Madex Twin, a new *Cydia Pomonella* Granulovirus Isolate for the Control of both Codling Moth *Cydia Pomonella* and Oriental Fruit Moth *Grapholita Molesta*

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Abstract

Madex Twin, a new Cydia pomonella *granulovirus (CpGV) isolate for the control of both codling moth (CM) and oriental fruit moth (OFM), has been developed and tested in bioassays and field trials by Andermatt Biocontrol, Switzerland. At field level, the product has been tested in a total of 44 field experiments in the Northern and Southern hemisphere. The product showed promising efficacy results against both pest species in the laboratory, as well as in the field. Applications for registration of Madex Twin in Southern Europe and in other countries all over the world have already been initiated.*

Keywords: CpGV, codling moth, oriental fruit moth, Madex Twin

Introduction

OFM is a key pest in peach and nectarine production all over the world. A possibility of residue free control of OFM would be highly appreciated as residue requirements are becoming more and more important. The Swiss company Andermatt Biocontrol selected a granulovirus isolate (Madex Twin) on OFM in the laboratory. Madex Twin was then tested against CM and OFM in laboratory bioassays and in numerous field trials in the relevant pome and stone fruit growing regions. Selected trial examples as well as a review of its average performance under field conditions will be presented here.

Material and Methods

DNA Restriction Endonuclease Analysis (REN)

The viral DNA of the test item (Madex Twin) and the reference item (CpGV, Neustadt Mexican isolate) was digested with the restriction enzymes *Bam*HI, *Eco*RI, *Sal*I and *Eco*RV. All digested DNAs were electrophoresed in a 0.8% agarose gel over night (25 V) using TAE as a buffer system.

PCR Amplification and Sequencing of Marker Genes

The partial sequences of late expression factor *lef-8* and the granulin (*polh/gran*) genes were amplified using the degenerate primer method described by Lange et al. (2004) and Jehle et al. (2006). PCR products used for direct sequencing were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham, Freiburg, Germany), and both DNA strands were sequenced using M13 universal, M13 reverse and T7 standard primers (MWG, Germany). The sequences were aligned using BioEdit with the corresponding sequences of further CpGV isolates determined previously and described by Eberle et al. (2009).

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Phylogenetic Analysis

Partial *polh/gran* and *lef-8* sequences determined for Madex Twin were concatenated and aligned with the corresponding sequences of Cryptophlebia leucotreta granulovirus (Cr1eGV) (Lange & Jehle, 2003) as an outgroup using Clustal W (Thompson et al., 1994) implemented in BioEdit 7.0.5.3 (Hall, 1999). A phylogenetic analysis using minimum evolution algorithms was performed using MEGA 4.1 (Kumar et al., 2004).

Genome Sequencing

Genome sequencing of Madex Twin was performed by a commercial supplier by 454 pyrosequencing (Genterprise Genomics, Mainz). Sequences obtained were assembled using DNAStar Lasergene Seqman NGen® Software. Analysis and joining of contigs was done with DNAStar Lasergene SeqMan Pro (Version 8.1.5). The trace files were checked by eye and minor sequencing mistakes were corrected if necessary. In case of sequence ambiguities, primers were designed using DNAStar Lasergene PrimerSelect and the sequence parts were re-sequenced by Sanger sequencing (Genterprise Genomics, Mainz). Methionine-initiated open reading frames (ORFs) encoding 40 amino acids or more were determined using GeneQuest (DNAStar Lasergene 8.1.4). ORFs were checked for homology to ORFs in other dsDNA viruses using the BLAST programs blastn and blastx; the consensus sequence of Madex Twin was aligned to CpGV-M (Eberle, 2010) on nucleotide level by bl2seq. The predicted ORFs were compared to the re-sequenced CpGV-M (Eberle, 2010) in BioEdit 7.0.5.3.

Bioassay

The bioactivity of Madex Twin was determined in a concentration-response bioassay on semisynthetic diet (WARD's) with CM and OFM larvae. A CpGV-M isolate of known activity was used as reference. Up to 6 different concentrations between $2x10^2$ and $2x10^6$ occlusion bodies per gram diet were tested on both insect species for each virus isolate. For each virus concentration, 50 neonate larvae were kept individually in darkness at 25°C. Mortality was assessed after 12-14 days. 200 larvae on virus-free diet were used as untreated control.

The median lethal concentration (LC50) and the relative potency (RP) were calculated by probit analysis (Finney, 1971) using Priprobit 1.63. Normal Distribution and the model "Preference (a,b,c: Natural Preference to Treatment Side), D=0" was used. Visualisation of the results was done with Microsoft Excel®.

Field Trials

Trial site 1, OFM-Peach: Madex Twin was applied at different rates and compared to the reference product Calypso (Thiacloprid). A total of 5 applications took place on 11th, 20th, and 28th of May (1st generation applications), 15th of July and 26th of August (2nd generation applications) in a well established peach orchard in Veselé, Slovakia in 2010.

Pest infestation damage was scored as "stopped damage" (superficial empty gallery where larvae have been killed) and "active damage" (deep damage to the core with living larva or signs that larva had emerged from the fruit and completed its development). "Stopped damage" was distinguished from "active damage" by cutting the fruit open.

Trial site 2, OFM-Pear: The pilot pear orchard was situated close to a peach field in the Bologna province of Italy, where the presence of OFM has been high throughout the summer 2011. Treatments were done during the 3rd OFM generation close to harvest. The

products were applied from beginning of August 2011 until harvest time. Madex Twin was applied at two dose rates of 50 (6 day interval) and 100 ml/ha (8 day interval) respectively.

The final assessment was carried out at harvest time by counting the number of damaged fruit on 200 randomly selected fruits per plot. The damaged fruits were sectioned to control the type of damage ("deep entries" or "stings" for *C. pomonella*, "deep entries" for *G. molesta*) and to classify the larvae inside the fruit.

Trial site 3, CM-Apple: In order to study the field performance of Madex Twin on CM in pome fruit, a trial site was selected in the Bologna region of Italy. Madex Twin was compared to the normal CpGV-M strain (Madex) and a chemical reference. The CpGV based products were applied at an 8 day interval (starting from 6th of May 2011) until the end of the first CM generation. Coragen (Rynaxypyr), a new larvicide highly effective against CM, was applied twice at a 15 day interval (29th of April and 14th of May 2011). The final assessment was carried out on 11th of June counting the number of damaged fruit on 300 randomly selected fruits per plot.

Results and Discussion

DNA Restriction Endonuclease Analysis

On the basis of DNA restriction analysis using *Sal*I, *Eco*RV, *Eco*RI and *Bam*HI, it could be concluded that Madex Twin is a CpGV isolate. The predominant genotype corresponds to CpGV-M (NW) and is therefore an A type genome isolate. However, there were some submolar bands observed in the *Eco*RI and *Eco*RV profiles, indicating that there is a second genome type present at a low level.

Phylogenetic Aalysis

On the basis of the concatenated *polh/gran* and *lef*-8 sequences, Madex Twin did not differ in its predominant genome type from CpGV-M. Madex Twin was found to contain a predominant A type genome.

Genome Sequencing

Whole genome sequencing of Madex Twin confirmed that it is a CpGV isolate with a predominant type A genome. With a genome size of 123,345 bp it is slightly shorter than CpGV-M (Eberle, 2010). All 142 ORFs annotated for CpGV-M were found in Madex Twin. No additional ORF could be observed. Five ORFs differ in their predicted amino acid sequence to CpGV-M due to insertions, deletions or SNPs. Sequence identity to CpGV-M on nucleotide level is 99%.

Beyond the predominant type A genome, a second genotype is visible at a ratio of about 50% in ten ORFs. In nine ORFs, this leads to amino acid sequence differences to CpGV-M. Analysis of the *polh/gran* and *lef-8* sequences revealed that a type D genome is also present in Madex Twin at a very low level of about 2%.

Bioassay

Reaching a 50% lethal concentration (LC50) of 2.9x103 OB/g diet in the laboratory bioassay on OFM, Madex Twin showed a 45 times higher activity than CpGV-M (Table 1). With a LC50 of 1.1x103 OB/g diet in the bioassay on CM larvae (Table 2), Madex Twin performed equally well compared to the highly effective CpGV-M reference isolate.

Table 1: Activity of CpGV-M and Madex Twin against OFM.

	LC50 [OB/g diet]	95% lower limit	95% upper limit	relative potency	95% lower limit	95% upper limit
CpGV-M	130'190	81'686	199'530	1.0	0.7	1.5
Madex Twin	2'892	1'799	4'444	45.0	29.5	69.1

Table 2: Activity of CpGV-M and Madex Twin against CM.

	LC50 [OB/g diet]	95% lower limit	95% upper limit	relative potency	95% lower limit	95% upper limit
CpGV-M	1'058	824	1'322	1.00	0.73	1.37
Madex Twin	1'107	863	1'382	0.96	0.70	1.31

Field Trial Results

Trial site1: OFM-Peach: Figure 1 shows the mean reduction of active and total fruit damage. All test products significantly reduced fruit attack in comparison to the untreated plot. Treatments with Madex Twin 100 and 200 ml/ha and Calypso showed equally good results of up to 84%. An increase in the dose rate from 100 to 200 ml/ ha did not result in a significant improvement of efficacy. This data reflects the well-known flat dose-response relationship of granulovirus products under field conditions (Kienzle et al., 2001).

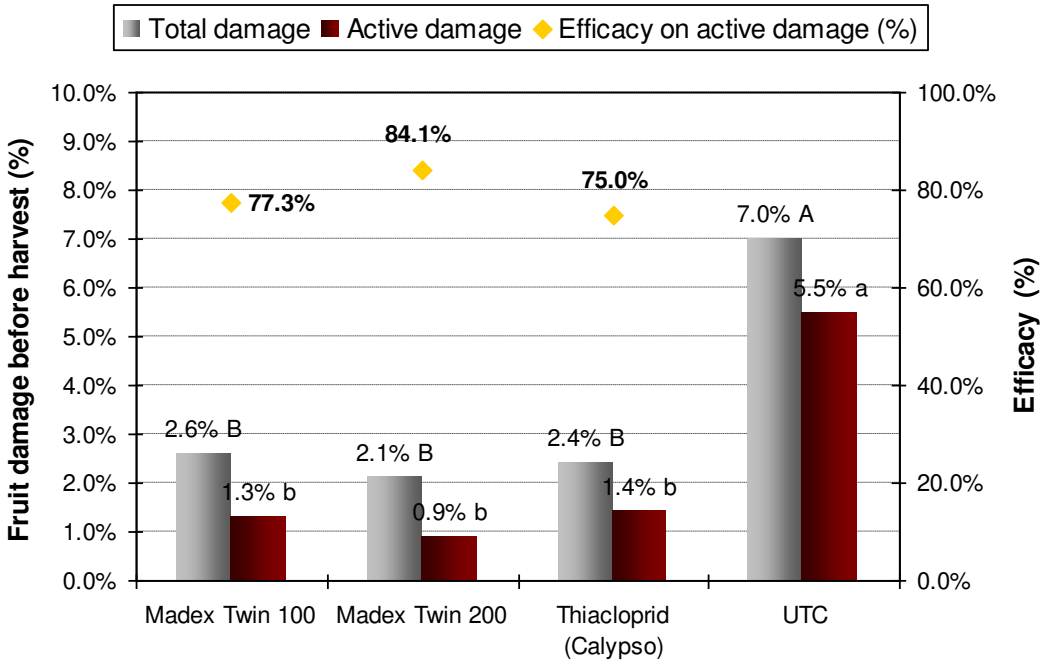

Figure 1: Evaluation of total and active damage of OFM before harvest on peach treated with Madex Twin at different dose rates and compared to a chemical reference product. (Tukey test; P<0.05)

Trial site 2, OFM-Pear: In the untreated control (UTC) the mean total fruit damage amounted to 4.4%, while in the other treatments mean total fruit damage ranged between 0.75% and 1.88% (Figure 2).

The percentage of deep entries caused by CM was lowest in plots treated with Madex Twin (100 ml/ha), intermediate in plots treated with Madex Twin (50 ml/ha), Madex (CpGV-M, 100 ml/ha) and the chemical standard, and highest in untreated control plots.

In the Madex Twin (100 ml/ha) treatment, the percentage of fruit damaged by OFM was significantly lower than in untreated control plots. In the Madex Twin (50 ml/ha) and Madex (100 ml/ha) treatments, the percentage of fruits damaged by OFM neither differed significantly from Madex Twin (100 ml/ha) nor from the untreated control, while in the chemical standard (Trebon Up) OFM damage was statistically comparable to that recorded in the untreated control. The percentage of total fruit damage (all fruits damaged by *C. pomonella* and *G. molesta*) was significantly lower in plots treated with Madex Twin (100 ml/ha) than in untreated control plots.

The efficacy of the Madex Twin treatment (100 ml/ha) was 80.9%, thus higher than the efficacy of the chemical reference treatment and the other CpGV-based treatments.

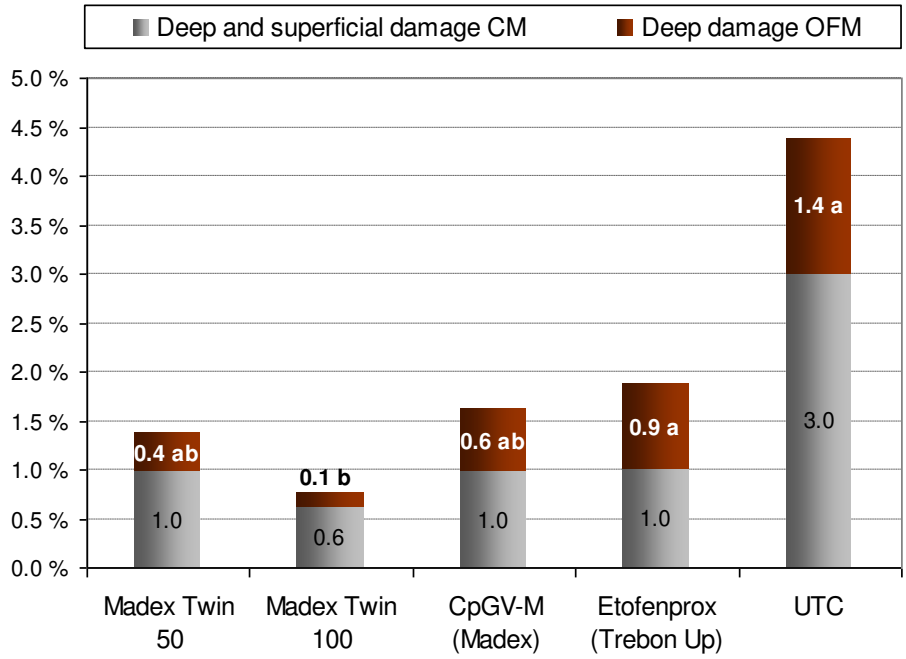

Figure 2: Mean percentage of fruits with deep entries and stings caused by CM and with deep entries caused by OFM. Italy 2011.

Trial site 3, CM-apple: In the untreated control (UTC) the mean fruit damage amounted to 8.7%, of which 7.2% were “deep entries” and 1.5% “stings” (Figure 3).

The percentage of fruits damaged with “deep entries and superficial damage” was significantly lower in treated than in untreated control plots, with differences among treated plots not being significant.

Madex Twin showed a very high efficacy level and was as effective as the normal CpGV-M isolate (Madex) against CM in apple.

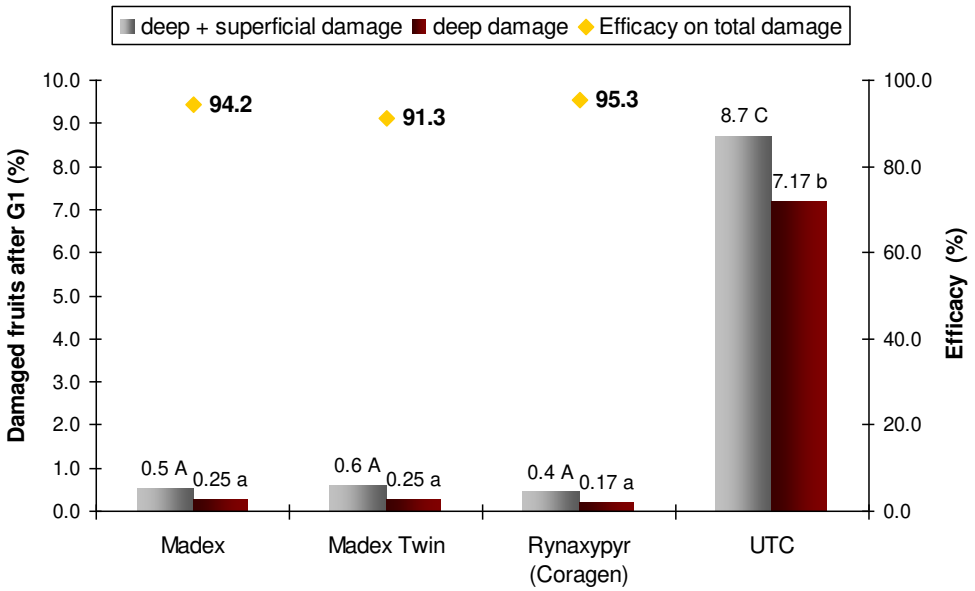

Figure 3: Mean percentage of fruits with deep damage, total fruit damage and efficacy level of Madex Twin compared to CpGV and chemical standard. Italy, 2011.

Conclusion

Madex Twin differs only slightly from CpGV-M in REN analysis and sequencing profile. However these differences seem to be the reason for the higher potency of Madex Twin towards OFM in the bioassays. Based on the extensive testing, Madex Twin can effectively be used for the control of OFM on stone and pome fruit as well as for control of CM on pome fruit (Table 3). The registration process has already been initiated in several countries all over the world.

Table 3: Average performance of Madex Twin at standard rate of 100 ml/ha. Review of field trial results from 2010/ 2011 (Min./Max. and number of trials)

	Apple (CM)	Peach (OFM)	Nectarine (OFM)	Apple (OFM/CM)
Average efficacy on shoot strike reduction		61.9% (50 - 83%; n=9)	76.3% (n=2)	
Average efficacy on reduction of total fruit damage	69% (45-91%; n=11)	64.5% (52-86%; n=10)	67.7% (64.3 - 70%; n=3)	66.2% (39 - 81%; n=3)
Average efficacy on reduction of deep damage	83% (61.5 - 95.4; n=12)	80.8% (n=2)		

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