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Evaluation of the *per os* insecticidal activity of baculoviruses by a nebulization method

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Abstract: Precise evaluation of the biological activity of Baculoviruses by the usual droplet method is not appropriate for boring insect larvae that remain on plant surfaces for a limited time before penetrating the substrate. Alternative systems using surface contamination of the substrate (Potter tower, immersion) are often expensive and/or difficult to implement and are not always sufficiently reliable because they produce non homogeneous and non reproducible results. A new system was developed for evaluating precisely the biological activity of several granulovirus isolates on potato tuber moths. In this system, the spraying of viral suspensions was carried out by a Pulmo-Aide Sunrise® Compressor/Nebulizer usually used for the treatment of pulmonary infections. This system delivered small drops by the Venturi effect. PVC tubes directed the aerosol to the potato surface where it is deposited homogeneously. The system worked well in different locations where the atmospheric pressures were significantly different. To provide an internal control, we used samples of purified viral suspensions at appropriate dilutions mixed with Coomassie blue. Spectophotometer measurements of the optical density of the colorant deposited on the surface after nebulization showed that the reproducibility of the method was always greater than 95 %. This method is reliable, inexpensive and easy to use.

Key words: aerosol, nebulization, surface contamination, granulovirus, insect borer

Introduction

The droplet method usually used for the evaluation of the biological activity of baculoviruses is not appropriate in the case of borers like the potato tuber moths (Lepidoptera; Gelechiidae) whose mining larvae remain on the infected surface only for a few minutes. So, the biological activity of the *Phthorimaea operculella* granulovirus (PhopGV) was generally evaluated by immersing the eggs (Sporleder *et al.*, 2005) or the potato tubers (Zeddam *et al.*, 1999) in viral solutions which contain a certain number of macerated PhopGV-infected larvae (larva-equivalents). This protocol has since been improved by the use of purified virus granules instead of larva-equivalents. However, these methods do not allow the precise determination of the granule concentration present on the potato surface, which could lead to a high variability between experiments. A more precise technique is the pulverization of purified granules using a Potter tower, but its use as a routine methodology is expensive and thus not adapted for emerging countries. A cheap, precise and repeatable method of the evaluation of biological activity of a virus isolate is required to allow proper comparisons and quality control. Small droplet generators are widely used in the treatment of pulmonary diseases and the technology to produce droplets of reproducible size is well developed. We have examined the possibility of using such devices to deliver precise and repeatable virus doses onto a given surface.

Material and methods

Contamination device

The contamination device is composed of a medical compressor/nebulizer (DeVilbiss Pulmo-Aide Sunrise® Compressor/Nebulizer, model 5650, Sunrise Medical, USA) coupled to a combination of 125 mm-diameter PVC tubes constituting the tower and the chamber for pulverisation of the viral suspensions (Fig 1). Different constructions were tested by modifying the horizontal and vertical lengths as well as the position of the decompression hole. The total cost of the whole device was less than 200 US$.

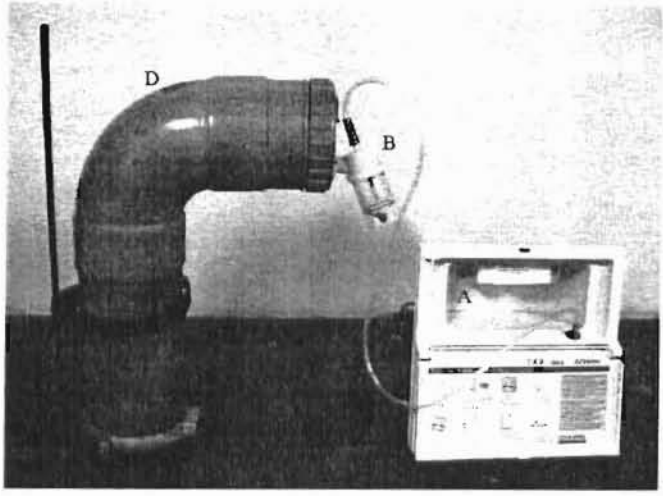

Figure 1. The nebulization device. A: Nebulizer, B: Nebulization chamber, C: Pulverization tower, D: Decompression hole

Repeatability and homogeneity of the spray

A Coomassie blue solution (500 mg/L) was used to calibrate our system. The nebulization chamber was filled with 1 to 5 ml of this colorant solution. Different volumes were tested to assess whether the system provided consistent results over this range. Five 16 mm-diameter plastic coverslips were randomly placed on the treated surface located at the base of the vertical PVC tube. The coverslips were used during the tests to collect the colorant solution deposited onto a known area and, therefore evaluate the homogeneity (i. e. the variability of the quantity of colorant among the 5 slipcovers corresponding to a single application) and the amount of solution recovered. After 10 to 20 min of pulverisation, the coverslips were recovered and dried. First, the homogeneity of the drop distribution was evaluated by light microscopy. Then, the dried coverslips were washed with 0,2 ml of water. The optical density (OD) of this solution was measured at 556 nm wavelength (corresponding to the maximum absorption of the Coomassie blue) using a spectrophotometer. The OD values were compared to a standard curve. Ten experiments were carried out for each device tested. The homogeneity of the experiments was estimated by the variation coefficient ($VC = S_x/x$) of the OD obtained for each coverslip. The repeatability was estimated by comparison of the

different VC obtained in the 10 experiments. A non parametric test of Kruskal-Wallis was used to compare the deposit of colorant onto the different coverslips for each construction. These results were compared with those obtained using a Potter tower.

Granulovirus application and yields estimation

Viral bio-assays were performed using purified supensions of the Tunisian isolate of PhopGV (Taha *et al.*, 2000). Different purified granule dilutions (corresponding to final deposits of 0,6, 6, 60, 600 and 6000 viral inclusion bodies/mm^2, respectively) were used with or without colorant to control the quantity of virus sprayed onto the treated surface by the system. For these controls, coverslips were laid and treated as previously described and the virus concentration was measured at OD 450 nm, corresponding to the wavelength at which the absorbance of the granulovirus solution is the greatest. The quantity of virus was evaluated using the formula derived from Tchang and Tanada (1978), modified by Zeddam *et al.* (2003): $6,8 \times 10^8 \times OD_{450}$ x dilution = Number of granules/ml.

Results and discussion

Nebulization device

Various configurations of the device were evaluated by nebulizing a Coomassie solution as a control of the quality of the deposits. The optimum homogeneity and repeatability were obtained using the PVC pulverisation device (Fig.1). At Saint-Christol-les-Alès (France, 140 m.a.s.l.), its dimensions were the following: Horizontal length: 385 mm. Vertical length: 345 mm. Decompression hole: 28 mm in diameter placed at 260 mm above the basis of the device. The system developed at Alès had to be adapted for use under different atmospheric pressure conditions, i. e. in Bogota (Colombia, 2600 m.a.s.l.) and Quito (Ecuador, 2800 m.a.s.l.). Namely, the place of the decompression hole and the length of the horizontal chamber were modified for these two latter locations. The hole was enlarged to 28 mm in diameter and displaced 175 mm above the device base and the length of the horizontal chamber was reduced to 290 mm.

Repeatability and homogeneity of the deposits

In Alès, a 94,0 % homogeneity and a repeatability of 97,5 % (i. e. the variability of the quantity of colorant deposited onto the 5 discs among the 10 repetitions) with variation coefficients of 0,0599 and 0,0252, respectively were obtained. No difference was observed between repetitions (Kruskal-Wallis test, Kc = 8,08; $P < 0,01$). One mg of Coomassie blue spray resulted in 8,065 ng of blue deposited/mm^2, representing a 9 % recovery (for an internal diameter of the pulverisation tower of 120 mm).The same protocol applied in Quito, with a slightly modified device, gave similar results with 94 % homogeneity and 95,8 % repetitivity with 0,0620 and 0,0419 VC, respectively, for a 10,4 % recovery. We obtained similar results for nebulized volumes in the range 1 to 5 ml. We carried out a comparison between pulverisations with a Potter tower and our system, before and after its calibration. Using a Potter tower a lumpy aspect was observed, indicating a bad dispersal due to a drop size too large. Light microscopical observations detected a great homogeneity in the sizes of the drops generated by the nebulization system.

Granulovirus application yields obtained with the device

After calibration with the Coomassie blue solution, the device was tested for its suitability for uniform application of the granulovirus onto the treated surface. The results were as good as those obtained with the colorant solution (data not shown). However, the yield was different

in the case of the PhopGV application. 2,5 ml of viral suspension containing 10^{10} granules nebulized with our protocol allowed us to deposit 10.000 granules/mm^2, representing a 1,13 % recovery. The difference between the quantities of blue and virus recovered may be due to a superior loss of virus in the horizontal part of the device. Using the immersion method, we estimated that only 1 of 10^6 granules were finally adsorbed onto the potato surface (Carrera *et al.*, 2002). Thus, with the same initial quantity of material, the yield of the virus deposited using the new device would be superior by more than 10^4 times to the yield given by the immersion method, which means that a much smaller amount of virus is required to perform the assays.

The method presented here appears very useful for the realization of standardized *per os* infection of potato tuber moth larvae. It is particularly suitable for the precise evaluation of the biological activity of granuloviruses and was successfully used for this purpose (Carrera *et al.*, 2002). This method presents three main advantages: it is cheap, repeatable, and easy to perform.

This device is presently used in laboratories located in several countries (France, Ecuador, Colombia and Costa Rica). It gave reliable results as the same lethal concentration values were obtained for the same viral isolate tested in each of these laboratories. One of the great advantages is the possibility of using a same calibrated method to compare the virulence of distinct PhopGV (or other granulovirus) isolates against laboratory colonies of potato moths maintained in different laboratories.

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