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Susceptibility of *Grapholita molesta* (Busck, 1916) to formulations of *Bacillus thuringiensis*, individual toxins and their mixtures

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ABSTRACT

The Oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae), is a major pest of fruit trees worldwide, such as peach and apple. *Bacillus thuringiensis* has been shown to be an efficient alternative to synthetic insecticides in the control of many agricultural pests. The objective of this study was to evaluate the effectiveness of *B. thuringiensis* individual toxins and their mixtures for the control of *G. molesta*. Bioassays were performed with Cry1Aa, Cry1Ac, Cry1Ca, Vip3Aa, Vip3Af and Vip3Ca, as well as with the commercial products DiPel® and XenTari®. The most active proteins were Vip3Aa and Cry1Aa, with LC_{50} values of 1.8 and 7.5 ng/cm^2, respectively. Vip3Ca was nontoxic to this insect species. Among the commercial products, DiPel® was slightly, but significantly, more toxic than XenTari®, with LC_{50} values of 13 and 33 ng commercial product/cm^2, respectively. Since Vip3A and Cry1 proteins are expressed together in some insect-resistant crops, we evaluated possible synergistic or antagonistic interactions among them. The results showed moderate to high antagonism in the combinations of Vip3Aa with Cry1Aa and Cry1Ca.

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1. Introduction

Bacillus thuringiensis is an entomopathogenic bacterium that produces several types of insecticidal proteins, such as Cry, Cyt, Vip, and Sip proteins, along with other virulence factors contributing to its pathogenicity. Among them, Cry and Vip3 proteins are the ones that have been best characterized (Vilas-Bôas et al., 2012; Palma et al., 2014). Cry proteins are produced during the sporulation phase as parasporal crystals and some of them exhibit a specific toxic effect to insects belonging to different orders, mainly Lepidoptera, Coleoptera and Diptera (Schnepf et al., 1998; Ricieto et al., 2013; Palma et al., 2014). Vip3 proteins are produced during the vegetative growth and are not concentrated in crystals since they are secreted to the environment or the culture medium (Estruch et al., 1996; Chakroun et al., 2016). Vip3 proteins are very toxic to Lepidoptera (Chakroun et al., 2012; Hernández-Martínez et al., 2013). The mode of action of Cry proteins involves solubilization and activation by gut proteases, recognition and binding to midgut receptors, pore formation, and cell lysis, finally causing the death of the insect (Bravo et al., 2007). Vip3 proteins are also cleaved by midgut proteases and bind to specific receptors in the insect midgut, which are different from those of Cry proteins (Chakroun et al., 2016).

Biopesticides based on *B. thuringiensis* have been used since the middle of the last century. They are widely used in organic farming and, compared to chemical insecticides, they have some advantages since they lack safety periods and are harmless to non-target organisms, including insect predators and other beneficial insects. On the other hand, due to the low persistence of the active ingredient in the environment, repeated applications is common practice. This has led to some outbreaks of resistance to *B. thuringiensis* commercial products (Ferré & Van Rie, 2002) and more recently, to Bt-crops (transgenic crops protected from insects by the expression of *cry* or/and *vip3* genes) expressing a single Cry protein (Tabashnik et al., 2009).

Grapholita molesta (Busck, 1916) (Lepidoptera: Tortricidae) is native from Asia, although it is present in all temperate zones of Europe, America, Africa and Australia. It is considered a pest of economic importance around the world since it causes damage to production of fruits like peaches, nectarines, apricots and apples, and can be associated with attacks in others crops (Myers et al., 2007; Piñero and Dorn, 2009; Kirk et al., 2013). Data on the insecticidal activity of *B. thuringiensis* Cry proteins for the control of other Tortricidae pests, such as *Cydia pomonella* and *Lobesia botrana*, have been reported (Boncheva et al., 2006; Ruiz de Escudero et al., 2007). However, despite the fact that formulations

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of *B. thuringiensis* have been tested in the field and laboratory for the control of *G. molesta* (Rama et al., 2001; Monteiro and Souza, 2010), there are no reports on the activity of individual *B. thuringiensis* proteins on this pest.

In the present study, we show the effectiveness of *B. thuringiensis* bioinsecticides for the control of *G. molesta*, test the insecticidal activity of some individual Cry1A and Vip3 proteins, and show an antagonistic effect in some of their combinations.

2. Materials and methods

2.1. Insects

A colony of *G. molesta* was established and maintained at the University of Valencia (Spain), originally obtained from Entomos AG (Switzerland). Insects were reared on semi-artificial diet (Guennelon et al., 1981) under controlled conditions of temperature (25 ± 2 °C), humidity (RH 70 ± 10%) and photoperiod (16:8 h light:dark) (Arioli et al., 2007). The same diet and rearing conditions were used in the bioassays.

2.2. Cry and Vip3 proteins

Escherichia coli clones carrying plasmids with *cry1Aa*, *cry1Ac* and *cry1Ca* genes were kindly provided by Ruud de Maagd (Plant Research International, Wageningen, Netherlands). The *vip3Aa16* gene was kindly provided by Slim Tounsi (Centre de Biotecnologie de Sfax) and *vip3Af1* by Jeroen Van Rie (Bayer CropScience, Ghent, Belgium). The *vip3Ca2* gene was isolated from an autochthonous *B. thuringiensis* strain (Palma et al., 2012).

Cry proteins were expressed, solubilized and trypsin activated as described elsewhere (Hernández-Martínez et al., 2008) and stored frozen in 20 mM Tris, 150 mM NaCl, pH 8.0. IPTG was used to induce expression of Vip3A proteins from *E. coli* BL21 cells and Vip3Ca from *E. coli* WK6 cells. The cells were then centrifuged and resuspended in lysis buffer (20 mM phosphate saline buffer, pH 8.0 with 3 mg/mL lysozyme, 10 μg/mL DNAse and 100 μM PMSF) and incubated with shaking for 30 min at 37 °C. The cells were lysed by sonication and the supernatant recovered by centrifugation at 27,000*g* and filtered through sterile 0.45 μm and 0.22 μm cellulose acetate filters. The Vip3 proteins were purified by isoelectric point precipitation (IPP) (Chakroun et al., 2012) with 0.1 M acetic acid to reach pH 5.5 (Vip3Aa), pH 5.4 (Vip3Af) or pH 5.95 (Vip3Ca). The partially purified Vip3 proteins were solubilized and stored frozen in 20 mM Tris, 150 mM NaCl, pH 9.0.

The concentration of Cry1 and Vip3 proteins was estimated by the Bradford method (Bradford, 1976). The quality of the expressed proteins was checked by 12% SDS-PAGE with Coomassie brilliant blue R-250 (Sigma-Aldrich) staining (Fig. 1). Cry1A activated proteins were observed as bands of around 62 kDa and Vip3 protoxins as bands of approximately 89 kDa.

2.3. Bacillus thuringiensis commercial products

DiPel DF® (*B. thuringiensis* subsp. *kurstaki*) and XenTari GD® (*B. thuringiensis* subsp. *aizawai*) (formulations as wettable granules) were kindly provided by Kenogard S.A. (Barcelona, Spain).

2.4. Bioassays

Different concentrations of formulations and protein solutions were dispensed on the diet surface. Prior to the sample application, the surface of the diet was sterilized under UV light for 10 min. A volume of 50 μL of each concentration was applied on the surface

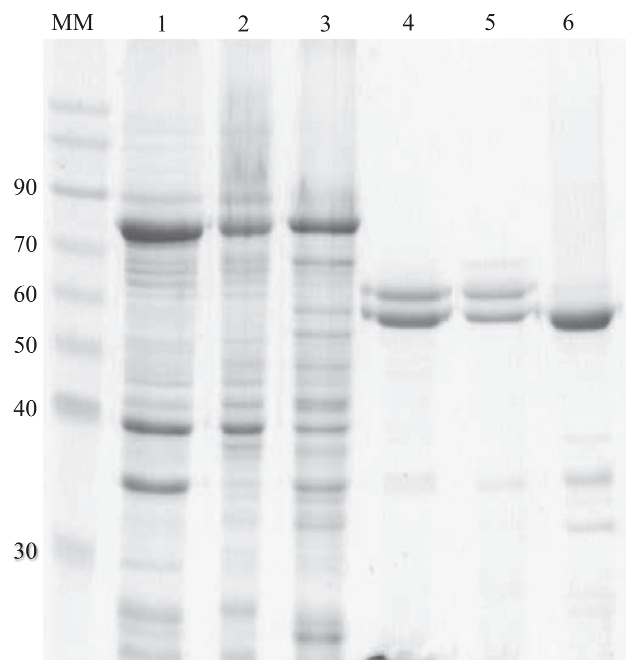

Fig. 1. SDS-PAGE of *Escherichia coli* lysates. MM, Molecular Mass Markers "PINK Plus Prestained Protein Ladder" (Genedirex); lane 1, Vip3Aa; lane 2, Vip3Af; lane 3 Vip3Ca; lane 4, Cry1Aa; lane 5, Cry1Ac; lane 6 Cry1Ca.

of solidified diet (2 cm^2 multiwell plates, Bio-Cv-16, C-D International) and let dry in a flow hood. Once dried, one larva was transferred to each well using 16 neonates per replicate, with two replicates per concentration. Preliminary assays were done at 100 and 1000 ng/cm^2 for DiPel®, XenTari® and Cry1 proteins, and at 2500 ng/cm^2 for Vip3 proteins. The larvae mortality was scored after 7 days.

Dose-response bioassays were performed only for those proteins causing a mortality higher to 90% in the preliminary assays. At least seven serial dilutions and a control with just buffer were tested for each protein. Bioassays were carried out in triplicate with sixteen neonate larvae per replicate (n = 48). Mortality was scored after 7 days. Only bioassays for which the mortality in the controls was lower than 12% were considered.

2.5. Statistical analyses

Estimates of LC_{50} and LC_{90} were obtained using the POLO-PC software (LeOra software, Berkeley, CA). LC_{50} and LC_{90} values were considered significantly different if their 95% fiducial limits (FL_{95}) did not overlap.

Tests for possible synergistic/antagonistic interactions between Vip3Aa and Cry1 proteins were initially performed at a single concentration of each protein. The expected mortality was estimated, assuming simple independent action, by the formula:

$$P = 1 - (1 - P_1)(1 - P_2)$$

which is equivalent to equation 11.33 of Finney (1971). P_1 and P_2 represent the proportions of dead larvae for toxins 1 and 2, respectively. Significance of deviations between the observed and expected mortality values was determined using Fisher's exact test and Chi-square test. A second type of experiment to test for interactions between Vip3Aa and Cry1 proteins was carried out with dose-response assays in which the proportions of two proteins in the mixture were close to the ratio between their LC_{50} values. The expected LC_{50} value of the mixture was estimated assuming simple

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