulation.

Anyway, being phytopagous, *Macrolophus pygmaeus* should induce some plant defences. This study proved that its induction doesn't influence the biocontrol of the aphids in natural conditions. However, further researches should determine which defences are induced and which insect they could influence.

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Annexes

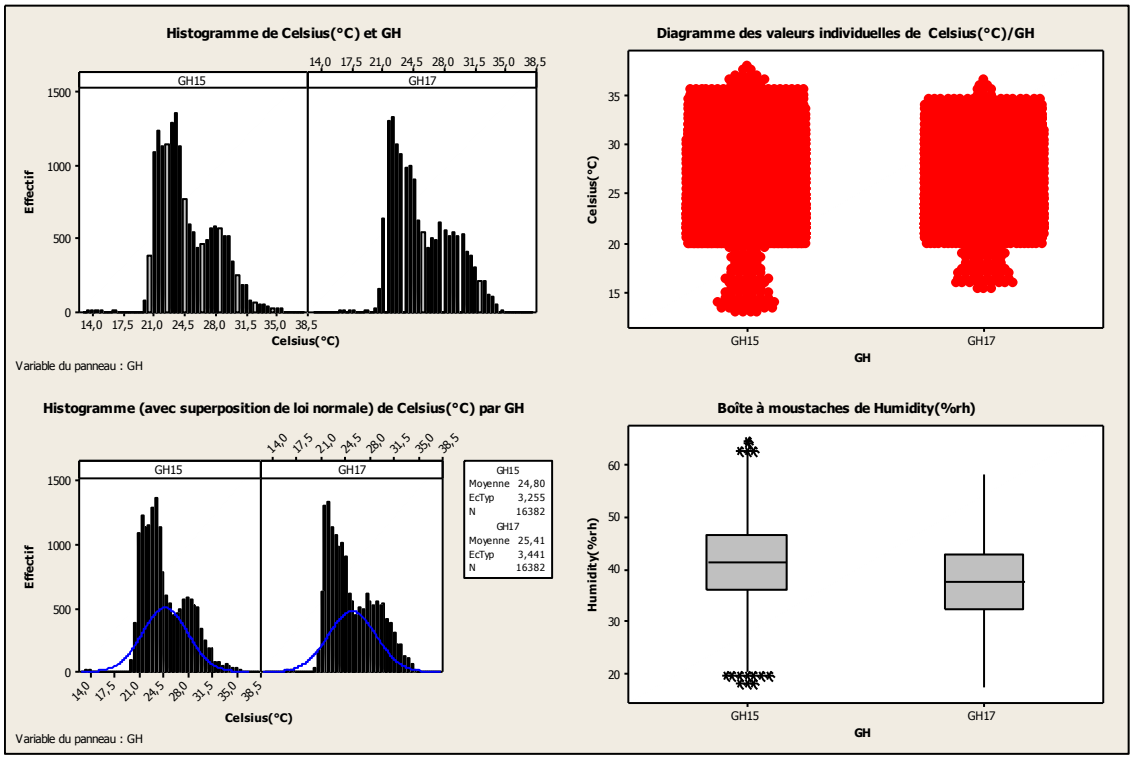

Figure 50 : statistics graph of the temperatures in the greenhouses

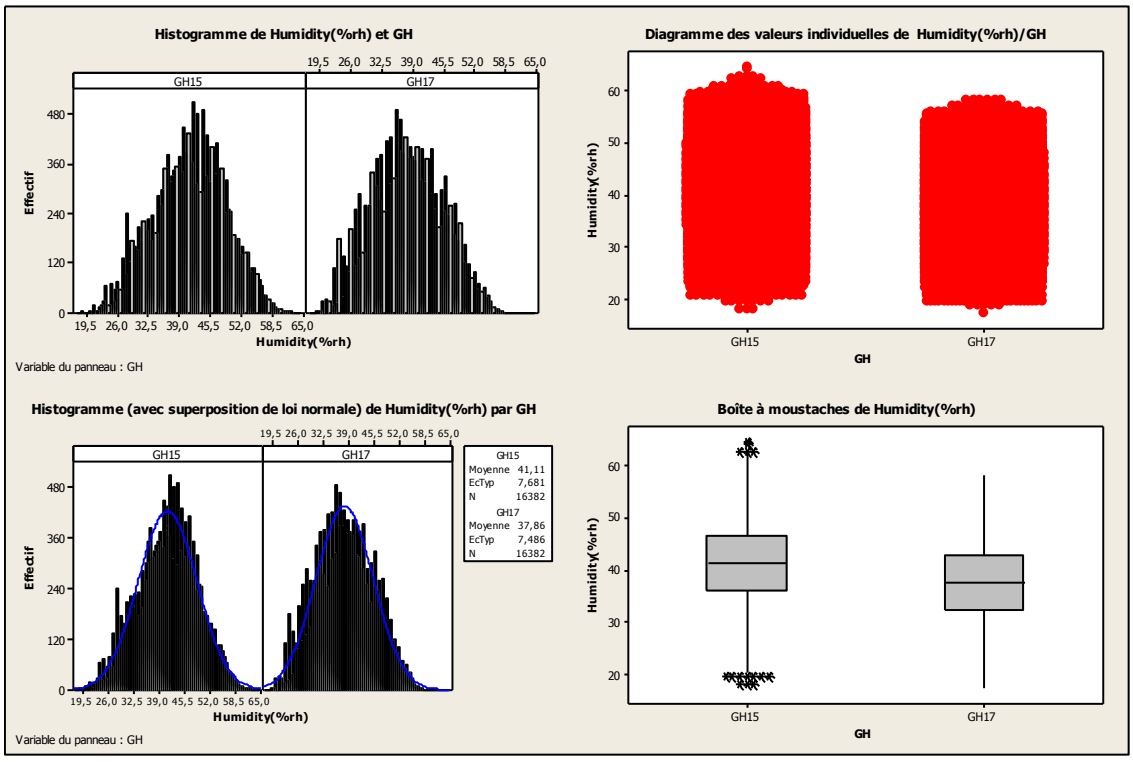

Figure 51: statistics graph of the humidity in the greenhouses

Table 24: *M. pygmaeus* number found in the net cages one week after their introduction for the experiment about the plant defence induction.

Treatment	Cage	*M. pygmaeus*
Females	1	3
Females	2	0
Females	3	2
Females	4	3
Females	5	3
Females	6	2
Females	7	0
Females	8	0
Females	9	0
Females	10	2
Males	1	1
Males	2	3
Males	3	0
Males	4	0
Males	5	4
Males	6	2
Males	7	1
Males	8	0
Males	9	0
Males	10	0
Females	**Mean**	1,5
Males	**Mean**	1,1
Females	**S.E.**	0,43
Males	**S.E.**	0,46