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Formulation Process Analysis of a Virus-based Biopesticide to Control the Tomato leafminer *Tuta absoluta*

Ginna Quiroga-Cubides[1*]
https://orcid.org/0000-0003-2138-2776

Juliána Gómez-Valderrama[2]
https://orcid.org/0000-0003-3963-1363

Gustavo Araque-Echeverry[2]
https://orcid.org/0000-0003-3703-3407

Paola Emilia Cuartas-Otálora[1]
https://orcid.org/0000-0001-8009-2600

Carolina Ruíz Moreno[2]
https://orcid.org/0000-0003-4015-6029

Martha Isabel Gómez Álvarez[1]
https://orcid.org/0000-0003-4919-2747

Gloria Patricia Barrera Cubillos[2]
https://orcid.org/0000-0001-8245-4388

Diego Cortés-Rojas[1]
https://orcid.org/0000-0002-5618-6284

[1]Corporación Colombiana de Investigación Agropecuaria (AGROSAVIA), Bioproducts Department, Mosquera, Colombia; [2]Corporación Colombiana de Investigación Agropecuaria (AGROSAVIA), Tibaitatá Research Center, Mosquera, Colombia.

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*Correspondence: gquiroga@agrosavia.co; Tel.: +57-1-3015187592 (G.Q.C.).

HIGHLIGHTS

- Technological analysis of the production for a virus-based biopesticide.
- Selection of technological conditions considered to have a significant influence.
- Scale-up strategy based on geometric and the dynamic similarities.
- Reproducibility and repeatability assessment of the formulation process.

Abstract: Virus-based biopesticides are effective biocontrol agents of crop insect pests. Development of suitable formulations and production processes are necessary to obtain high-quality products easily adopted by farmers. A detailed unit operation study was carried out for the production process of a *Phthorimaea operculella* granulovirus-based biopesticide to control the tomato leafminer, *Tuta absoluta*, one of the most important pests affecting this crop. Physicochemical, microbiological, and insecticidal parameters were implemented in the process and applied to the finished product, and a scaling strategy was developed. A Quantitative Polymerase Chain Reaction (Q-PCR) technique was implemented to quantify viral concentrations in the active ingredient ($5.34 \pm 1.44 \times 10^9$ Occlusion Bodies mL^{-1}) and in the finished product ($>1.6 \times 10^9$ OB mL^{-1}), without contaminant interferences. The Q-PCR methodology was also useful to select the appropriate solid mixing time following Lacey´s mixing index (8 min). Factors and similarity principles influencing the liquid mixing process were identified in the scaling evaluation. Furthermore, the drying kinetics analysis enabled identifying a drying temperature of 35 °C, with an efficacy under controlled conditions higher

than 97%. Contaminant concentration was lower than 1%, indicating controlled and aseptic formulation process conditions. A simple statistical method was used to estimate the reproducibility and repeatability of the parameters assessed in the finished product. These results enable to establish and extrapolate important parameters in the standardization, scale-up, and quality control for the granulovirus-based biopesticide.

Keywords: viral pesticide; process analysis; scale-up; Q-PCR quantification.

INTRODUCTION

Negative impacts on the environment and on human health from the use of chemical insecticides have promoted strict global government regulation of these products [1,2]. Worldwide, the development of biological pesticides for sustainable agriculture programs have been implemented and the registration process have been promoted in order to have greater access to them [2,3].

Virus-based formulations have been broadly used for their efficacy in controlling different pest insects, reducing their population below the threshold of economic damage [2,3]. Furthermore, their high selectivity facilitates safe handling and do not affect non-target organisms [3,4].

Recently, the Colombian Corporation for Agricultural Research (*Corporación colombiana de investigación agropecuaria,* AGROSAVIA) has been working on the development of a biopesticide based on a *Phthorimaea operculella* granulovirus (PhopGV isolate VG013) for the control of one of the most important pests of tomato crops, the armyworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) [5]. The biopesticide prototype developed has demonstrated high efficacy for controlling insect larvae in laboratory conditions, causing mortality rates greater than 80%. Dispersible solid prototypes tend to be more stable under storage and avoid the increase of contaminant concentration [6]. Furthermore, proper adjuvants need to be selected to preserve the efficacy of the viral active ingredients under field conditions [7].

Practical production processes, easy to operate and with acceptable production volumes, are required to facilitate the viral biopesticide production [8]. Likewise, the unit operations involved in the formulation process such as drying, mixing, granulation and milling must be carefully designed. Nevertheless, the implementation of processes to produce baculovirus-based bioproducts is affected by poor standardization of unit operations and their validation, and therefore, leads to the lack of a strategy for scaling up production and formulation [1]. Establishing quality controls in processing and in the finished product are essential to obtain reliable products. Reports on unit operations and process standardization of biopesticides are scarce and require special attention due to their sensitivity.

The present work aimed to develop a production process of a granulovirus-based biopesticide as well as the selection of technological conditions considered to have a significant influence on product efficacy. Here, we assessed key production parameters such as a scale-up strategy for the liquid mixing operation, the solid mixing time by Lacey´s mixing index, a drying kinetics analysis, and the insecticidal activity at different drying temperatures. Similarly, the active ingredient and finished product characterization, reproducibility and repeatability variables were evaluated.

MATERIAL AND METHODS

Viral active ingredient production

The Colombian virus strain used in this study was PhopGV encoded as VG013 which was previously isolated from *T. absoluta* larvae [5], and was deposited at the RNC (Registro Nacional de Colecciones Biológicas), with an accession number 129-Banco de Germoplasma de Microorganismos-AGROSAVIA (Germplasm Bank of Microorganisms-AGROSAVIA). This work was carried out under the Contract to Access Genetic Resources and its Derived Products No. 168 of 2017. PhopGV VG013 virus was multiplied in *Tecia solanivora* larvae from the rearing unit of the Entomology Laboratory of AGROSAVIA's Tibaitatá Research Center. For this purpose, *T. solanivora* eggs were inoculated with a viral suspension adjusted to $3x10^8$ occlusion bodies per mL (OB mL^{-1}) using a soft brush. The inoculated eggs were placed over potato tubers (*Solanum tuberosum sp. andigena* var. Pastusa suprema) inside plastic container and maintained under controlled laboratory conditions at a temperature of 20 ± 3 ºC and a relative humidity of 60 ± 5%. After 30 days, a destructive evaluation of the tubers was carried out and larvae with symptoms of viral infection were collected and stored at -20 ± 2 ºC (9). To prepare the active ingredient, 100 g of infected larvae were weighed and added to a 0.1% w/v SDS solution (MolLabs LTDA, Colombia). The mixture obtained was homogenized with a high efficiency homogenizer (T25 Ultra-Turrax™, IKA-Werke GmbH & Co, Germany) and subsequently

filtered through muslin cloth. The suspension obtained was centrifuged at 2900 *g* for 2 min and the supernatant recovered was centrifuged at 26900 *g* for 15 min. The pellet obtained was resuspended in 100 mL of sterile water. Viral suspension was quantified by absorbance measurements at 280 nm in a spectrophotometer (NanoDrop™ 1000, ThermoFischer Scientific, U.S.A.) and interpolated from a standard curve (R^2=0.987) previously constructed with purified OBs of VG013 counted in a dark-field-microscope (400X) using a Neubauer chamber, according to the methodology described by Zeddam and coauthors, 2003 [10]. The concentration was adjusted to $1x10^{10}$ OB mL^{-1}. The viral suspension was frozen at -20 °C until required for formulation (Cool-Lab™, Barnstead Lab- Line, Thermo Fisher Scientific, U.S.A.).

Viral active ingredient characterization

The viral suspension was analyzed to determine physicochemical, microbiological, and insecticidal parameters, per triplicate. Physicochemical characterization of the viral suspension was performed to determine pH (-), viscosity (kg m^{-1} s^{-1}), and relative density (-). The pH was measured taking 150 mL of the viral suspension in a 250 mL beaker and kept under constant agitation by turbine stirrer Bar-spin (Orto Alresa™, Spain). The pH was assessed using a potentiometer (C860, Consort™, Belgium). For viscosity evaluation, the viral suspension was assessed with the Hoppler ball drop viscometer Gilmont™ Instruments 2004 (Cole-Parner™, USA). Approximately 5 mL of the viral suspension was used to fill the tube in a vertical position and a glass ball was added. The time that the ball spent to decent between the two sets of fiduciary lines was measured with a stop-watch. Viscosity was calculated by the following formula:

$$\mu = \frac{k \cdot t(\rho_t - \rho)}{100}, \quad (1)$$

Where: μ is the viscosity (kg m^{-1} s^{-1}), k is a viscometer constant (35 min^{-1} seg^{-1}), t is time of descent (min), ρ_t is the ball density (g mL^{-1}), and ρ is the viral suspension density (g mL^{-1}).

The relative density was determined as the relation between the viral suspension weighted of 1 mL and sample volume.

Viral concentration and total contaminant content were assessed as microbiological parameters. Viral concentration was estimated by the granulin gene-based Q-PCR technique with a Taqman probe system. A calibration curve was used, elaborated with different concentrations of plasmid DNA with an insert corresponding to the ORF (open reading frame) of the granulin of the *Phthorimaea operculella* granulovirus [11]. The samples of viral suspension used in the amplification reaction were diluted in ultrapure water (1:100) and an aliquot of 1 mL was denatured at 100°C for 5 min. The supernatants obtained after centrifugation at 10,000 *g* for 15 s were used as template in the PCR reaction, as described by Cuartas and coauthors, 2018 [12]. The occlusion body concentration was based on calculation of gene copies obtained in the calibration curve. The VG013 granulovirus has one nucleocapsid per virion, indicating one genome per occlusion body [12].

The total contaminat content was determined by the spread plate method (CFU mL^{-1}). A volume of 5 mL of the viral suspension was diluted in 0.1% v/v Tween 80 solution, and decimal dilutions were made serially until 10^{-7}. For bacteria determination, 0.1 mL of 10^{-3}, 10^{-5}, and 10^{-7} dilutions were plated in Nutrient-Agar (Oxoid™, Thermo Fischer Scientific, USA), and incubated at 28 ± 0.5 °C for 24 h. For fungi content, 0.1 mL of 10^{-3} and 10^{-5} dilutions were plated in Potato Dextrose-Agar (PDA; Oxoid™, Thermo Fischer Scientific, USA) and incubated at 25 ± 0.5 °C for 72 h. For yeast content, 0.1 mL of 10^{-1} dilution was plated in Yeast-Malt-Agar (YMA; Oxoid™, Thermo Fischer Scientific, USA) and incubated at 25 ± 0.5 °C for 36 h. Results were expressed as the average number of colony-forming units per gram (CFU g^{-1}) [13].

For the insecticidal activity, a bioassay was carried out following the methodology described by [5]. A weight of 1 g of the viral suspension was suspended in sterile water, adjusting the concentration to $1x10^7$ OB mL^{-1}. Subsequently, tomato leaflets (*Solanum lycopersicum* var. Santa Clara) were cut into 6.25 cm^2 fragments. Each fragment was inoculated with the viral suspension, applying 200 µL of the suspension in the adaxial and 200 µL in the abaxial surfaces, using a nebulizer [14]. The inoculated leaflets were placed in 15 mL plastic cups which had a round piece of napkin inside, moistened with sterile water to maintain the container's humidity. Two artificially-reared, neonatal *T. absoluta* larvae were placed in each plastic container using a stereoscope (Stemi™ DV4 Series Stereomicroscope, Carl Zeiss™, Germany). The absolute control consisted of individuals fed with non-inoculated tomato leaflets. Each experimental unit comprised ten *T. absoluta* larvae (two per container). The treatments were maintained in a bioassay room at a temperature of 25 ± 2 °C and a relative humidity of 60 ± 5%. Individuals were checked daily from the fifth-day post-

inoculation, collecting the dead individuals. The corrected mortality or efficacy percentage was calculated based on mortality data using the Schneider-Orelli formula [15] (2).

$$Efficacy = \frac{b-k}{100-k} \cdot 100, \quad (2)$$

Where: *b* is the percentage of mortality in the treatment and *k* corresponds to percentage mortality of the control treatment.

Granulovirus-based biopesticide formulation process

Formulation was carried out in four-unit operations: liquid mixing, solid-liquid mixing, granulation, forced convection drying. Equipment specifications and operating conditions are described in Table 1.

Table 1. Unit operations for the production of PhopGV VG013 granulovirus-based biopesticide.

Unit operation	Equipment	Control parameter	Operating conditions
Active ingredient suspension with adjuvants	Turbine stirrer Bar-spin (Orto Alresa™, Spain). Flat blade, 5 cm diameter pH meter (Consort model CC860, Belgium)	pH of the mixture	Stage 1: viral suspension was mixed with a first sunscreen protector (UV No.1) at 0.78 m s^{-1} for 7 min. Stage 2: the phosphate buffer pH 8 and the polymer Eudragit S100™ (Evonik Industries, Germany) were mixed at 0.78 m s^{-1} for 10 min. Afterward, a pH stabilizer and a non-toxic organic solvent was added to the mixture for 3 min. Stage 3: mixture in Stage 2 was added to the suspension obtained in Stage 1 and mixed at 300 rpm for 3 min.
Solid-liquid mixing	Planetary mixer Ecomax (Hobart™, Germany). Flat beater (33 cm x 22 cm)	Viral concentration (OB mL^{-1}) Moisture content (kg water kg^{-1} dry matter)	An inorganic silicate-based diluent and a second sunscreen protector (UV No. 2) were mixed for 2 min. The stirring conditions were rotational agitation rate 136 rpm and translational rate 60 rpm. Subsequently, the liquid mixture obtained previously was added to the solid mixture, under the same stirring conditions for 2, 4, and 8 min.
Granulation	Oscillating granulator YK160A (Target Pharmatech CO., China) Moisture analyzer MLS503 (Kern™, Germany).	Moisture content (kg water kg^{-1} dry matter)	The solid mixture was homogenized using a mesh opening of 2 mm.
Forced convection drying	Drying chamber (AGROSAVIA, Colombia) Stainless steel trays (70 cm x 40 cm)	Moisture content (kg water kg^{-1} dry matter)	Drying temperature and relative humidity: 25 ± 2 °C, 48-53%; 35 ± 2 °C, 27-30%; 45 ± 2 °C, 13-17%

Scale-up strategy for the liquid mixing operation

Geometric and dynamic similarities were used as scale-up criteria and evaluated at laboratory and benchtop scales. A volumetric ratio between both scales were used as a scale-up factor for liquid mixing operation (3). This factor is given by the design characteristics of the formulation, to preserve the proportionality of the excipients and active ingredient used.

$$V_2 = k \cdot V_1, \quad (3)$$

Where: V_1 corresponds to the variable on the model scale; V_2 the variable at the prototype scale; and *k* is the scale factor. This value was used to determine the capacity of the mixing containers for the unit operation.

Geometric similarity was given by the relation height to diameter (*H/D*), and the dynamic similarity was related to power consumption per unit of volume or volumetric power input (*P/V*) for agitated tanks without

baffles [16,17]. The *P/V* criterion was developed from the power number (4), assuming the fluids mixed in each process have the same density and impeller diameters are similar. Agitation speed was determined from the expression obtained at the prototype scale -in this case-, at the benchtop scale (5):

$$N_p = \frac{P}{n^3 \cdot d^5 \cdot \rho}, \quad (4)$$

$$P/V = \frac{N_p \cdot n^3 \cdot d^5 \cdot \rho}{V}$$

$$P_1/V_1 = P_2/V_2$$

$$\frac{N_{p1} \cdot n_1^3 \cdot d_1^5 \cdot \rho}{V_1} = \frac{N_{p2} \cdot n_2^3 \cdot d_2^5 \cdot \rho}{V_2}$$

$$\frac{N_{p1} \cdot n_1^3}{V_1} = \frac{N_{p2} \cdot n_2^3}{V_2}$$

$$n_2 = \sqrt[3]{\frac{n_1^3 \cdot N_{p1} \cdot V_2}{N_{p2} \cdot V_1}}, \quad (5)$$

Where: N_p is the power number (-); P is the electric power (W); V, is the mixing volume (m^3); n, agitation speed ($m\ s^{-1}$); d, impeller diameter (m); and ρ, fluid density ($Kg\ m^{-3}$). Fluid density was calculated as the factor among the relative density and water density at 4°C. Numbers 1 and 2 refer to the model and prototype scale, respectively. The *Np* value is defined from the Reynolds number (*Re*; 6) and becomes constant for *Re* > 5000 values [16,18,19].

$$Re = \frac{\rho \cdot n \cdot d}{\mu}, \quad (6)$$

Where: μ is the fluid viscosity ($kg\ m^{-1}\ s^{-1}$).

For the Reynolds number and agitation speed at benchtop scale calculations, two laboratory-scale batches of liquid mixing were prepared with 15 mL of the viral suspension and performed into plastic containers (5.3 cm x 7.9 cm). The liquid mixtures obtained in each stage (Table 1) were characterized by measuring pH, viscosity, and relative density, according to the methodologies described previously. Geometric and dynamic similarities used as scale-up criteria and evaluated during the production of three batches of 1000 g finished product at benchtop scale.

Lacey´s mixing index

This parameter was determined to define the most favorable mixing time required in the solid-liquid mixing operation (Table 1). During the production of three batches at benchtop scale, three samplings were taken at 2, 4, and 8 min, at three mixer heights: bottom (0-3 cm), middle (3-6 cm), and surface (6-9 cm). The real viral concentration of the active ingredient measured by Q-PCR was used as a tracer. To do this, the samples were suspended in ultrapure water at a final concentration of 0.05 g mL^{-1}. In the amplification reaction, a 1 mL aliquot was taken and processed in the manner described above. The mixing index was calculated using (7) proposed by Lacey [20],

$$M = \frac{\sigma_0^2 - \sigma^2}{\sigma_0^2 - \sigma_R^2}, \quad (7)$$

Where: M is the mixing index (-); σ^2 is the population variance of the data taken for the same time at the different sampling positions; and σ_0^2 is the maximum variance (upper limit) of a completely segregated mixture calculated according to (8):

$$\sigma_0^2 = \frac{p(q-p)}{n}, \quad (8)$$

p is the concentration of the tracer in the final mixture and q corresponds to the maximum value of the tracer in the mixture; σ_R^2 is the minimum variance (lower limit) and corresponds to a completely random mixture; and n is the number of tracer particles.

Drying kinetics and insecticidal activity

The viral subproduct of the three batches at benchtop scale were distributed homogeneously in stainless steel trays (70 cm x 40 cm) with a maximum load of 230 ± 5 g, 1 ± 0.2 mm of thickness, and dried at a forced

air convection drying chamber. Three drying conditions of dry-bulb temperature and relative humidity were evaluated: 1) 25 ± 2 °C, 48-53%; 2) 35 ± 2 °C, 27-30%; and 3) 45 ± 2 °C, 13-17%. Drying curves (moisture content vs. drying time), and drying rate curves (drying rate vs. drying time) of each temperature was established, measuring moisture content and weight of three samples every 30 minutes. Moisture content (kg water kg^{-1} dry matter) was determined by means of a halogen balance (MLS503, Kern & Sohn GmbH, Germany), and dried at 121 °C to constant weight. The thin layer drying process ended when the moisture content was below 0.05. The drying rate was calculated with (9) [21].

$$DR = \frac{dM_t}{dt} = \frac{M_{t+\Delta t} - M_t}{\Delta t}; \quad (9)$$

Where *DR* is the drying rate between time Δt (h)*;* $M_{t+\Delta t}$ and M_t are moisture content at times *t* and $t+\Delta t$ (kg water kg^{-1} dry matter).

Finally, samples of 5 g were taken from each batch and each drying temperature to evaluate their insecticidal activity, according to the methodology described previously. The samples of finished product from each drying temperature were diluted in distilled water until obtaining $1x10^7$ OB mL^{-1} viral concentration. The percentage of efficacy was calculated with (2).

Finished product characterization.

Viral concentrations by the Q-PCR molecular technique (OB g^{-1}), total contaminant concentration (CFU g^{-1}), and moisture content (kg water kg^{-1} dry matter) of each finished batch at the benchtop scale were determined using the techniques previously described. For pH measures, 1 g sample was diluted in 99 mL of deionized water. The suspension produced was stirred by a magnetic agitator at 100 rpm (ST15, Torrey Pines Scientific, U.S.A.) and the pH was measured with a potentiometer (C860, Consort™, Belgium).

Statistical analysis

Experiments were carried on at three batches in independent trials done by triplicate. Data determined was compared by coefficient of variation analysis. For biological activity data, the analysis of variance was performed followed by Tukey HSD test for comparison between the means with a significance level of 5%, using the software Statistix™ 8.1 (Analytical Software, U.S.A.).

R & r test

In order to standardize the production system, the methodology described by [22] was employed to determine reproducibility and repeatability - known as the R & r test (Repeatability and reproducibility, respectively) - or the method of ranges and means. Viral concentrations (OB g^{-1}), total contaminant concentration (CFU g^{-1}), moisture content (kg water kg^{-1} dry matter), and pH of the finished product batches were used as evaluation variables. The quantification of repeatability, reproducibility, and the *R & r* test was performed using the following equations:

$$R\ (\%) = \frac{K_1 \cdot \bar{R}}{T} \cdot 100, \quad (10)$$

$$r\ (\%) = \frac{\sqrt{(K_2 \cdot x_D) - \frac{(K_1 \cdot \bar{R})^2}{n \cdot r^*}}}{T} \cdot 100, \quad (11)$$

$$R\&r = \sqrt{R^2 + r^2}, \quad (12)$$

Where: K_1 is a constant that depends on the number of replicates of the measured variable and provides a 99% confidence interval [22]; $\bar{R}$ corresponds to the average of the ranges, which are determined from the difference between the highest and lowest measured value; and *T* is tolerance, defined as the difference between the expected minimum and maximum values of the variable measured. K_2 is a constant that depends on the number of repetitions over time and provides a 99% confidence interval; x_D is the difference between the values of the highest average and the lowest average of the measurements made in the repetitions over time; *n* corresponds to the number of measurements per repetition; and *r** is the number of repetitions.

RESULTS

Viral active ingredient production

The average pH was 6.59 ± 0.03 (CV=0.03%), lower than the critical value defined as 8, to avoid virions release in subsequent processes. Viscosity was 0.0032 ± 0.0003 kg m^{-1} s^{-1} (CV=6.56%), value that was three times higher than the reference substance (water: 0.001 kg m^{-1} s^{-1}), while relative density was 1.06 ± 0.01 (CV=1.46%), close to that reported for water (0.988 at 20°C).

The viral concentration of the active ingredient estimated by quantitative PCR was 5.34 ± 1.44 x10^9 OB mL^{-1}, with a coefficient of variation (CV) minor to 1.5%. The quantification methodology was validated under previously-established parameters for the type of analysis, which include the correlation coefficient R^2 (0.99), efficiency (91.6%), and slope of the standard calibration curve (-3.66) [23,24]. The microbial contaminant concentration was 4.72 ± 3.60 x10^6 CFU mL^{-1} (CV=0.51%). Efficacy of the active ingredient for *T. absoluta* larvae under laboratory conditions was 95.2%.

Granulovirus-based biopesticide formulation process

Scale-up strategy for the liquid mixing operation

Analysis of the active ingredient with adjuvants was done in three stages: 1) mixing of the viral suspension with a protective agent; 2) mixing the adjuvants; and 3) mixing 1 and 2 mixtures (Table 2). The pH of the different liquid mixtures was greater than the values found for viral suspension and within expected values (pH<8). The three stages viscosities were similar among each other and comparing with the viral suspension viscosity (0.0032 kg m^{-1} s^{-1}), the results showed an increase of 1.5-fold. The mixtures relative densities were higher in the first stage and a 7% higher to the viral suspension.

Table 2. Physicochemical characterization of the active ingredient suspension with adjuvants and determination of the Reynolds number *(Re)* for the production at laboratory scale of PhopGV VG013 granulovirus-based biopesticide (Mean ± SD).

Stage	pH (-)	Viscosity (kg m^{-1} s^{-1})	Relative density (-)	Reynolds number (-)
1	6.80 ± 0.00	0.0057 ± 0.0003	1,139.9 ± 8.6	7,932 ± 387
2	7.20 ± 0.00	0.0061 ± 0.0001	1,129.2 ± 3.0	7,263 ± 73
3	6.80 ± 0.00	0.0062 ± 0.0002	1,115.9 ± 5.8	7,135 ± 68

The *H/D* value for the container used at the laboratory scale (5.3 cm x 7.9 cm) was 0.83. Therefore, to comply with this relationship, a container with a height of 9.8 cm and a diameter of 11.8 cm was used at the benchtop scale. Likewise, the volumetric scale factor presented a value close to 6.3 (parameters for equation 3: V_{lab}=170.5 cm^3; $V_{benchtop}$=1,071.7 cm^3).

The Reynolds number was required to calculate the volumetric power input (*P/V*), selected as a dynamic similarity criterion. The Reynolds number for the three stages of this operation ranged between 7,000 and 8,000 (Table 2). *Re* values provided to calculate agitation speed at the benchtop scale and equivalency in the power number was established for the two scales, according to the methodology described by Treybal´s [16], indicating similarity in regimes. The 6.3 value of the V_2/V_1 ratio was determined from the expression given by equation (5). Agitation speed at the benchtop scale was calculated to be 1.45 m s^{-1}, based on an agitation speed at laboratory scale of 0.78 m s^{-1}.

During preparation of the three batches at the benchtop scale and applying the agitation speed calculated by the *P/V* criteria, pH of liquid mixtures was monitored to ensure that this parameter did not exceed the value of 8 by the scale-up strategy. Stage 1 pH values were: 6.87 for batch 1, 6.90 for batch 2 and 6.91 for batch 3. Stage 2 pH values were: 7.39 for batch 1, 7.27 for batch 2 and 7.21 for batch 3. Stage 3 pH values were: 6.87 for batch 1, 6.90 for batch 2 and 6.91 for batch 3. In all three stages, pH values were below the critical value for the controlled release mechanism established for the formulation to be effective (pH=8). Also, pH values of stages 1 and 3 were close to the values reported in Table 2, while stage 2 pH values were between 5 and 8% higher than those reported at the laboratory scale.

Lacey´s mixing index

The mixing index was measured for the liquid-solid mixing unit operation, using the virus concentration (OB g^{-1}) as a tracer. Figure 1 shows that mixing index values varied from 0.93 to 1.07 (all close to one), meaning that theoretical values were equivalent to experimental ones. The virus concentration values converged at the mixing time of 4 minutes, i. e., when they were more homogeneous and precise. After 2 min, the mixture was heterogeneous with the presence of white particles, and at 4 minutes of mixing, most of the values tended to diverge, which may be related to a segregation phenomenon. Over time, the color of the mixture was observed by visual inspection of this unit operation to become darker and no white particles (probably corresponding to the excipient used as a diluent) were detected. Total mixture homogeneity and a more intense red color were observed in sampling times of 4 and 8 min.

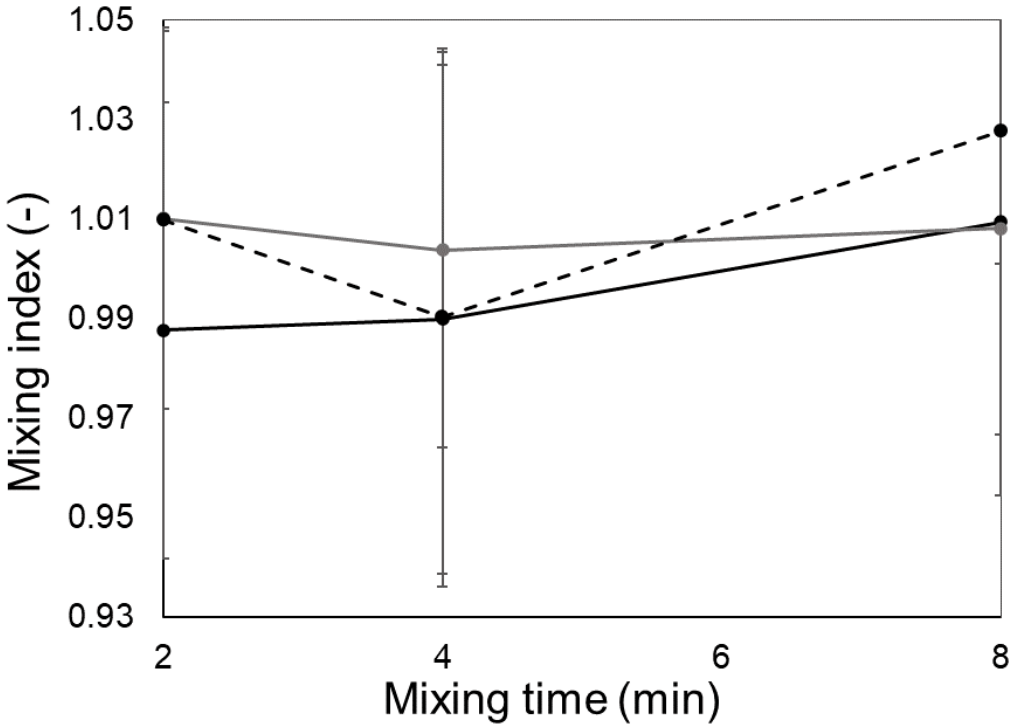

Figure 1. Mixing index in the solid-liquid mixing stage for the production at the benchtop scale of PhopGV VG013 granulovirus-based biopesticide: Bottom (**—**); Middle (—); and Surface (**- -**).

Drying kinetics and insecticidal activity

At the end of the granulation operation, the moisture content of the granulated product was less than 0.27, with average values of 0.26 ± 0.01, 0.22 ± 0.01, and 0.27 ± 0.01 for batches 1, 2 and 3, respectively, with a coefficient of variation less than 2.5%. Moisture content values found for batches 1 and 3 were very close to the theoretical value of 0.27, while batch 2 showed a reduction of 16%. Each batch was divided into three equal fractions, and each fraction was subjected to three drying temperatures (25 ± 2 °C, 35 ± 2 °C, and 45 ± 2 °C). The final time of the drying process was determined to guarantee better microbiological preservation, which is a moisture content below 5% [25]. The average moisture content of the finished product for each temperature showed CV values of 27.09%, 19.07% and 20.27%, respectively.

The divergence over moisture content and drying time at the three temperatures evaluated are showed in the Figure 2. Drying curves represent the variation of moisture with drying time. As seen in Figure 2a, the drying time decreased when drying air temperature increased, and revealed different average operating times: 25 ± 2 °C for 2.5 h, 35 ± 2 °C for 1.5 h, and 45 ± 2 °C for 1 h.

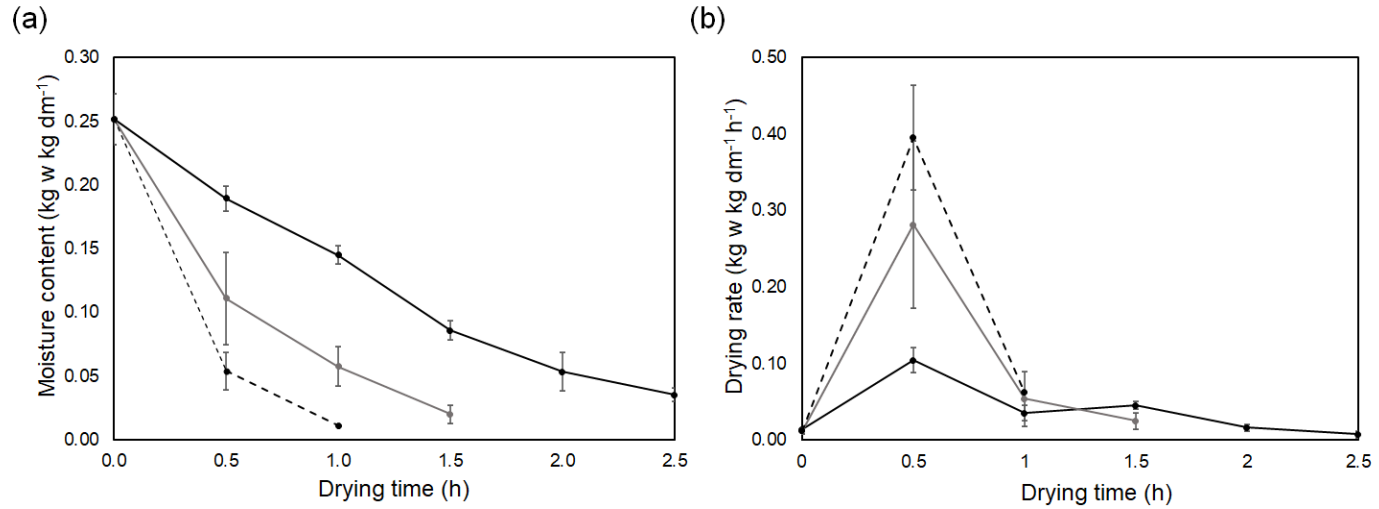

Figure 2. Drying kinetics for the production at the benchtop scale of PhopGV VG013 granulovirus-based biopesticide at different drying temperatures 25 ± 2 °C (—), 35 ± 2 °C (—), and 45 ± 2 °C (- -): (a) Moisture content (kg water kg^{-1} dry matter); (b) Drying rate (kg water kg^{-1} dry matter h^{-1}).

For drying rate curves, we assumed uniform initial moisture distribution, negligible external resistance, constant diffusivity, and uniform temperature. As expected, drying process at a temperature of 25 ± 2 °C was slower and instable; the rate estimated for the temperatures of 35 ± 2 °C and 45 ± 2 °C demonstrates an acceleration of the superficial water evaporation process (Figure 2b). The mean maximum drying rates for each temperature found after 0.5 h of drying, with high variation among temperatures: 0.10 kg w kg dm^{-1} h^{-1} (25 ± 2 °C), 0.28 kg w kg dm^{-1} h^{-1} (35 ± 2 °C), and 0.40 kg w kg dm^{-1} h^{-1} (45 ± 2 °C).

To determine the effect of drying temperature on biological activity, the mortality of *T. absoluta* larvae was evaluated for the product dried at the three temperatures, and the treatments mortality was corrected with the mortality in the absolute control (10%), obtaining the results presented in Table 3 (mean of the three replicates). Statistical differences were observed among the three treatments ($F_{3,8}$=3.83; p<0.0001). The viral concentration evaluated corresponds to LC_{90}, previously reported [5]. Therefore, an efficacy higher than 80% at the three drying temperatures, guarantee the insecticidal activity of the virus in the finished product. Additionally, the results after drying process were similar to that obtained with the unformulated virus (active ingredient: 95.2%), thus, the drying process did not affect the viral activity, at least in the newly manufactured product.

Table 3. Effect of drying temperature on the biological activity of the PhopGV VG013 granulovirus-based biopesticide.

Parameter	Batch No.	Drying temperature			Acceptance limits
		25°C	35°C	45°C	
Efficacy (%)	1	82.9	100.0	92.6	>80%[1]
	2	80.4	100.0	84.1	
	3	93.1	91.5	88.3	
	Mean	**85.5**	**97.2**	**88.3**	
	SD	6.7	4.9	4.2	

[1]Established by the manufacturer (AGROSAVIA).

Finished product characterization

The variables described in Table 4 were measured to verify that the three batches of the biopesticide complied with the quality parameters established for its use [8,25–27]. The coefficient of variation in each of these parameters did not exceed 15% for the physicochemical variables (CV between 0.23 and 12.4%), and 3% for the microbiological variables (CV between 0.09 and 2.90%). Viral concentrations of active ingredient of the three batches were higher than $1x10^9$ OB g^{-1}, which is the lower limit established by the manufacturer (AGROSAVIA). Viral concentrations were found within a range from $1.61x10^9$ to $4.21x10^9$ OB g^{-1}, with CV values of 1.05, 1.33 and 1.32 for the three batches, respectively. Therefore, the process formulation and the selected scaling parameters could be contributing to the reproducibility of the viral concentration.

Table 4. Physicochemical and microbiological characterization of the finished product for the production at the benchtop scale of PhopGV VG013 granulovirus-based biopesticide (Mean ± SD).

Parameter	Batch No.	Drying temperature			Acceptance limits
		25°C	35°C	45°C	
Viral concentration (10^9 OB g^{-1})	1	1.61 ± 0.07	2.80 ± 0.20	2.04 ± 0.22	1.0 ± 0.3[1]
	2	1.80 ± 0.15	1.93 ± 0.03	3.40 ± 0.92	
	3	2.36 ± 0.13	2.21 ± 0.13	4.21 ± 0.40	
Total contaminant content (10^5 UFC g^{-1})	1	1.08 ± 0.04	1.19 ± 0.03	1.20 ± 0.00	≤ 500 [8,26]
	2	1.29 ± 0.20	1.45 ± 0.09	0.85 ± 0.04	
	3	2.25 ± 0.10	2.68 ± 0.04	1.65 ± 0.22	
Moisture content (kg water kg^{-1} dry matter)	1	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	≤ 0.10 [25]
	2	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	
	3	0.04 ± 0.00	0.02 ± 0.01	0.03 ± 0.01	
pH (-)	1	7.33 ± 0.02	7.41 ± 0.07	7.35 ± 0.04	4 - 8 [27]
	2	7.29 ± 0.03	7.50 ± 0.02	7.45 ± 0.02	
	3	7.56 ± 0.03	7.45 ± 0.03	7.37 ± 0.04	

[1]Established by the manufacturer (AGROSAVIA).

The finished product showed an exponential reduction in contaminant content, compared to the value reported for the active ingredient. Results demonstrate the reproducibility of this variable when finding coefficients of variation of less than 2%. An inverse relationship of this variable with the drying temperature was observed, i.e., the higher the temperature, the lower the contaminant concentration.

As previously mentioned, this biopesticide must maintain a solution pH value below 8. In this case, this parameter was in a range between 7.29 and 7.56, complying with the previous premise. In addition, this variable presented a low variation in the three batches, with CV values of 0.47, 1.20, and 1.04%, respectively. Likewise, moisture content values lower than 0.04 were found in all the batches (Table 4), which ensures that there will be no increase in the contaminant burden [28]. In this case, the variability was greater (CV = 20-27%) due to the heat treatment that the samples received.

R & r test

Repeatability, reproducibility, and *R & r* values determined using equations (10-12) and the response variables of the finished product batches are shown in Table 5 (K_1=3.05, K_2=2.70, r=3, n=9). The parameters evaluated using viral concentration ($\hat{R}$=0.06, T=1.00, x_D=0.13), total contaminant content ($\hat{R}$=0.06, T=1.50, x_D=0.27), moisture content ($\hat{R}$=0.01, T=4.00, x_D=0.004), and pH ($\hat{R}$=0.16, T=2.00, x_D=0.1) were below 10%. *R & r* values less than 30% are acceptable to variables of a biological nature.

Table 5. *R & r* test evaluation in the production at the benchtop scale of PhopGV VG013 granulovirus-based biopesticide.

	Viral concentration (OB g^{-1})	Total contaminant content (CFU g^{-1})	Moisture content (kg water kg^{-1} dry matter)	pH
Repeatability (%)	2.41	2.50	3.76	8.13
Reproducibility (%)	3.94	9.60	0.90	4.22
R & r (%)	4.62	9.92	3.87	9.16

DISCUSSION

Baculoviruses, as an active ingredient of biopesticides, are a very promising insect control strategy. In the case of the tomato leafminer *T. absoluta*, one of the most important insect pests worldwide, a Colombian granulovirus (betabaculovirus) has demonstrated potential against this insect due to its efficacy in controlling

the insect larvae, its low impact on the ecosystem, and its high specificity and persistence in the environment [29–31]. As a result of these previous research, the granulovirus was selected as the active ingredient of a wettable powder biopesticide for the control of this insect. In the present work, the standardization of the formulation process, scaling, and quality control of the granulovirus-based biopesticide was carried out, important steps to ensure the conditions for an industrial production level.

Production standardization allows replication and uniformity of the quality parameters of the batches, maintains, or increases productivity, and reduces the variability of the physicochemical, microbiological, and biological characteristics of the biopesticide. The expected results are processes that systematically achieve their production and performance objectives, using well-defined conditions and quality standards to reduce the risk of the finished product being rejected. Besides, results confirmed that the scaling criteria employed in this study could be used for larger scales. In addition, the use of reliable and reproducible techniques for the quantitative analysis of quality parameters guarantees adequate control of the standards required for commercialization of the finished product.

Here, the microbiological, physicochemical, and efficacy parameters were considered in the standardization process for the biopesticide production. Microbiological characterization was focused on viral concentration and contaminant content. One of the most important steps in standardizing the production process of a viral biopesticide is the estimation of viral concentration, where the hemocytometer chamber and spectrophotometric techniques, based on extrapolation of calibration curves, are useful in the quantification of viral particles [10]. These methods have some limitations because require highly purified viral suspensions, hindering their use in production processes where formulation adjuvants or contaminants are present. Alternative nucleic acid-based methodologies could be used under these conditions to accurately quantify the virus particles. The quantitative PCR methodology using with the Taqman probe guarantees sensitivity and specificity, allowing the amplification of a specific region of the viral genome [11]. The differences observed in the concentration of viral particles could be related to different factors such as the validity of the analysis technique or even intrinsic characteristics of the sample. In the first case, the validation parameters of the technique were found within the established quality ranges [23]. However, it is important to consider the sensitivity of the technique due to small variations in the threshold cycle represent variations in the quantification of the viral particles. Another factor is the type of sample, where the distribution of the viral particles is not homogeneous, causing viral particle aggregates that could interfere in the quantification, especially in granuloviruses. The quantitative PCR methodology was useful to quantify viruses both in the active ingredient and formulated.

It is important to clarify that the quantification methodologies do not guarantee the presence of biologically active viruses; therefore, biological assay methodologies using susceptible hosts or *in vitro* viral titration in insect cells are necessary for viral estimation. Maximum efficacy of 85% and occlusion bodies concentration found in this bioproduct had the expected insecticidal capacity.

Quantification of contaminating microorganisms is also a relevant component in quality control, and the evidence of any change in their concentration may be associated with changes in the production process [4,26]. A contaminant content of $5x10^8$ CFU g^{-1} has been established as acceptable for virus-based solid biopesticide formulations [26], considering that the microbial content of the larvae used in the active ingredient propagation can increase contamination to values higher than $2x10^8$ CFU mL^{-1} (3.3% of the concentration of the active ingredient). Therefore, the upper contaminant limit is defined as 5% of the viral concentration. The three batches produced in this study guaranteed a contaminant content below this limit (<0.01%) and much lower than those suggested by the authors previously mentioned. The inverse relationship between the decrease in contaminant content and the increase in drying temperature causes cell inactivation by dehydration and heat damage [32]. In this process, water molecules are removed from the cells and surroundings, limiting chemical reactions and metabolic activities [33]. Due to the dilution effect by the addition of the formulation excipients, contaminant content in the finished product is expected to be less than $1x10^6$ CFU g^{-1}.

Physicochemical characterization was determined with pH, viscosity, and relative density parameters. The pH values of the active ingredient were within the range required to maintain stability of both the viral active ingredient and the finished product [8,26,27]. Values close to neutrality must be maintained for the pH variable throughout the production process in order to avoid dissolution of occlusion bodies, which occurs under conditions of alkaline pH present in the digestive tract of the host insects [27].

Viscosity and relative density parameters of the viral active ingredient and of the liquid mixing are important factors to consider in the design of production processes, since the choice of equipment required for mixing operations depends on these parameters [18]. Due to the nature of the adjuvants used, the

viscosity determined in the mixing stages was approximate twice the value found for the active ingredient (Results Section, Table 2). Regarding the relative density of the mixtures, an increase of 7% was observed compared to the density of the viral suspension (Results Section, Table 2).

Selection of scaling batch sizes was based on the availability of supplies and equipment, the operational capacity, the physical characteristics of intermediate products, and the finished product size [34]. Based on this diagnosis, two similarity criteria were established for scaling the mixture of the active ingredient suspension with adjuvants: geometric and dynamic similarities. In liquid mixtures, geometric similarity at both benchtop and laboratory scales is related to the dimensions of the mixing containers [18]. For scaling-up purposes, the difference between the linear relationships such as height over diameter relation (*H/D*), must be a maximum of 20% to maintain a satisfactory geometric similarity [35]. In this study, the containers were selected to fit this criterion, with a difference in the linear relationships of only 0.29%.

In general, a scale-up process enables fulfilling production needs and meeting demand for the product; corroboration of the process requires methodologies that use mathematical modeling and an empirical component. The proper selection of criteria reduces errors due to inaccurate correlations or lack of information, achieving efficient use of resources and time [36–38]. The dimensionless parameter volumetric power input (*P/V*) was used to support dynamic similarity and correlate impeller speed, relative density, and viscosity. It corresponds to a parameter used in load-controlled mixing processes, such as the dispersion of solids in liquids and is related to physical phenomena the mixture (i.e. oxygen transfer, turbulence intensity, mixing uniformity, and hydrodynamic stress) [36,39,40].

The scale-up criteria *P/V* enables reaching an interfacial area per unit volume similarity in liquid or solid-liquid dispersions. Other scale-up factors require different fluid-dynamic conditions from those exposed in this study [36,39]. On the other hand, the impeller speed determined by the *P/V* parameter increased by 85% with respect to laboratory scale, since an impeller with the same geometric characteristics was used for both scales. Nevertheless, an additional impeller with a larger diameter is recommended for larger scales, keeping the diameter ratio of the impeller and the mixing container equal in all scales [18].

In solid mixtures, homogeneity was verified by means of the mixing index factor, since it enables defining the degree of magnitude of the mixture, evaluating the mixer efficiency, and determining operational effective time [41]. In an ideal mixing process, concentration dispersion values are expected to decrease as time passes and, therefore, the mixing index approaches 1. However, excessive mixing times can cause segregation of the active ingredient, from a random to a non-random mixture. This phenomenon is more likely to occur in solids mixtures with particles of different size, shape and density [41]. In a mixing operation, the proportion of the active ingredient in the formulation must also be considered. In this case, the active ingredient represents 10% of the wet-based formulation and a mixing time of 4 min can be considered acceptable for this type of processes.

Final time of the drying process was determined based on the moisture content of the product (<0.05), which is recommended to slow microbial metabolism and prevent the proliferation of contaminants [28]. High moisture contents in the product can cause a reduction in the glass transition temperature and the matrix can change to an elastic state where molecule mobility and chemical reaction speed is greater. Greater molecule mobility destabilizes the biological material and changes the solid prototype characteristics, causing DNA denaturation and reducing shelf life [32].

The drying curves define the variable and constant rate periods in which superficial and internal moisture content is removed [42]. In the variable rate periods, drying temperature affects vapor pressure in the solids and causes the removal of moisture from the inside faster to surface [21]. Initial high drying rates caused a reduction in virus viability measured as efficacy and coefficients of variation greater than 10% [21,43]. Figure 2 shows a first variable rate period that corresponds to an increase of water loss associated with an accelerated reduction of moisture content. The solid surface contains free liquid with a constant vapor pressure at the surface and equal to the saturated vapor pressure [44]. Afterward, the curves described a deceleration (Figure 2b), associated with the slope reduction in moisture content curves or constant water content (Figure 2a). Falling rate periods could be associated with moisture diffusion and represented by Fick's second law of diffusion; solid surface enters the hygroscopic phase, in which the water activity decreases by increasing the saturated vapor pressure and keeps a positive vapor flux at surface [21,44].

Insecticidal activity results showed that the drying temperature did not affect the efficacy of the virus formulated under the conditions evaluated. Baculoviruses have previously been shown to be more resistant to adverse conditions compared to microorganisms such as fungi and bacteria because viruses are protected by protein occlusion bodies [45]. This advantage enables the selection of different technologies for unit operations in the formulation process, optimize costs and production time. In our study, the tolerance of a

drying temperature of 35 ± 2 °C decreased operating time concerning to 25 ± 2 °C (Figure 2) and showed the highest biological efficacy (97.2%).

The interpretation of the *R & r* test was based on Llamosa and coauthors, 2007 [22], whose method allows decomposing the variability of a system into repeatability and reproducibility. *R & r* test values between 10 and 30% indicate that the system is considered acceptable due to the biological nature of variables used and, therefore, the production of the biopesticide would be considered as standardized [22]. The parameters assessed at benchtop scale showed *R & r* test values less than 10% for contaminant content and pH, while viral concentration and moisture content had vales less than 5% (Table 4).

CONCLUSION

The scale-up strategy of the viral biopesticide based on granulovirus for *T. absoluta* control, enabled the identification of the factors and principles of similarity that influence the dispersion process. Furthermore, the physicochemical, microbiological, and biological parameters monitored in the formulation process resulted in a finished product within the expected limits to comply with pesticide activity under benchtop scale conditions. This methodology could be extrapolated for generating production parameters at a larger-scale and the required adjustments to reduce costs, energy consumption, and process time.

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