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# An immunological method for granulovirus detection in larvae of *Tuta absoluta*: searching for isolates with prospects for biological control of this pest in Colombia

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The tomato leafminer *Tuta absoluta* is an important insect pest of tomato crops worldwide. Biological control using entomopathogenic viruses such as isolates of *Phthorimaea operculella* granulovirus (PhopGV) can be a promising tool for suitably controlling this pest. However to date, few viral isolates from *T. absoluta* have been reported. Therefore, the aim of this study was to develop an economic, fast and accurate immunological technique for granulovirus detection and to evaluate viral presence in *T. absoluta* larvae from field samples collected in Colombia. Polyclonal antibodies against PhopGV were produced by using hens and rabbits. The production system in hen was selected as the most efficient and simple method. Then, a dot-blot test was developed, demonstrating the ability of antibodies to recognize granulovirus. Finally, granulovirus presence in 1186 *T. absoluta* larvae collected in the most important tomato production areas in Colombia was evaluated, and eight isolates were found in Boyacá and Cundinamarca (0.7% of occurrence). These results constitute the first report of a granulovirus isolated from *T. absoluta* larvae which had been naturally infected in Colombia's tomato producing-fields, and thereby validates the use of the immunological technique to detect granulovirus in field conditions.

**Keywords:** biological control; granulovirus occurrence; immunological technique; *Phthorimaea operculella*; polyclonal antibodies; tomato leafminer

## 1. Introduction

The tomato leafminer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), currently present in America, Europe, Africa and Asia, is considered economically to be one of the most significant pests of tomatoes in greenhouses (Desneux et al. 2010; Guedes and Picanço 2012). The larvae of this insect cause damage to leaves, flowers, stems and most seriously to fruits by feeding inside them, resulting in commercial losses of up to 100% and reductions in crop yield of up to 90% (Desneux et al. 2010; Potting 2010; Urbaneja et al. 2012). In Colombia, *T. absoluta* has been found in most greenhouse tomato crops, and its control is carried out mainly with chemical methods, although the development of resistance is leading to control failure (Guedes and Picanço 2012). This insect has increased its resistance to chemical pesticides that were previously effective, such as permethrin and cartap (Siqueira et al. 2000; Lietti et al. 2005; Silva et al. 2011), which has motivated the development of alternative methods.

An important and increasingly used strategy for the management of *T. absoluta* is biological control using bacteria, fungi, viruses, nematodes, as well as insect parasitoids and predators (Desneux et al. 2010; Chailleux et al. 2012; Zappalà et al. 2012). Among these, the use of entomopathogenic viruses such as those of the Baculoviridae family has been little explored. Baculoviruses include

betabaculoviruses (lepidopteran-specific granuloviruses), which are organisms with high pathogenicity, virulence, and specificity that have demonstrated their efficacy in controlling other Lepidoptera such as *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) and *Tecia solanivora* (Povolny) (Lepidoptera: Gelechiidae) (Zeddarn et al. 2003; Mascarín et al. 2010). However, to date, there are no records of the use of baculoviruses for the control of *T. absoluta*.

Baculoviruses represent a family of viruses with double-stranded DNA that infect invertebrates, especially insects (Caballero et al. 2001). The great majority of baculovirus strains have a narrow host-spectrum, which means that they may be used as bioinsecticides, without affecting beneficial insects such as parasitoids and/or predators of the pest (Caballero et al. 2001) or plants and vertebrates including humans. The most important characteristic of baculovirus is the presence of protein occlusion bodies (OBs) that stabilize them during long periods (Rohrman 2011).

Although granuloviruses isolated from *T. absoluta* larvae in Colombia have not been reported, the susceptibility of insects to a granulovirus isolated from *P. operculella* has been demonstrated (Mascarín et al. 2010). However, the isolation of virus from tomato leafminer larvae might provide a virus which is better adapted to this host and

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more efficient for its control. In addition, isolates obtained from the location where *T. absoluta* control is required may be better adapted to the environmental conditions of the ecosystem (Cory et al. 2005).

Granulovirus detection and quantification by conventional methods such as microscopy, or by reproducing disease symptoms in the host, prove to be wasteful, expensive, and sometimes require a significant sample (Arneodo et al. 2012), which may deter the search in *T. absoluta* larvae. In this sense, immunological or molecular methods have proven to be a good option for the detection and quantification of granulovirus, both in the search for new isolates and in quality control testing of formulated products (Parola et al. 2003; Arneodo et al. 2012). A variety of serological tests are used to identify antigens, among which the dot-blot assay stands out. In this technique, the antigen is passively adsorbed (blotting) when a droplet (dot) is placed on a nitrocellulose paper, and recognition is performed using specific antibodies with a substrate that allows visualization of color on the paper. The dot-blot assay is a rapid, accurate, specific, sensitive, and easy-to-perform test, and it is generally used in qualitative studies (Lange et al. 2010).

Considering the importance of *T. absoluta* as a worldwide pest infesting tomato crops and the need to implement improved biological pest management programs for its control, the objective of the present work was to develop an economical, rapid, and accurate immunological technique allowing the detection of granulovirus in *T. absoluta* larvae. In addition, we explored the presence of the virus in insect larvae collected from different regions in Colombia, in order to select an isolate with potential to be used for the development of a biopesticide.

## 2. Materials and methods

### 2.1. Viruses

Pure viral suspensions of the following isolates were used: a granulovirus from *P. operculella* (named as VG006) (Léry et al. 2008) and a nucleopolyhedrovirus from *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) (named as VG008 and NPV003, respectively) (Gómez et al. 2010).

### 2.2. Production of polyclonal antibodies for granulovirus detection

The efficiency and specificity of polyclonal antibody production were evaluated in two domestic animal species (hens and rabbits), by inoculating them with a viral suspension of granulovirus from *Phthorimaea operculella*, adjusted to a concentration of 9.2 µg of total soluble protein per milliliter, equivalent to  $2.46 \times 10^7$  of OBs per milliliter (OBs/mL). Rabbits were selected because they are the most commonly used mammals for polyclonal antibodies production, and hens were selected because they represent a refinement and a reduction in animal use, avoiding painful and invasive blood sampling that is replaced by collecting eggs (Chalghoumi et al. 2009).

#### 2.2.1. Production of antibodies in hens

Two, 16-week-old Hy-Line Brown hens were inoculated intramuscularly. A final volume of 1 mL of virus/adjuvant (complete or incomplete Freund Sigma F5881 and F5506) mixture was injected into individual hens. This volume was divided into two, 0.5-mL inoculation volumes, each of which was injected in either the left or the right side of the breast, following the inoculation scheme described by Schade et al. (2005). Eggs produced at 7 and 15 days after inoculation (DAI) were collected, and antibodies purification was made using the commercial kit, EGGstractIgY Purification System, from Promega®.

#### 2.2.2. Production of antibodies in rabbits

Two, 4-month-old New Zealand White rabbits were inoculated intramuscularly. A final volume of 0.7 mL of virus/adjuvant (complete or incomplete Freund Sigma F5881 and F5506) mixture was injected into the rabbit's leg, following the same inoculation scheme employed in the production of polyclonal antibodies in the hen. At 15 DAI, the rabbits were bled by cardiac puncture and the antibodies were purified following the protocol described by Josic and Lim (2001), consisting in a purification with ammonium sulfate and dialysis.

### 2.3. Sensitivity of the antibodies by ELISA

An indirect enzyme-linked immunosorbent assay (ELISA) to determine the minimum concentration of antigen (viral OBs), and needed to recognize both types of antibodies, was developed. The antigen used was PhopGV VG006 in total protein concentrations ranging from 20 ng/mL to 250 ng/mL ( $5 \times 10^3$  to  $1 \times 10^8$  OBs/mL). The methodology used, as described by Hornbeck et al. (2001), was as follows: briefly, 50 µL of antigen was attached to 96-well ELISA plates and incubated overnight at 4°C, followed by three washes with phosphate buffered saline solution (PBS). Then, 50 µL of the antibodies from either the hen or rabbit were added at dilutions of 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and 1:2048, and the plates were incubated at 37°C for 90 min. Subsequently, 50 µL of conjugate (antibodies: anti-chicken IgY Alkaline Phosphatase Sigma A9171® or anti-rabbit IgG Alkaline Phosphatase Sigma A3687®) were added at a dilution of 1:1000 and incubated for 90 min at 37°C. The reaction was done by adding 50 µL of *p*-nitrophenylphosphate 0.1% in diethanolamine-HCl 10% (pH 9.8) to each well. Plates were read on an ELISA reader (Expert 96, AsysHi-tech GmbH), at 405 nm. PBS was used as a blank. Antibodies with the highest sensitivity for detecting granulovirus were selected.

### 2.4. Affinity of the selected antibodies for the viral antigen

Once the antibodies were selected, a dot-blot test was performed to determine the affinity of the antibodies for the

antigen. For this purpose, the following samples were used as the antigens: purified OBs from PhopGV VG006 adjusted to  $6 \times 10^7$  OBs/mL, the macerate of 10 infected *T. solanivora* larvae, and a suspension of purified OBs from granulovirus isolated from *S. frugiperda* (VG008) adjusted to the same concentration as that of PhopGV.

To check the specificity of the antibodies, two negative control treatments were employed: the macerate of 10 healthy *T. absoluta* larvae and a suspension of a virus of the baculovirus family, genus alphabaculovirus (*S. frugiperda* nucleopolyhedrovirus NPV003; Gómez et al. 2010), which was adjusted to the same concentration as described above. Sterile water was used as a negative control of the technique.

The sensitivity of the technique was determined by evaluating 10 PhopGV suspensions, adjusted to concentrations ranging from 0.87 ng/mL to 7.84 µg/mL.

For dot-blot development, the following methodology was used: drops of 2 µL of the antigens and the controls were placed onto a nitrocellulose membrane. Membranes were soaked in transfer buffer for 15 min and then were allowed to dry. Afterwards, blocking was performed by immersing the membranes in a buffer containing 5% skimmed milk for 1 h at 37°C and rinsed three times with a wash buffer (Tris-HCl 1 mol/L 10%; EDTA 0.2%; Tween 20, 0.1%; NaCl 0.9%). Membranes were incubated for 60 min in a solution containing the purified antibodies at a 1:100 dilution, and after this time the membranes were washed. A second block was performed for 30 min at 37°C, and the membranes were rinsed again. Immediately, the conjugate was added at a dilution of 1:5000 and the color development was carried out by immersing the membranes in a solution of the chromogenic reagent (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; SigmaB5655®). This test was replicated three times.

### 2.5. Granulovirus detection in *Tuta absoluta* larvae

Larvae of *T. absoluta* were obtained from greenhouse tomato crops located in two municipalities in each of the four most important tomato production departments in Colombia: Cundinamarca and Boyacá (located in Central Region, 4°36' north latitude and 74°5' east longitude), Norte de Santander (located in Central Northern Region, 8°00' north latitude and 76°00' east longitude) and Caldas (located in Central Western Region, 06°05' north latitude and 75°38' west longitude). One to three *T. absoluta*-infested greenhouses in each town were checked. In each of these greenhouses, all larvae that were present on leaves from the middle and upper part of approximately 1000 tomato plants were collected. Each greenhouse was visited twice, once per semester (February and August 2012). Each larva was individually placed in microcentrifuge tubes (Eppendorf®) containing 100 µL of saline solution (NaCl 0.9%), macerating the tissue using a micropestle.

A drop of 2 µL of each larval macerate was dispensed in a nitrocellulose membrane in order to perform

granulovirus detection, following the dot-blot methodology described above. Positive samples were subsequently used to multiply the infectious agent and confirm the disease, using a subsample of 20 µL of larval macerate for the surface contamination of 20 *T. absoluta* eggs. The eggs were placed on a tomato leaflet in a Petri dish, and were then incubated for 20 days (temperature:  $25 \pm 3^\circ\text{C}$ ; relative humidity:  $50 \pm 10\%$ ; 12:12 light:dark photoperiod). The insect larvae were checked daily and those showing symptoms of viral infection were collected. The samples in which symptoms of viral disease were observed, as an intense whiteness, were considered as positive and were classified as viral isolate. Granulovirus presence was confirmed by a polymerase chain reaction (PCR) test, using degenerate primers for granulin gene amplification, previously designed by Jehle et al. (2006) based on a similarities analysis conducted between highly conserved sequences of this gene that were reported for seven different insect species (Lange et al. 2004).

## 3. Results and discussion

### 3.1. Production of polyclonal antibodies for granulovirus detection

From two selected hen eggs, 14 mL of IgY antibodies was obtained at a concentration of 4.47 mg/mL, whereas 28.5 mL of serum at a concentration of 7.31 mg/mL was obtained from an immunized, selected rabbit. Although there were greater quantities and concentrations of antibodies from the rabbit serum (208 mg), one hen can produce four times more antibodies than one rabbit, taking into account the number of eggs that can be collected. In the present work only two eggs were used, but five to seven eggs from one hen can be collected per week for 4 weeks (Schade et al. 2005; Reyes-Montaña et al. 2012), which means that approximately 876 mg of antibodies could be obtained. The amount of antibodies produced from hen eggs or rabbit serum in the present study is within the range reported by other authors, which is between 4 mg and 10 mg of antibodies per milliliter of egg yolk (Reyes-Montaña et al. 2012) and from 8 mg to 13 mg of antibodies per milliliter of rabbit serum (Michael et al. 2010). Moreover, the extraction of antibodies from rabbits was more labor intensive and costly compared with the purification of antibodies from hens, because overnight processing after obtaining rabbit serum is required whereas antibody purification from hens is a two-hour process. In addition, antibody production in rabbits involves some steps that could cause distress to the animal, such as cardiac puncture for animal bleeding in order to obtain the serum (Shin et al. 2009; Michael et al. 2010).

### 3.2. Sensitivity of the antibodies by ELISA

The minimum concentrations of the antigen that were detected by the antibodies produced in hen and rabbit were determined by the ELISA test. A higher sensitivity of the antibodies obtained from hen (IgY) was observed in comparison to the antibodies obtained from rabbit (IgG). For

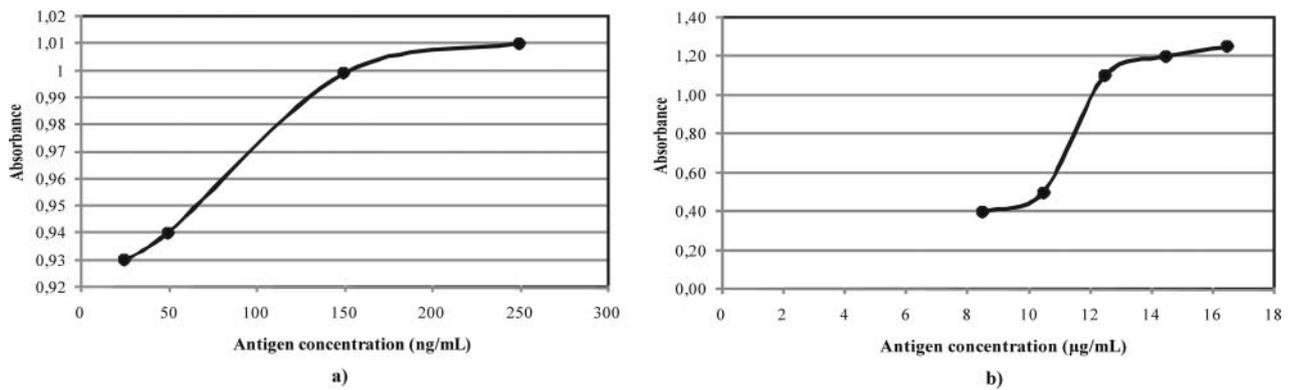


Figure 1. Absorbance at 405 nm using different antigen concentrations (virus) in ELISA test with antibodies produced in hen (a) and in rabbit (b).

instance, with the use of IgY it was possible to observe a proportional increase in optical density values for evaluated virus (from 25 ng/mL to 250 ng/mL). In contrast, higher antigen concentrations (from 8 µg/mL to 17 µg/mL) were necessary to detect optical density values when IgG was used. IgY antibodies (produced in hen) detected a minimum amount of 25 ng/mL (Figure 1a), which is equivalent to  $6.7 \times 10^4$  OBs/mL, whereas IgG antibodies (produced in rabbit) detected a minimum amount of 8 µg/mL, equivalent to  $2.14 \times 10^7$  OBs/mL (Figure 1b). The results obtained provided evidence that IgY produced in hen eggs had higher affinity for the viral OBs than IgG produced in rabbit serum. In addition, IgY could have higher multivalency than IgG. (Birds can often produce antibodies which recognize epitopes that are different from mammalian antibodies, as reported by Grzywa et al. [2014.]). This characteristic of the IgY antibodies could be related to the fact that birds attain antibody diversity using sequences of pseudogenes in a process of gene conversion, and this process produces a wide range of immune responses and more diverse antibody molecules compared with mammals (Kovacs-Nolan and Mine 2004). In the present work, the complete viral particle was used to immunize hens and rabbits, which makes possible the

production of antibodies that recognize different kinds of molecules, which leads to a greater immune response.

As a consequence of the results discussed above – and considering that IgY provides a more hygienic, cost efficient, convenient, and plentiful source of antibodies as compared to the traditional method of obtaining antibodies from mammalian serum (Kovacs-Nolan and Mine 2004) – IgY antibodies were selected for continuing with development of the immunological technique for granulovirus detection.

### 3.3. Affinity of the selected antibodies for the viral antigen

The present study revealed that IgY antibodies recognized the 10 samples of granulovirus-infected larvae, but they did not recognize the 10 samples of healthy larvae, suggesting specificity in recognizing the presence of granulovirus in the larvae but not the larval proteins. In addition, antibodies also recognized *S. frugiperda* granulovirus and presented a slight degree of cross reactivity with *S. frugiperda* nucleopolyhedrovirus (Figure 2). This result suggests that the antibodies so produced mainly recognize the protein granulin, which is quite variable between species

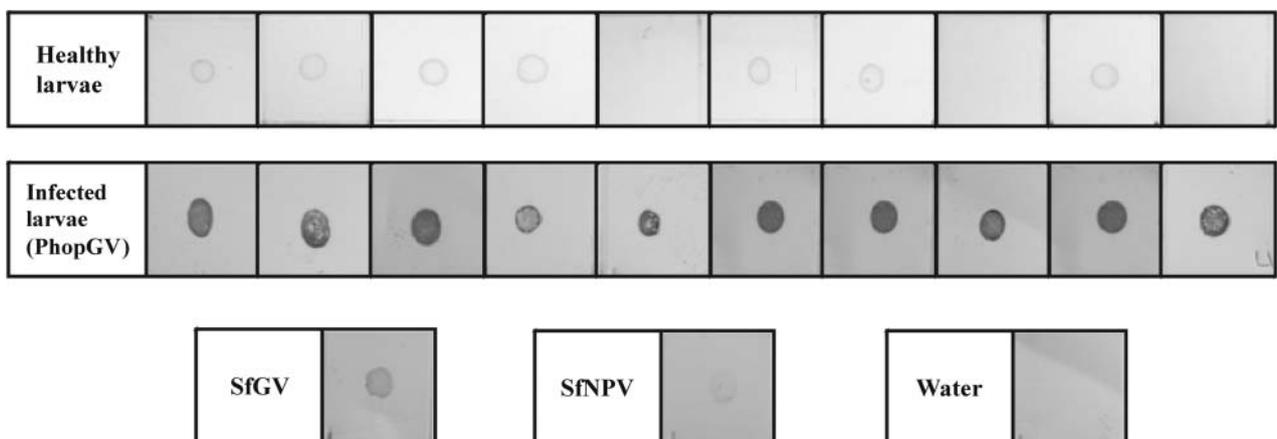


Figure 2. Dot-blot membranes for recognizing different antigens with the antibodies produced in chicken against granulovirus. SfGV: *Spodoptera frugiperda* granulovirus. SfNPV: *Spodoptera frugiperda* nucleopolyhedrovirus.

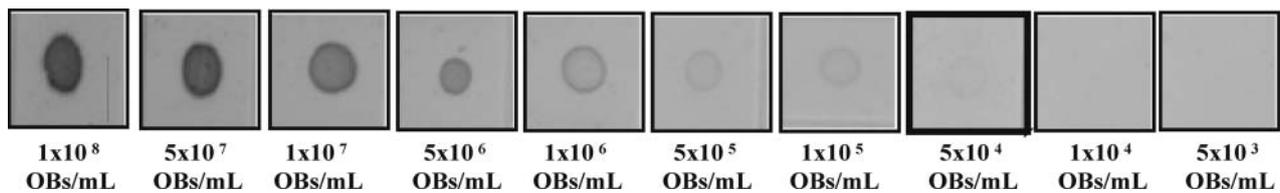


Figure 3. Dot-blot membranes for recognizing different concentrations of granulovirus (sensitivity of the method). In the box is highlighted the minimum concentration detected.

of granulovirus (Oh et al. 2013). However, produced antibodies are polyclonal, so they may also recognize some of the 30 proteins that are shared between granulovirus and nucleopolyhedrovirus (Miele et al. 2011).

Results of the dot-blot test performed to determine the sensitivity of IgY antibodies showed that the intensity of the spot generated by the immunological reaction decreased as the concentration of the sample decreased, with a concentration of  $5 \times 10^4$  OBs/mL (18.4 ng/mL of viral protein) being the most diluted sample that showed a positive response, a similar result to that obtained with the ELISA test (Figure 3). The detection range of samples obtained in this study (over  $5 \times 10^4$  OBs/mL) is within the range reported by other authors for immunological techniques such as ELISA. For instance, Parola et al. (2003) and Thorne et al. (2007), using baculovirus as an antigen, were able to develop antibodies for the detection of granulovirus from insect larvae and complex samples of granulovirus formulations.

Antibodies produced in hen showed a high affinity for viral proteins with no cross-reactivity with larval proteins. Hence, the use of these antibodies could be an adequate tool for detecting granulovirus in *T. absoluta* larvae.

### 3.4. Granulovirus detection in *Tuta absoluta* larvae

A total of 1186 *T. absoluta* larvae from 12 municipalities located in the four major tomato-producing departments in Colombia (Boyacá, Caldas, Cundinamarca, and Santander) were analyzed. They were collected in 22 commercial greenhouses visited during February 2012 and 20 commercial greenhouses visited in August 2012.

Larvae samples collected in the field were asymptomatic, suggesting that symptoms of viral infection exhibited in the field are hard to detect, which could be the result of absence of the virus in the larvae collected, or else because there may have been some sick individuals that presented latent infection (Laarif et al. 2003).

Only eight samples showed a positive response in the dot-blot test, indicating a possible granulovirus infection. Of these positive samples, five were found in the department of Boyacá and three in the department of Cundinamarca, and all samples from the departments of Caldas and Santander were negative. This result suggests a higher occurrence of granulovirus in *T. absoluta* larvae in the area known as Cundiboyacense high plain, although generally the number of positive samples was very low. This result is similar to those obtained by Gómez et al. (2005) and Espinel-Correal et al. (2010), who evaluated the

occurrence of granulovirus in *T. solanivora* larvae collected from major potato-producing departments in Colombia. These authors found greater presence of the virus in samples from Cundinamarca. The departments of Caldas and Norte de Santander showed the lowest levels of pest occurrence, which could also affect the presence of baculovirus, because natural epizootics caused by these viruses depend, of course, on host population density (Espinel-Correal et al. 2010).

The eight samples that gave a positive result to the presence of granulovirus using the dot-blot test were used to infect *T. absoluta* larvae in order to reproduce the typical symptoms of a viral infection. The samples coded as VG012 (from Choachí, Cundinamarca) and VG013 (from Pasca, Cundinamarca) produced viral disease symptoms, obtaining larvae with bright milky white color, sluggish behavior, decreased feeding rate, and flaccid body. Furthermore, it was observed that sick individuals changed their behavior, coming out from mines and dying while attached to the surface of leaves. The remaining six samples did not produce clear signs of infection on *T. absoluta* larvae despite being reactive in the immunoassay, which could suggest a low viral titer that was not enough to amplify and cause apparent disease in the evaluated host (Szewczyk et al. 2006).

In order to confirm the presence of granulovirus, VG012 and VG013 samples were analyzed by PCR. With the DNA from the two samples, a 500 base pair (bp) fragment was obtained, corresponding to the expected size for the *gran* gene that encodes granulatin (Figure 4), confirming that these samples correspond to granulovirus. This

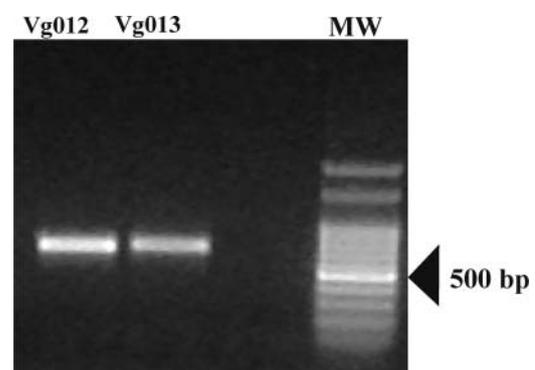


Figure 4. PCR gel of samples VG012 and VG013, using a pair of primers of *gran* gene. MW: molecular weight marker. Indicated with an arrow the molecular weight of the expected band (500 base pairs).

finding constitutes the first report of granulovirus isolated from naturally infected *T. absoluta* larvae from a field in Colombia, although other authors previously reported that this insect is susceptible to a granulovirus isolated from *P. operculella* (Angeles Rivas and Alcázar 1996; Mascarín et al. 2010; Carpio et al. 2012). A key point for understanding the finding of granulovirus in *T. absoluta* larvae is the origin of the virus. *T. absoluta* can use potato as an alternative host plant to tomato crops (Unlu 2012), hence *P. operculella*, *T. solanivora* and *T. absoluta* can be found in the same field. In the 1990s, a *P. operculella* granulovirus isolate from Peru (originally isolated from *P. operculella*) was introduced and applied on a large scale in Colombia in order to respond to sanitary problems caused by *T. solanivora* in potato crops. As a consequence, this virus could become adapted to the three species of insects (Espinell-Correal et al. 2010), and it might have then established by itself in tomato crops in the area. Soil can also contain an important reservoir of baculovirus, usually with high genetic diversity and the ability to adapt to different types of hosts (Gómez-Bonilla et al. 2011; Ríos-velasco et al. 2012).

Due to the weather conditions and rich environmental supply in the Cundiboyacense high plain, the soil in that area allows for the maintenance of high biodiversity (Baptiste et al. 2004), possibly also favoring viral diversity and adaptation to agroclimatic conditions of potato and tomato crops. This was demonstrated for other viruses such as potato mop-top virus in Colombia, in which four different viral strains were isolated from soil samples from potato crops, mainly in Cundinamarca and Antioquia departments (Gil et al. 2011). Moreover, soils from Cundiboyacense high plain are formed from deposits of clay and silt (Baptiste et al. 2004), which favors the maintenance of baculoviruses in the soil due to the adsorption of viral particles to clays by mineral electrostatic forces, resulting in higher effective titers (Christian et al. 2006). These hypotheses could support the assumption that the viruses isolated from *T. absoluta* and *P. operculella* may have been derived from the adaptation of PhopGV to each insect host. However, more detailed molecular studies are being conducted to support this hypothesis in a more robust way.

The virus occurrence found in the studied *T. absoluta* populations was 0.3%. This value is relatively low compared with that corresponding to larvae of other species of Lepidoptera. Indeed, other authors found a higher occurrence: a 15% rate in Indonesia (Zeddám et al. 1999) and 25% in Australia (Briese 1981) for *P. operculella*; and 2% in Colombia in the case of *T. solanivora* (Espinell-Correal et al. 2010). This may be explained by local conditions that might favor the development of this type of entomopathogen, or else *T. absoluta* in these areas could be more resistant to granulovirus infection than other species of the same family. However, the occurrence of insect baculoviruses collected in field populations is generally low, as has been reported for other lepidopteran insects with values from 0.001% to 25% (Valicente and Barreto 1999; Douglas 2002; Gómez et al. 2010; de Araujo Coutinho

et al. 2012). These values may relate to low insect densities in given seasons that probably affected baculovirus prevalence, since viral epizootics are density-dependent (Moscardi et al. 2011). The distribution of OBs from baculovirus also varies depending on the different baculovirus–insect combinations and the plants on which the host insects feed (Fuxa 2004).

#### 4. Conclusions

The dot-blot methodology developed in this work allowed us to detect granulovirus in a fast and accurate way, with a capacity for simultaneous analysis of up to 300 samples and with a high degree of sensitivity, showing great potential for finding viruses in field samples and for monitoring viral infections and determining the persistence of baculovirus under commercial crops.

The use of granulovirus isolated from *T. absoluta* in Colombia, which is a virus that is possibly better adapted to tomato crops in the country, may be the basis for the development of a biopesticide for pest control in commercial conditions.

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