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# Promoting mixed genotype infections in CpGV: analysis on field and laboratory sprayed apple leaves

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The success of biological control of the codling moth, *Cydia pomonella*, with commercial products based on *C. pomonella* granulovirus (CpGV) isolates relies both on the ingestion of a sufficient amount of occlusion bodies (OBs) between their hatching from the egg and the penetration inside the fruits, and the correct replication of the virus in the insect larvae. Larvae resistant to one isolate, CpGV-M are frequently found in European orchards. In recent years, efforts have been devoted to obtaining virus isolates able to control resistant insects, among them, CpGV-R5. Ingestion of CpGV-M by a resistant larva does not allow replication of CpGV-M. Ingestion of CpGV-R5 results in virus replication and larval death. Ingestion of a mixture of CpGV-M and CpGV-R5 allows replication of both genotypes and better insect control. Both CpGV-M and CpGV-R5 are present in the commercial product Carpovirusine® Evo 2 (NPP, Arysta LifeSciences). No recent data have been collected concerning the actual amount of virus ingested by the neonate larvae. As mixed infection CpGV-M and CpGV-R5 provide better protection, this is an interesting property to explore to improve the strategy of treatment in orchards. We analysed the distribution of virus droplets on leaves for two orchards using two different dispersion techniques and leaves sprayed in laboratory conditions. We observed the mortality of larvae allowed to feed on these contaminated leaves for selected amounts of time. We then used High resolution melting (HRM) to estimate the occurrence of each genotype alone and mixed genotypes in individual larvae.

## KEYWORDS

Baculovirus; resistance;  
synergism; genotype  
diversity; biological control

## Introduction

Following its discovery in the 1960s (Tanada, 1964), the Mexican isolate of *Cydia pomonella* granulovirus (CpGV-M) was distributed to various laboratories in the world and tested for controlling the codling moth (CM), *Cydia pomonella* populations in orchards (Huber, 2007). After proving their efficiency, various CpGV-M-based bioinsecticides were progressively homologated. In Europe, during the '90s the orchard area protected with these products increased to more than 1,00,000 ha, about 20% being organic orchards (Berling et al., 2009). The Lethal dose 50 (LC<sub>50</sub>) of CpGV-M to first instar CM larvae is below 5 Occlusion Bodies (OBs) in laboratory conditions (Harvey & Volkman, 1983;

Huber, 1986; Sheppard & Stairs, 1977). Field tests were carried out to determine the appropriate doses to be applied in the field. Doses of  $9 \times 10^{13}$  OB/ha resulted in almost complete control (Glen & Payne, 1984). Reduction in the doses 100 times did not reduce significantly the mortality in bioassays, but resulted in a rapid increase in CM damage (Huber & Dickler, 1978), due to delayed death. The doses preconised nowadays by the bioinsecticide producers ranges between  $3 \times 10^{12}$  and  $1 \times 10^{13}$  OB/ha.

CM females oviposition behaviour varies in function of the fruit development. In the first generation, females lay eggs mainly in the upper side of leaves (75%) close to the fruits, or on fruits surfaces (18%) (Subinprasert & Svensson, 1988). In the following generations the proportion of eggs laid on the fruit surface increases (Burgerjon, 1986). Neonate larvae move towards the fruits, biting on leaf and fruit surfaces, before entering into the fruit (Ballard et al., 2000). They become contaminated when biting (Glen & Clark, 1985).

A recent work concludes that the majority of eggs (90%) are laid within 10 cm of the fruit, and more than 50% of eggs are laid onto the leaves close to the fruit (Wearing, 2016). Newly hatched larvae move towards the fruit, ingesting the virus while biting on leaves. Geier observed that neonate larvae penetrate the fruit between 10 min and 24 h after hatching (Geier, 1963).

From 2005, a resistance specific to CpGV-M has been observed in European orchards (Schmitt et al., 2013). The replication of CpGV-M is blocked while that of other genotypes is not compromised (Asser-Kaiser et al., 2011). Nowadays, commercial product as Madex<sup>®</sup> Pro or Carpovirusine<sup>®</sup> Evo2 is composed of several genotypes, the major components are two viral genotypes, group A (CpGV-M) and group E (CpGV-R5) (Jehle et al., 2016). The presence of more than one genotypic variant in a single CM larva has been observed in nature (Rezapanah et al., 2008), but the frequency of such an event is not known. It has been recently observed that mixtures of CpGV-M and CpGV-R5 allow replication of both genotypes in resistant or susceptible larvae (Graillot et al., 2016). The determination of the frequency of single or double infections in CM larvae following treatment of orchards can be determined using the high-resolution melting point approach (Hinsberger et al., 2019).

In this paper, we describe the results obtained using leaves collected after orchard treatment and compare them with leaves sprayed in the laboratory.

## **Material and methods**

### ***Insect rearing***

CpNPP is our reference laboratory colony susceptible to CpGV-M. It originates from the Northern part of France, and it has been maintained in the laboratory for almost 30 years. It is used for the industrial production of Carpovirusine<sup>®</sup> and has been provided by Natural Plant Protection SA (Pau, France) (Graillot et al., 2016).

### ***Larvae infection***

To compare laboratory and field conditions, larvae were infected with leaves from apple orchards (sprayed with Carpovirusine<sup>®</sup> Evo2 and collected immediately after) or leaves sprayed in the laboratory.

Leaves treated with Carpovirusine® Evo2 were collected in two apple orchards in Lamotte-du-Rhône (Vaucluse, France) and Graveson (Bouches-du-Rhône, France). The two apple growers use different spraying systems: low volume (300L/ha) and medium volume (500L/ha), respectively, and the CpGV dosage was  $10^{13}$  OBs/ha.

Pure CpGV-M and CpGV-R5 genotype stocks were mixed in proportion 36%-64%, respectively, to mimic the relative frequencies on the commercial product, and sprayed at final concentrations of  $2.16 \times 10^4$ ,  $2.16 \times 10^5$ ,  $1.29 \times 10^6$  and  $6.02 \times 10^6$  OBs $\cdot$ cm $^{-2}$ .

Leaves were sprayed in the laboratory using a nebulisator (Carrera et al., 2008) to obtain quantified CpGV concentrations. Leaf discs (9.6 cm $^2$ ) were cut from the leaves and transferred onto Petri dishes ( $d = 3.5$  cm). Neonate larvae (CpNPP susceptible to CpGV-M) were deposited onto the upper surface of the discs (around 100 larvae per virus concentration in four independent assays). Neonate larvae were placed onto leaf discs and allowed to feed during 10, 30, 60, 120 and 1140 min. Then, they were transferred onto 24 wells plates filled with *Heliothis Stonefly* medium (Ward's Science, USA) for individual rearing at 26°C during seventy-two hours.

### ***Infection analysis***

Larvae were collected, crushed onto 200  $\mu$ L distilled water and stored frozen for later use or processed. qPCR was performed with 5  $\mu$ L of larval suspension, followed by HRM step as previously described (Hinsberger et al., 2019).

### ***Scanning electron microscopy of apple leaves***

Drop dispersion and larvae biting holes were observed using an environmental-scanning electron microscope (Quanta FEG 200 ESEM, FEI) (no staining nor desiccation of the leaves), with leaves treated with medium or low volume, or those treated in laboratory conditions. Drop density and the bites size were measured.

## **Results**

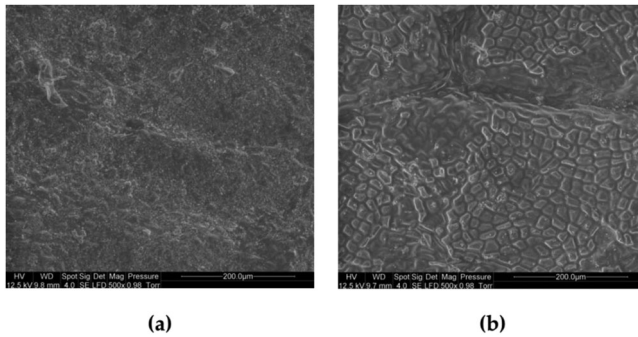
### ***Comparison between two dispersion methods of CpGV used in orchards***

Leaves sprayed in apple orchards at  $10^{13}$  OBs/ha (Arysta LifeScience, 2019) with two dispersion methods: medium volume (500L/ha) and low volume (300L/ha) were observed by scanning electron microscope. Even in the medium volume dispersion, no runoff was observed on leaves. Both were quite homogenous and covered the entire leaf surface (Figure 1).

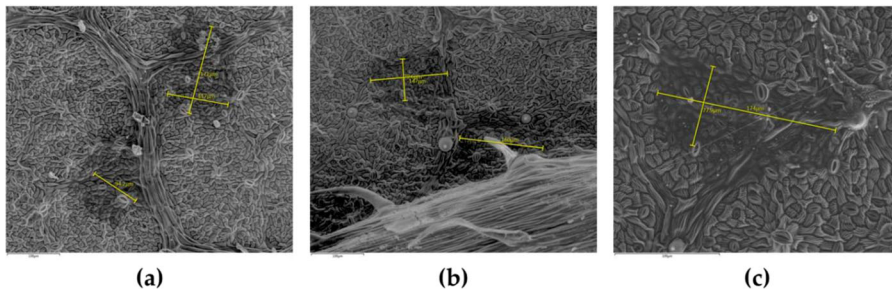
### ***Probability distribution of infections***

The leaf surface of each bite was calculated using a scanning electron microscope (Figure 2). The mean surface of each bite was around 0.012 mm $^2$ .

The probability of infection adjusts satisfactorily to a binomial distribution, suggesting that larvae eat the virus in a non-specific way while eating the leaf. If more than one virus genotype is present, the probability of multiple infections in fully susceptible hosts will be



**Figure 1.** Commercial CpGV drop dispersion on apple leaves collected from two orchards, observed by Scanning Electron Microscopy. (a) Low volume dispersion (300 L/ha) and (b) medium volume dispersion (500 L/ha).



**Figure 2.** Apple leaves surface bitten by a larva observed by SEM.

the combined probability of encountering each genotype. To avoid the bias that resistance could introduce, the feeding experiments were conducted using fully susceptible larvae.

### ***Larvae infection with orchard leaves***

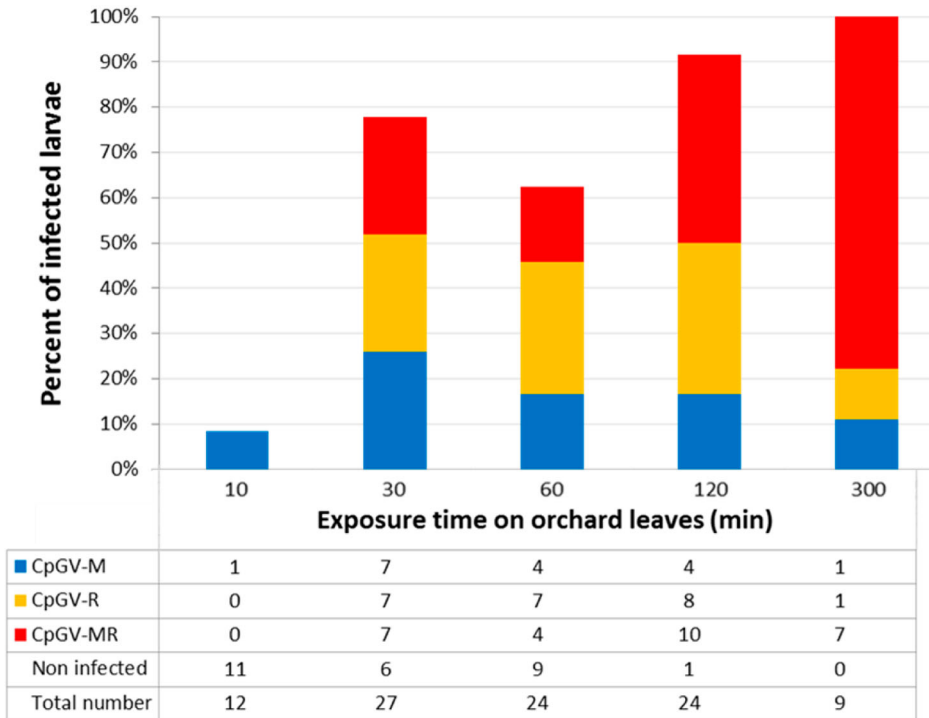
Orchards were sprayed with Carpovirusine® Evo2. Fully susceptible (CpNPP) neonate larvae were fed on discs cut from leaves collected on apple orchards immediately after being sprayed. CpGV infection was quantified by qPCR and HRM (Figure 3).

The number of infected larvae increased with contact time. After 30 min feeding on infected leaves, almost 80% of larvae were infected and 100% were infected after 300 min.

The frequency of double infections increased with the time of contact, after 30 min both genotypes were detected in 26% of larvae; after 2 h 42% and 78% after 5 h.

### ***Larvae fed on leaves sprayed with CpGV in laboratory***

Larval infections followed the same trends as observed in orchards. The number of infected larvae increased both with exposure time and virus concentration. The occurrence of mixed infections followed the same trends. If the time of exposure to the virus is long enough (1140 min), the number of mixed infections reaches 100%. Similarly, when increasing the virus concentration on the leaf surface, the level of infection increased



**Figure 3.** CpGV genotypes found 72 hpi in CpNPP neonates infected on orchards leaves sprayed with Carpovirusine® Evo2. The numbers of larvae are shown in the table under the graph.

for a given time. At  $1.29 \times 10^6$  OBs.cm<sup>-2</sup>, 100% of the larvae were infected regardless of the time of exposure to CpGV, and the relative proportion of mixed infections increased, to reach 100% at the highest doses (Figure 4).

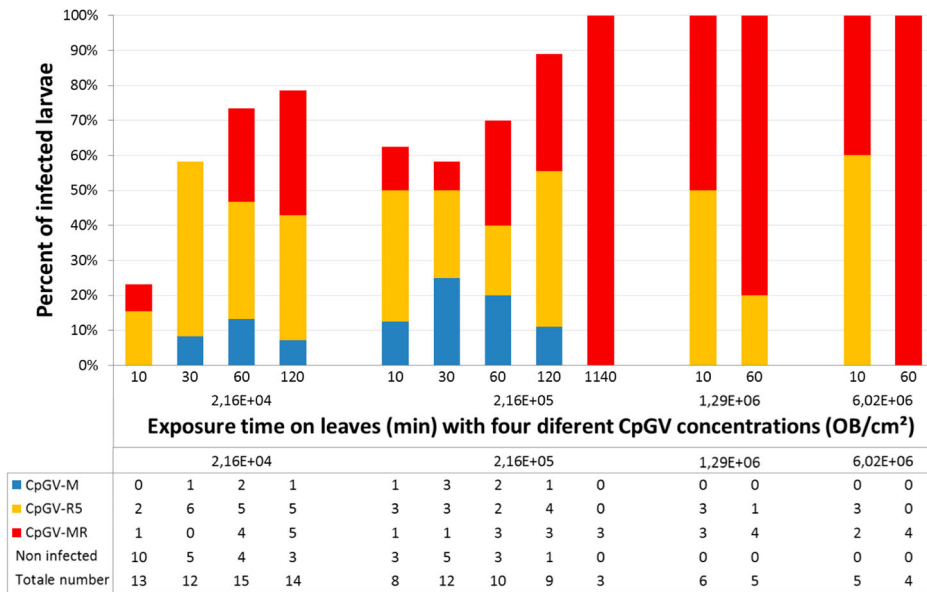
By comparing these laboratory results with those obtained previously in the orchard, the viral concentration found on the surface of the leaves is certainly comprised between  $2.16 \times 10^4$  OB.cm<sup>-2</sup> and  $2.16 \times 10^5$  OB.cm<sup>-2</sup>.

## Discussion

The two orchards use different spraying equipment (medium and low volume), resulting in a different amount of liquid deposited onto the leaf surface. SEM observations revealed this difference on the dried drops covering the leaves, and no runoff or uncovered surface were observed, confirming that both systems can distribute evenly the virus solution.

Ballard et al. (2000) analysed both the number and the surface of bites made by neonate CM larvae upon the time. Their results show that feeding holes are found after only 3.5 min of contact with leaves, and their number and surface increase with time. In their conditions, at  $4.32 \times 10^3$  OBs.cm<sup>-2</sup> the exposure time required to kill 50% (LET<sub>50</sub>) was about 60 min, and the surface eaten was 0.32 mm<sup>2</sup>, that is, about 14 OBs. The mean area we observed is coherent with their observations.

In laboratory conditions, the lethal concentration has been estimated between 1 and 5 OBs for 1st instar larvae (Huber, 1986; Sheppard & Stairs, 1977).



**Figure 4.** CpGV genotypes found 72 hpi in CpNPP neonates infected by feeding on apple leaves sprayed in laboratory conditions. The effective is represented in the table under the graph.

Although the number of larvae used in these experiments is low, it appears that in the apple orchards treated with Carpovirusine® Evo2, following the producer’s recommendations, the concentration of the virus is high enough to allow larvae ingesting many OBs when feeding the sprayed leaves. To obtain a protection level > 90%, at least 2 h were required. The virus cover is thus sufficient if larvae stay long enough crawling and eating before entering the fruit. This will be the situation in the first CM generation, but it might not be the same in the second or third generations. It has been previously observed that the level of protection when using CpGV is higher on the first generation of insects compared to the following ones (Burgerjon, 1986), probably because on the first generation the fruits are not yet formed, and females lay eggs on the leaves.

The composition of the Carpovirusine® Evo2 comprises two main genotypes, CpGV-M:36%/CpGV-R5:64% (Jehle et al., 2016). When testing individually on fully susceptible larvae, both genotypes are detected, and mixed infection frequency increases over time. A model was constructed based on the feeding behaviour of the larvae and the genotype composition of Carpovirusine® Evo 2 under independent distribution hypothesis. The predictions adjust to results obtained. However, only one larva from a natural isolate was found to carry a mixed infection (Rezapanah et al., 2008), suggesting that in natural conditions the density of OBs is low, and thus the mixed infections would be rare events. As it has been demonstrated that positive interactions may occur between genotypes, it would be interesting to ensure a maximum number of double infections in orchard conditions. This objective can be attained in the first generation only by using today’s strategies, but not in the following generations, that would require increasing the concentration on the eaten surfaces.

A step forward will be to analyse the variability of the response using a higher number of larvae per treatment and to reproduce similar experiments using larvae resistant to CpGV-M.



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## Disclosure statement

No potential conflict of interest was reported by the authors

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