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Field Trials of CpGV Virus Isolates Overcoming Resistance to CpGV-M

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Abstract: The *Cydia pomonella granulovirus* (CpGV) has been used for many years as biological agent for codling moth control in apple orchards. Resistance to the Mexican strain of CpGV was detected in orchards in Germany, France and Italy. A laboratory insect colony was started from insects collected in a French resistant orchard. It was named RGV. Various virus isolates were identified as active against this resistant insect colony. Field tests were carried out in 2007 to test if the two virus isolates CpGV-I12 and NPP-R1 were effective in the field. Although these virus isolates were not able to reduce insect caused fruit damages, they significantly reduced the overwintering insect populations. NPP-R1 was subjected to eight passages on RGV larvae (NPP-R1.8) that improved its biological activity on RGV larvae. 2008 field trials were set up to test this improved virus strain, compared to CpGV-I12 and Madex plus active on RGV. These tests confirmed the ability to control both in susceptible and resistant insect populations.

Key words : *Cydia pomonella*; Codling moth; Biological control; Genotype selection; Field trials

The potential for the control of *Cydia pomonella* populations in apple orchards with the *Cydia pomonella granulovirus* (CpGV) was realised soon after the discovery of the virus in Mexico (CpGV-M) (11). In 1969 the first field trials were carried out in the USA, in Australia, and in Europe, followed by the Canada (6). In Europe, the use of the CpGV-M isolate as a biological control agent was registered in the late 1980's (8). Thereafter, different commercial products based on it have been intensively used not only in organic orchards, but also in Integrated Pest Management (IPM) strategies. *C. pomonella* populations gradually became resistant to most chemical insecticides, making the control by the CpGV an attractive issue.

Failure of *C. pomonella* control by the virus preparations raised suspicion of resistance back in 2003 in Germany (5) and in France (10). Putatively resistant larvae were collected from orchards in Germany and France, and two independent laboratory colonies were started from these larvae, the CpR and the RGV colonies, respectively. Bioassays on these populations with CpGV-M confirmed the resistance, allowing a more precise study on the characteristics of

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its transmission (1, 4). The resistance factor appears to be higher than 13000 in the French population (10).

Three different approaches were undertaken in the search for virus isolates able to control these resistant insect populations: i) Search for new virus isolates was carried out in regions where no previous treatments with CpGV-M were recorded. This approach allowed the identification of new isolates from Iran (9); ii) Search in laboratory virus collections and iii) use the laboratory resistant insect to select genotypes able to replicate and kill resistant larvae.

Three virus isolates were found that overcome the resistance, the CpGV-I12 isolate (3), the Madex + isolate (12) and the NPP-R1 isolate (2). Analysis of the biological activities of these virus isolates against the resistant laboratory colonies does not guarantee their effectiveness in the field. As a further step towards the control of resistant insects, we describe here the results of the field trials carried out in 2007 and 2008 in various orchards in France and Italy.

MATERIALS AND METHODS

Virus isolates

The CpGV-M strain, the usual active ingredient in the commercial formulations, was systematically included as a control in all the trials.

The virus isolates I12 (9) and NPP-R1 strain (2) are able to overcome the resistance in laboratory colonies (2, 3). Similarly, the Madex+ isolate, obtained by selection in resistant insects starting from the CpGV-M is also able to overcome the resistance (12). The NPP-R1 isolate was also subjected to selection through successive passages in RGV insects as described previously (2). The eight passage (NPP-R1.8) was used for the 2008 field trials.

All virus isolates were amplified on resistant insects from the original inoculums provided, to constitute a stock for the test production that was made on the same susceptible insects as the industrial production of Carpovirusine®. The different virus isolates were formulated by Natural Plant Protection in the same way as Carpovirusine®, at a final concentration of 1×10^{13} OBs/L, and used at 1 L/ha or 5 L/ha.

These formulated virus preparations were called Carpovirusine 2000 for the standard formulation using CpGV-M, Carpovirusine I12 for the CpGV-I12 formulation, Carpovirusine R1 for the NPP-R1 formulation, Carpovirusine M+ for the Madex Plus containing virus formulation, and Carpovirusine R1.8 for the NPP-R1.8 formulation.

Each of these formulations was tested for its ability to control both the Sv (susceptible) and the RGV larvae in standard bioassays.

2007 field trials

Field trials were conducted in France in 2007, using large plots in five apple orchards with confirmed resistance on four different apple varieties (Akane, Golden, Fuji and Granny Smith). Three virus formulations were tested, Carpovirusine 2000, Carpovirusine I12 and Carpovirusine R1. Virus preparations were applied at the usual concentration of 10^{13} OB/ha. 11 to 13 treatments were applied during the season (from 30 April to 8 September).

Efficacy assessment: Three assessments of the damage of codling moth were carried out, at the end of each codling moth generation (end of June, July and September) except for Akane, where fruits were collected at the end of the second codling moth generation.

Damages were classified as stopped superficial damage

Table 1. Results for the 2007 French field trial.

Variety	Virus	G1			G2			G3		
		Total damage (%)	Deep damage (%)	Stopped damage (%)	Total damage (%)	Deep damage (%)	Stopped damage (%)	Total damage (%)	Deep damage (%)	Stopped damage (%)
Akane	C2000	0.7	0.1	0.6	***2.4***	***0.1***	***2.3***			
	R01	1.6	0.1	1.5	***1.7***	***0.4***	***1.3***			
Golden	C2000	4.7	1.7	2.9	9.1	1.0	8.1	***13.2***	***3.7***	***9.5***
	R01	6.4	0.6	5.8	8.0	0.5	7.5	***15.9***	***3.7***	***12.2***
	I-12	4.0	0.5	3.5	3.7	0.0	3.7	***12.4***	***2.9***	***9.5***
Fuji	C2000	1.7	0.2	1.5	2.9	0.1	2.8	***5.5***	***1.2***	***4.3***
	I-12	2.0	0.1	1.9	1.5	0.1	1.4	***3.9***	***0.8***	***3.1***
Granny	C2000	2.8	0.4	2.4	4.1	0.4	3.7	***7***	***1.4***	***5.6***
	R01	3.4	0.4	3.0	6.6	0.3	6.2	***10.2***	***2***	***8.2***

Virus isolates were formulated as for the standard Carpovirusine 2000 (C2000). R01 is the isolate NPP-R1; I12 is the CpGV-I12 isolate. In bold, the post-harvest evaluation.

(marketable fruits) or deep damage (fruits attacked and not marketable). For the latest variety, Golden, a weekly survey of the overwintering larvae was carried out (from 13 August to 17 September, using 30 corrugated cardboards for each treated parcel).

2008 field trials

The 2008 field trials were conducted in three localities, in Italy (Spinimbeco and Villa Bartolomea, Emilia-Romagna) and in France, (Saint Aubin, Loire Valley), using Carpovirusine 2000 as a control for the resistance, Carpovirusine I12, Carpovirusine M+ and Carpovirusine R1.8 at the standard doses of 1×10^{13} OB/ha (that is, 1L /ha), and the last three viruses at five times higher doses. For each assay, untreated plots were used as damage controls. The trials were carried out in small plots, with a minimum of 250 fruits per plot at harvest and four replicates for each treatment. Treatments were applied every 10-12 days from 22nd of May to the 29th August.

Efficacy assessment: Damage was classified as stopped superficial damage (marketable fruits) or deep damage (fruits attacked and not marketable). Healthy and virus-infected codling moth larvae in the cardboards were counted at the end of the first generation and at harvest, to evaluate the level of population control.

RESULTS

Laboratory tests were conducted prior to the field test, both on the susceptible (Sv) and the resistant (RGV) insect colonies. All the virus isolates tested (CpGV-M, CpGV-I12, Madex +, NPP-R1 and NPP-R1.8) had similar activities on the Sv colony (data not show). In contrast, significant differences appeared concerning their activities on the RGV laboratory colony (Fig.1). Carpovirusine I12 and Carpovirusine M+ partially overcame the resistance, with a LC_{50} of 5.01×10^3 (1.85×10^3-1.03×10^4) and 9.24×10^3 (2.27×10^3-2.25×10^4) OB/μL, respectively, while Carpovirusine R1 LC_{50} is 166 (91-278) and Carpovirusine R1.8 LC_{50} is 74 (43-114).

In the 2007 field trials in France, no better protection of the fruit was observed in parcels sprayed with Carpovirusine I12 or Carpovirusine R1, compared to those where Carpovirusine 2000 was used (data not shown). The final level of damage at harvest increased over time, reaching 15% in the Golden parcel (the latest to be harvested). However, the number of overwintering larvae collected was clearly reduced in the parcels treated with Carpovirusine-I12 or Carpovirusine R1 compared to Carpovirusine 2000 (Fig. 2). In the last two dates, the differences between Car-

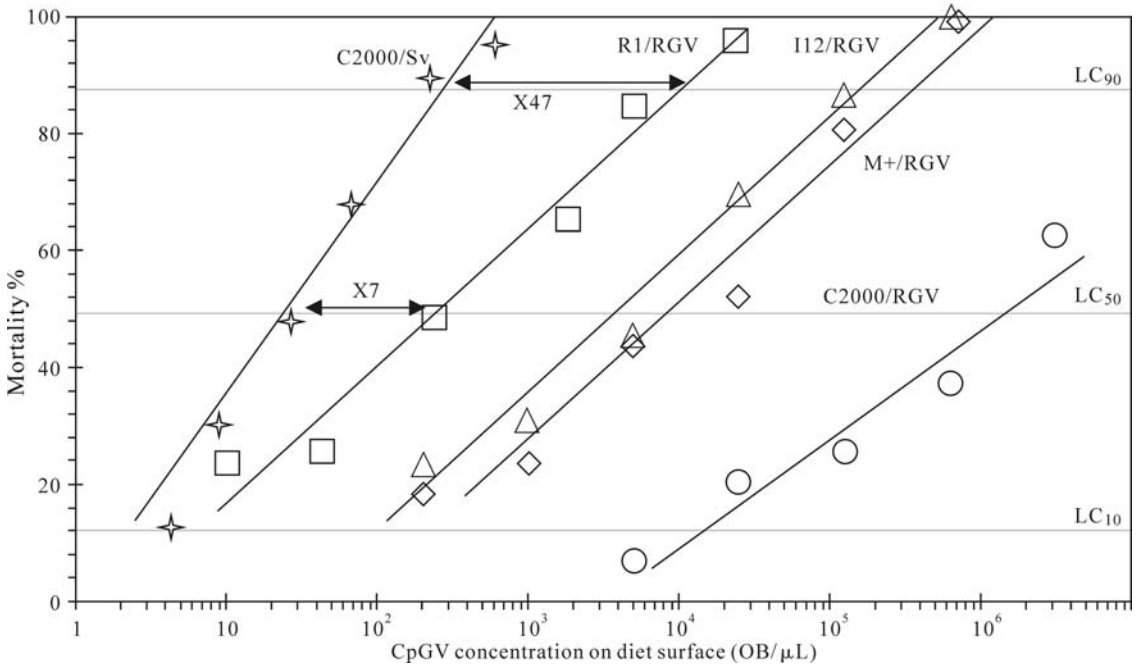

Fig. 1. Biological activity of the virus isolates on the laboratory insect colonies, susceptible (Sv) and resistant (RGV). The activity of all virus isolates on the susceptible Sv colony is similar to that of CpGV-M. C2000 is the CpGV-M formulation. R1 is based on the NPP-R1 virus isolate. M+ is based on the Madex+ virus isolate, and I12 is based on the I12 isolate.

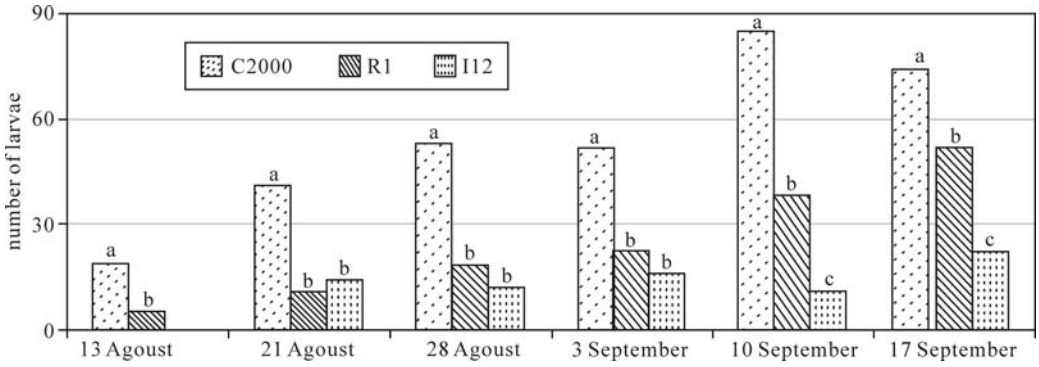

Fig. 2. Number of larvae on 30 corrugated cardboard traps at each date for the Golden orchard. Statistically significant differences are represented by letters (a, b, c).

povirusine R1 and Carpovirusine I12 were statistically significant.

Analysis of the laboratory results show that the dose/mortality regression lines for NPP-R1 on the Sv and RGV larvae are not parallel (Fig. 1). A much higher doses than expected is required to kill over 90% of the resistant insects, compromising the expected efficacy of the virus preparation in the field.

A process of selection for a more active virus was carried out by successive cycles of replication of the NPP-R1 virus isolate on RGV insect larvae. This process lead to an enhancement of the activity of the virus, both for the LC_{50} and the LC_{90}, the later being more important, although it did not reach the same level as that of the reference CpGV-M on Sv (Fig.3). For the 2008 field trials, the virus isolate NPP-R1.8, corresponding to the 8th passage, was used. The formulated product was named Carpovirusine R1.8.

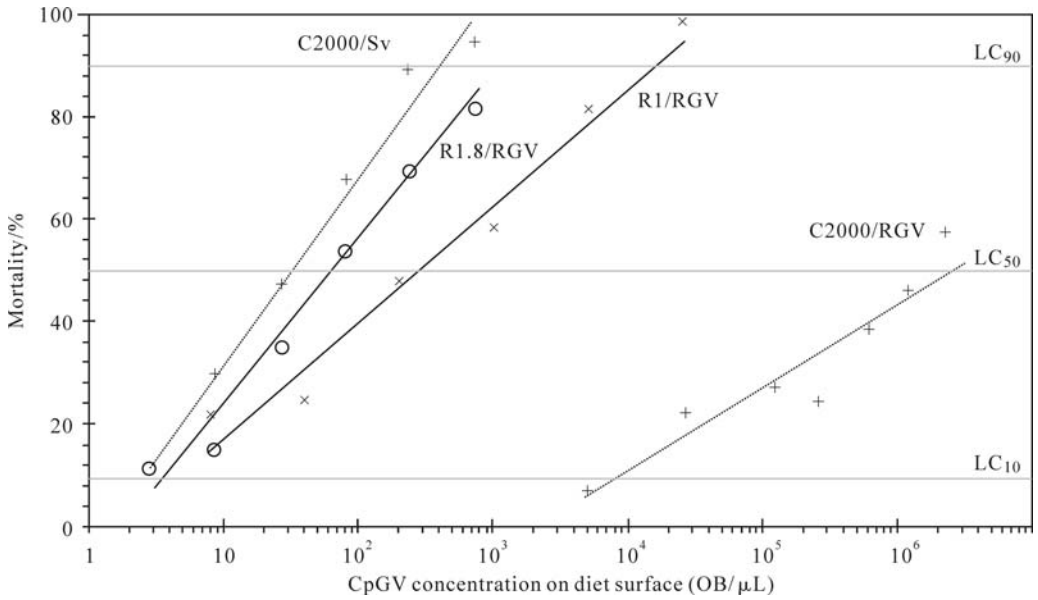

Fig. 3 . Enhancement of biological activity of virus isolate NPP-R1.8 by successive passages on RGV larvae. As a reference, the biological activity of the reference virus isolate CpGV-M formulation (C2000) is indicated both for susceptible (Sv) and resistant larvae

The action of the CpGV-M (Carpovirusine 2000) provided a control for the presence or absence and the level of the resistance in the trial locations. In all the locations, the insect pressure on the orchard was very high, far more than the pressure required for optimal efficacy of insect control by CpGV. In Spinimbeco (Table 2A), CpGV-M is effective, with a reduction in the damage from 45 to 15%. The other virus preparations performed as well as CpGV-M, confirming the results obtained in the laboratory concerning the equivalent activity of all viruses in susceptible insects. Increasing the doses to 5×10^{13} OB/ha resulted in a non significant reduction of damage. An important reduction in the overwintering population density was observed for all the virus preparations. However, the CpGV-M treatment did not reduce the population as much as the other virus isolates (3.6 compared to less than 1.1 in average in the traps). A small proportion of resistant insects might be at the origin of this result.

In the Villa Bartolomea field trial (Table 2B), there was a small damage reduction with the CpGV-M preparation, suggesting that part of the insect population were susceptible. A higher reduction is observed with all the other preparations. Curiously, in this location there was a significant difference of efficacy in function of the doses for the Carpovirusine R1.8 formulation, but not for the others.

In the Saint Aubin le Dépeint trial (Table 2C), the level of damage in the CpGV-M treated parcel was not different from the untreated control parcel, indicating a very high prevalence of resistant insects. The other virus preparations significantly reduced the level of deep damage at harvest, while the level of superficial damage was not affected. As for the other locations, the overwintering populations of insects, estimated by the larvae captured in corrugated cardboard traps, were reduced in the plots treated with the new virus isolates.

DISCUSSION

Table 2. Results of the 2008 field trials

A: Spinimbeco, Emilia Romagna. Italy

Treatment	Damage 23/6	Efficacy G1	Incidence at harvest	Efficacy harvest	Traps healthy	Traps infected
Untreated	12.1 ^{a}		45.0 ^{a}		6.5	0.5
C2000	3.3 ^{b}	67.3	15.6 ^{b}	65.6	1.8	1.8
I12	2.4 ^{b}	78.9	13.8 ^{b}	69.7	0.8	0.3
I12×5	1.3 ^{b}	89.6	9.7 ^{b}	78.7	0.3	0.5
M+	2.8 ^{b}	68.7	14.8 ^{b}	67.3	0.5	0.3
M+×5	3.4 ^{b}	71.6	9.7 ^{b}	78.5	0.3	0.3
R1.8	1.3 ^{b}	88.2	12.8 ^{b}	71.7	0.3	0.0
R1.8×5	0.7 ^{b}	93.7	15.1 ^{B}	66.8	0.0	0.0

B: Villa Bartolomea, Emilia Romagna. Italy

Treatment	Damage 27/6	Efficacy G1	Incidence at harvest	Efficacy harvest	Traps healthy	Traps infected
Untreated	7.0 ^{ns}		20.6 ^{a}		0.5	0.0
C2000	5.3 ^{ns}	36.0	17.5 ^{ab}	15.0	1.0	0.8
I12	2.5 ^{ns}	48.5	9.6 ^{ab}	53.4	0.8	0.5
I12×5	1.6 ^{ns}	60.3	10.1 ^{ab}	51.0	1.5	0.0
M+	4.3 ^{ns}	37.5	11.2 ^{ab}	45.6	0.0	0.8
M+×5	2.5 ^{ns}	58.3	13.8 ^{ab}	33.0	1.5	1.0
R1.8	1.2 ^{ns}	60.9	12.8 ^{ab}	37.9	0.0	0.3
R1.8×5	0.3 ^{ns}	70.6	7.1 ^{b}	65.5	0.0	0.0

C : Saint Aubin le Dépeint, Loire Valley. France

Treatment	Total damage	Deep damage	Superf. Damage	Efficacy on total	Efficacy on deep	Traps healthy	Traps infected
Untreated	26.8 ^{a}	23.3 ^{a}	3.4 ^{ns}			3.3	0.0
C2000	25.7 ^{a}	21.6 ^{a}	4.1 ^{ns}	3.8	7.4	2.3	0.3
I12	16.3 ^{b}	13.1 ^{b}	3.2 ^{ns}	39.1	43.7	1.3	0.0
I12×5	13.9 ^{b}	11.3 ^{b}	2.6 ^{ns}	48.2	51.7	0.3	0.0
M+	16.2 ^{b}	13.3 ^{b}	2.9 ^{ns}	39.6	43.2	1.3	0.3
M+×5	18.2 ^{b}	14.4 ^{b}	3.7 ^{ns}	32.0	38.1	1.0	0.0
R1.8	16.0 ^{b}	12.3 ^{b}	3.6 ^{ns}	40.4	47.1	0.5	0.0
R1.8×5	12.8 ^{b}	10.0 ^{b}	2.8 ^{ns}	52.3	57.2	0.8	0.3

Individual significant differences are represented by letters (a, b, c). ns: not significant.

Screening of resistant laboratory colonies allowed the isolation and selection of viruses that are able to replicate in insects resistant to CpGV-M. However, the activity of these virus isolates in the resistant laboratory colony RGV was significantly lower than that of CpGV-M on the Sv susceptible insects. In field trial conducted in 2007 in parcels where resistance was previously confirmed, neither I12 nor NPP-R1 were able to satisfactorily control the insect populations. As the assay was conducted on large plots, no untreated parcel was available. Lack of protection with CpGV-M, together with the lack of any protection with CpGV-I12 and NPP-R1, confirmed that resistance is well established.

Estimates of the insect population can be deduced from the number of larvae collected in the traps. Probably, the insect pressure in our 2007 assays was too high for the virus to be able to control the insects, regardless of their susceptible or resistant phenotypes.

A reason for the absence of difference between virus efficacies may be related to differences in evaluation of the efficacies of the virus in the laboratory and in field conditions. The LC_{50} is used as a measure of the virus effectiveness in the laboratory. Using this

measure, NPP-R1 is only 7 times less effective on RGV larvae than on Sv larvae. However, killing 50% of the larvae in the field will not modify the level of damage if the insect pressure is moderate to high. Better evaluation (although not yet perfect) should be obtained by using LC_{90}. The same comparison for LC_{90}s gives a ratio of 47 times less active on resistant larvae than on susceptible larvae.

Accordingly, between the 2007 and the 2008 trials, the NPP-R1 isolate was selected by successive rounds of replication on RGV larvae. In the 2008 trial, the NPP-R1.8 isolate was used, that corresponded to 8 passages on RGV larvae. A significant improvement of the pathogenicity of the virus isolate was observed for the RGV larvae, while maintaining the same level on susceptible larvae (Fig. 3).

The 2008 field trials in France and in Italy were designed to test the improvement of the virus after such selection process. The small plot design was used, allowing the introduction of untreated plots. The locations presented an insect pressure even higher than that of the 2007 trials, as shown by the high level of damage in the untreated plots (15% to 45%). In two locations, the resistance was confirmed, as no difference was observed between the plots untreated or treated with the formulation containing CpGV-M.

In the location with no resistance, all virus isolates performed as well as CpGV-M when applied at the recommended doses of 1×10^{13} OB/ha, reducing the global damage from 45% to 13% on average. No significant further reduction was observed by increasing the viral dose (up to 5×10^{13} OB/ha), again suggesting that the relatively low level of protection is in fact not due to an absence of efficacious virus but to a too high insect pressure in the orchard.

In the locations where resistance is present, a significant reduction is observed for Carpovirusine I12, Carpovirusine M+ and Carpovirusine R1.8 (Table 2B and C) Small differences were observed between the virus isolates but with no clear trend. However, the level of protection was not satisfactory, probably due to the high insect pressure (more than 20% damage in the untreated control).

These results contrast with those published for CpGV-I12 and Madex Plus (12) where a significant increase of the protection level was obtained compared to the untreated (control) samples. The difference may be related to a higher level of resistance or/and to the high degree of infestation. (more than 15% damage in the untreated controls in comparison with 2.5% in test plots). Field trials were also conducted in two German locations using Madex 3 (based on CpGV-M) and Madex Plus (7). They also obtained a significantly better protection with Madex Plus than with Madex 3. The two locations differed in the level of insect resistance that could be measured by the effectiveness of the Madex 3 preparation. In the BW-HI location, Madex 3 was ineffective-confirming the presence of the resistant phenotype-; while Madex Plus reduced the damage by one fold (from 2.5% to 1.3%). The second location, BW-FN presented a susceptible population, as Madex 3 was effective in controlling the damage. Again, in both locations, the insect pressure was relatively low. However, the authors notice a lower efficacy of both Madex Plus and CpGV-I12 on the CpGV-resistant insect populations in comparison to the behaviour of CpGV-M on susceptible populations. This observation can be correlated to our laboratory tests (Fig. 1).

The reduction of the overwintering population,

observed in 2007 field trials, was confirmed in 2008 for all the locations, with Carpovirusine R1.8 systematically performing the best. As the overwintering insect population remaining in each orchard is significantly reduced, it can be expected that in a continuous reduction in the level of damage will be obtained in subsequent years.

The differences in LC_{90} observed in the laboratory tests are thus clearly relevant in the view of developing a biological control agent. Selection of the virus by successive passages on resistant insects appears to be a suitable approach to improve the efficacy of natural virus populations facing new resistant hosts, as far as these resistant insects faithfully represent the insect populations in the field. The selection process will be continued with the objective to obtain a virus performing equally well on susceptible Sv and resistant RGV larvae. Once this virus isolate is obtained, new field test will be required to validate the laboratory results.

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