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Original Article

Screening *Cydia pomonella* granulovirus (CpGV) isolates via comparative bioassays

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Abstract

Background and Aims: The *Cydia pomonella* granulovirus (CpGV) is a baculovirus and very effective biological control agent against apple pest, codling moth, *Cydia pomonella* L. especially for healthy organic apple production and protection. Not only, screening of CpGV isolates via biochemical variation, but also their biological variation is practically necessary. However, the bioassay methods and biological activity parameters, such as LC50, LD50 and LT50 are different, sensitive and usually time and expense consuming; the right chosen method of bioassay can show differences and variation for applied screening purposes.
Materials and Methods: In this study, an appropriate double bioassay method have been successfully set to express biological variation of CpGV isolates on neonate larvae under laboratory conditions (26±0.2 ˚C, RH: 60%±2, 16:8 L: D). The diet incorporation bioassays (LC50) with 0 to 16000 occlusion bodies/ml usually determine the rate of larval mortality on 7th day.
Results: The LC50 of CpGV isolates were evaluated 4454 to 2277 occlusion bodies (OBs) per ml of artificial diet.
Conclusions: Tthat show the double bioassay method have been successfully set and comparable with other results that can enhance biocontrol of codling moth especially in organic apple orchards.

Keywords: codling moth, *Cydia pomonella* granulovirus, bioassay.

Introduction

The *Cydia pomonella* granulovirus (CpGV) is a main member of Baculoviridae family. The CpGV is a dsDNA virus that is highly virulent to codling moth (CM), *Cydia pomonella* L. (Lep.: Tortricidae), one of the most destructive insect pests in apple, pear and walnut orchards. Since CpGV is harmless to non-target animals and has no detrimental impact on the environment (1, 2); so, reliable and effective alternative methods against *C. pomonella* will become a prerequisite for integrated production of apple or organic apple production and protection. Mostly, the control strategy of this pest is still focused not only on the immediate prevention of damage by using synthetic insecticides but also on the long term reduction of the pest's population to an acceptable level (3). The CpGV preparations meet all the demands of a modern, sustainable and environmentally friendly insect control agent for codling moth, especially in organic gardening. The CpGV products are registered as biocontrol agents in at least 34 countries worldwide (4). They are harmless to any other form of life in the apple

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plantation and to users and consumers. Most of CpGV products that are commercialized and used for codling moth control are based on the so-called Mexican (M) isolate, which was isolated and identified in 1963 (5). Two further described isolates are a Russian (R) isolate in 1974 (6) and an English (E) isolate from laboratory (7). The geographic isolates of CpGV have been reported from different locations in the world (5-8). These isolates can be distinguished by restriction fragment length polymorphisms (RFLPs) (7-9), single nucleotide polymorphisms (SNPs) in highly conserved genes (10) and in their phylogenetic position based on genome sequencing (4). All known CpGV isolates are classified into five phylogenetic genome groups A – E (4, 10).

The biological activities of the CpGV isolates have been evaluated in field and, under laboratory conditions mostly on neonate larvae or 5th instar larvae (11-13). The CpGV's biological activity parameters, such as LC50, LD50 and LT50 were discussed (1, 6, 7, 11). The LC50 bioassays have been conducted not only on surface of artificial media, leaves (13) or fruits, but also in whole volume of media (11) during bioassay for 7 or 14 days permanently or partially. The variation of LD50 bioassays is due to feeding method of the larvae with fixed dosage of virus. Also, the LT50 bioassays have been differently conducted due to the fixed dosage or concentration of virus. In this study, the biological activities of some isolates have been evaluated on a recognised *C. Pomonella* laboratory population via comparative bioassays for faster screening of CpGV isolates due to the organic apple orchards need to new CpGV preparations nowadays.

Methods

Propagation and purification of virus occlusion bodies. Fourth or early fifth instar larvae of *C. pomonella* were used to propagate the isolates separately. Infected larvae were ground in 50mM Tris-HCl buffer, filtered through tea filter including cotton tissue and centrifuged in final concentration 50% (W/W) sucrose for 60 min at 40000g. After two times wash in the buffer, they loaded onto a 20-80% (v/v) glycerol gradient and centrifuged for 40 min at 13000g. The virus band was recovered, washed twice and suspended in ultra-pure water. Counting of occlusion bodies was performed using a Helber counting chamber (0.02 mm depth) and Olympus phase-contrast light microscope (8). 20 ml of 105 OBs/ml (G/ml) concentration was prepared for each isolates. The prepared concentrations were stored at refrigerator with 6˚C and diluted just before using. The CpGV isolates 68 and 70 were introduced (1) biochemically characterized (8) and biologically evaluated in this investigation. The light and electron microscopic studies reviled for the presence confirmation of granulovirus in samples by TEM electron micrograph of a partially dissolved capsule of CpGV isolates by 0.1 N NaOH and stained with PTA 2% aqueous (1).

Rearing conditions and *Cydia pomonella* colony establishment. A monotonous laboratory colony with efficient neonate larvae needed for Bioassays. The *Cydia pomonella* larvae used for bioassays derived from insect rearing at the JKI-Darmstadt, Germany. Insects and eggs were incubated at 26±0.2°C, 60±2% relative humidity and a 16/8 hours light/dark photoperiod. The larvae were reared individually in the 2.5*2.5*2.5 cm^3 plastic cubes.

Bioassay procedure. The diet incorporation bioassay (LC50) was carried out for freshly hatched *C. pomonella* larvae (11). The 50 individually held larvae were put on artificial diet containing the virus concentration (each 1*1*1 cm^3).

The bioassays formed using 5 concentration of virus that produced mortality ranging about 10 to 90%. The concentration of 105 OBs/ml of isolates was incorporated into the artificial diet in different amounts so that different concentrations of virus from 0 (control) to 16000 OBs was obtained each in one dish with 50 individually held larvae (11). So, 50 neonate larvae were used for each concentration of virus control. The top of the dishes were covered by a lid preventing larvae escape. Larvae were incubated at 26˚C, 18:6 L: D, and 60% RH for 6 days before

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determination of mortality. The response of the larvae was determined through observable physical characteristics which occur in an infected larva. All the bioassays conducted as double bioassays using the Mexican isolate as control. Bioassays were performed with selected isolates and CpGV-M1 isolate as positive control. The investigation was carried out the first day following the experimental setup and on seventh day. Larvae which did not react to tactile stimuli were regarded as dead. Replicate tests were performed on 3 separate dates and totally about 750 to 900 larvae were examined for each isolates. Mortality data were evaluated by probit analysis using the POLO-PC software and the hypothesis about similarity of isolates probit lines were checked too as well as parallel slope hypothesis (LeOra software, 1987 and 1994) and figures were obtained using excel.

Also, the LT50 Bioassay was conducted with the LC50 dosage of all isolates against the neonate larvae. The 2-ml Ependorf vials filled with about 1 cm of artificial diet .A 2-mm diameter hole in the cap of each vial covered with stainless steel screen eliminated condensation (12). The LC50 dosage was performed for the experiment. The dead larvae were counted every day until they all died.

Another examination was carried out, determining the effect of the storage period in the refrigerator (6 ˚C), by using the virus concentrations stored for 0, 1 and 3 months. The LC50 degree was calculated in each experiment and compared to each other for the isolate No. 68.

Results

The TEM Electron micrographes of CpGV isolates have proved the presence of virus via occlusion bodies (OBs) and sometimes the presences of Nucleocapsid in OBs (Fig 1). Laboratory data shows the reared codling moth under laboratory condition was susceptible to all virus isolates. Among the tested isolates, the greatest virulence according to its LC50 value was 2277 OB/ml, and the LC10 and LC90 values were 230 and 23493 OB/ml. The LC50 for isolate 68 was 4454 OB/ml. Despite the acceptance of the similarity of the lines, the LC50 was nearly half of others and 0.6 of the Mexican isolate, however, that was not significant. The calculated LC50 value for the Mexican isolate in the present experiment was 2724 OB/ml, comparable with 2600 OB/ml (7). The slopes of probit lines were promising and had no significant difference, but the similarity hypothesis of probit lines rejected. However, considerable differences in the virulence and biological activity of the isolates of the CpGV isolates for neonante *C. pomonella* larvae were found (Table 1) is hopefulness.

Fig. 1. TEM Electron micrographes of occlusion bodies (OBs) of CpGV and the nucleocapsid inside granulin, stained with PTA 2% aqueous, (Original, X12,000).

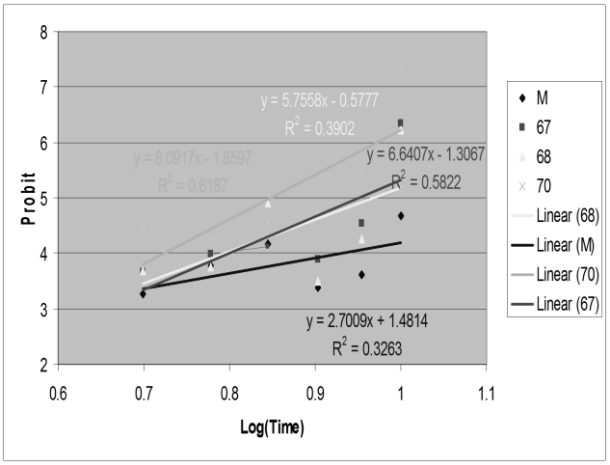

Fig. 2. LT50 bioassay and probit lines of CpGV isolates. The probits of 3-7 indicates the mortalities of 2, 16, 50, 84 and 98 percentages.

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Table 1: Comparative LC50 of CpGV isolates, slope and constant of probit lines.

CpGV isolates	OB/ml	slope	constant	Reference
M	2600	1.21		7
R	3300	1.03		7
E	2500	0.9		7
68	4454	1.83	-1.68	
70	2277	1.29	0.67	
M1	2724	2.07	-2.1	

The results of the LT50 bioassay which conducted to determine the rate of the mortality among the isolates and also in comparison with Mexican isolate summarized in the figure 2.

Discussion

It is interesting that there is not a cited report about isolation of CpGV from an experimental orchard in Georgia (1 cited from Chkubianishvili and Mdzinarashvili, 1972), but there is no naturally collected isolate from Europe even in natural outbreak of CpGV in Hungary (1 cited from Szalay-Marzso, 1975). However, there is a laboratory strain of CpGV in Canada (1 cited from Eastwell et al., 1999) characterized. Since the Caucasus region is thought to be the evolutionary origin of codling moth; it is most likely that this region is also the origin of CpGV and a pool of naturally occurring biodiversity for this virus (1). It can be concluded that the studied isolates have a high potential of pathogenisity on the codling moth and further modification for bioassay studies (11), in vivo cloning (11) and more field examinations (11) in order to use this isolates for the new formulation of CpGV is recommended especially for orchards may dealing with low susceptibility to CpGV isolates (1, 3, 10) even more than the yield satisfactory of codling moth control (12, 13). The double bioassay method has been successfully set and comparable with other results that can enhance biocontrol of codling moth especially in organic apple orchards.

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