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Toxicity of six *Bacillus thuringiensis* Cry proteins against the olive moth *Prays oleae*

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Abstract

The activity of six *Bacillus thuringiensis* (Berliner) Cry proteins against third-instar *Prays oleae* (Bernard) larvae was examined. Five of the toxins tested (Cry1Aa, Cry1Ab, Cry1Da, Cry1Ja and Cry2Aa) proved active against *P. oleae*, the highest larval percentage mortality being obtained with by Cry2Aa and Cry1Ja. LC_{50} values were 0.12, 1.2 and 6.17 µg/ml for Cry1Ca, Cry2Aa and Cry1Ja, respectively. Relative potencies were calculated and Cry1Ca was found to be significantly (44-fold) more toxic than Cry1Ja.

Key words: δ-endotoxins, crystals, spores, pest control, mortality, bioassay.

Introduction

The olive moth *Prays oleae* (Bernard) (Lepidoptera Praydidae) is one of the most destructive pests in Mediterranean olive orchards. The life cycle of this olive moth is synchronized with that of the olive tree, and there are three generations per year, feeding on leaves (phyllophagous), flowers (anthophagous) and fruits (carpophagous), respectively (Arambourg, 1986). The greatest damage is prompted by the carpophagous generation, when *P. oleae* larvae give rise to a high percentage of fruit fall (De Andrés Cantero, 1991), prompting production losses of up 45-50% in some areas (Patanita *et al.*, 1998). While chemical insecticides are widely used for the control of this pest (Arias *et al.*, 1990), *Bacillus thuringiensis* (Berliner) has also been included in Integrated Pest Management programs as an effective biological control agent (Civantos and Sánchez, 1993; Cortes and Borrero, 1998). *B. thuringiensis* is a Gram-positive bacterium that produces parasporal crystals, which are formed by proteins with specific insecticidal properties (δ-endotoxins) against several insect species (Schnepf *et al.*, 1998).

Commercial bio-insecticides extensively used for the control of *P. oleae* are based on wild-type strains of *B. thuringiensis* subsp. *kurstaki* and contain a mixture of Cry1 and Cry2 proteins (Aldebis *et al.*, 2004), which exhibit high toxicity to various lepidopteran larvae. However, the relative toxicity of each Cry protein varies widely among insect species (Hofte and Whiteley, 1989; Van Frankenhuyzen *et al.*, 1991). Since rearing and laboratory maintenance of *P. oleae* is difficult, toxicity data for individual Cry proteins are scarce. The toxicity of Cry1Ac, Cry1Fa and Cry1Ca against *P. oleae* was evaluated by Hernández-Rodríguez *et al.* (2009), although LC_{50} values were not estimated. Dammak *et al.* (2010) recently reported LC_{50} values of 189 and 116 ng/cm^2 for Cry1Ia and Cry1Aa, respectively, against third-instar larvae. The present study examined the insecticidal activity of six Cry proteins against *P. oleae*, using spore and crystal mixtures to provide data that might help to improve the effective and rational control of *P. oleae.*

Materials and methods

B. thuringiensis Cry proteins were produced and supplied by Prof. Dr. Juan Ferré (Department of Genetics, University of Valencia, Spain) in the form of aqueous solutions, which were maintained in the laboratory at −18 °C. Spore and crystal suspensions had been prepared by Prof. Ferré as follows: recombinant *B. thuringiensis* strains expressing the single toxins Cry1Aa, Cry1Ab, Cry1Ba, Cry1Da and Cry1Ja were obtained from Ecogen (Langhorne, PA), while a strain expressing Cry2Aa was obtained from Monsanto (Chesterfield, MO). They were grown in medium supplemented with the appropriate antibiotic for 48 h at 29 °C. Spores and crystals were separated by centrifugation at 9,700 *g* for 12 min and then washed four times with 1 M NaCl–10 mM EDTA (Estela *et al.*, 2004). The pellet was finally suspended in 10 mM KCl. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976). Since *P. oleae* cannot be reared in laboratory conditions, third-instar larvae collected from natural populations were used for bioassays. All larvae were collected during the phyllophagous and anthophagous generations, from olive orchards in Cabra, in the province of Córdoba (Andalusia, Spain).

Bioassays were conducted using a semi-artificial diet comprising 800 ml water, 80 g fresh olive leaf flour, 34 g brewer's yeast, 32 g wheat germ, 18 g agar, 14 g casein, 4.5 g ascorbic acid, 1.3 g benzoic acid, and 1.1 g nipagin. Three millilitres of diet were placed in a tube (11 mm diameter) and a 15 µl drop of Cry protein solution was spread on the surface of the diet. After drying, single larvae were transferred to different tubes. In the first bioassay, larvae were treated with a single concentration (16 µg/ml) of Cry protein solution. Since *P. oleae* cannot be reared in laboratory conditions and the number of larvae available for bioassays was limited, two replicates of 30 larvae were used per toxin, plus a control treatment. Moreover, in order to calculate LC_{50} values for the most toxic proteins, a second set of bioassays was performed using four concentrations for

each toxin in a geometrical progression. Two replicates of 30 larvae per concentration plus a control treatment were used. Employing the same bioassay method, LC_{50} values were also calculated for Cry1Ca, which in an earlier study (Hernández-Rodríguez *et al.*, 2009) demonstrated strong activity against *P. oleae*. Based on results of preliminary bioassays, the concentrations used for each Cry protein ranged from 0.01 to 16.25 µg/ml. For all bioassays, larvae were maintained under laboratory conditions (25 ± 2 ºC, 65% relative humidity, and 14:10 h light/dark photoperiod) and mortality was recorded after seven days. The larval percentage mortality prompted by each Cry protein was compared with control mortality using the χ^2 test contained in the Statistix v. 9.0 statistical software package. Abbott-corrected mortalities were used to compare larval mortality rates. The relationship between dose and mortality was analysed using log-dose probit regression (Finney, 1971). The POLO-PC software package (LeOra Software, 1987) was used to calculate the mean lethal concentration (LC_{50}) of the most toxic proteins. Finally, a parallelism test was performed using the relative potency estimation method, in which the relative potency of two stimuli is defined as the ratio of equally effective doses (Finney, 1971); where two series of mortality data yield parallel probit regressions against log dose, the difference between doses producing the same response rate is constant.

Results and discussion

Results showed that five of the six toxins tested (Cry1Aa, Cry1Ab, Cry1Da, Cry1Ja and Cry2Aa) were active against *P. oleae* at the concentration tested, and gave rise to significantly higher average mortality rates than those recorded for controls ($\chi^2_{(1)}$ = 20.83, 13.58, 37.12, 38.07 and 80.52 respectively; $P < 0.05$ in all cases). Percentage mortality for Cry1Ba did not differ significantly from control values ($\chi^2_{(1)} = 3.76$; $P > 0.05$). The proteins associated with the highest Abbott-corrected percentage mortality were Cry2Aa (88.97 ± 1.03 %) followed by Cry1Ja and Cry1Da, both of which prompted around 60% mortality (59.02 ± 17.64 and 58.33 ± 13.51 %, respectively). The two remaining Cry proteins (Cry1Aa and Cry1Ab) gave rise to mortality rates below 40% (figure 1).

Susceptibility of *P. oleae* to Cry1Ca, Cry1Fa and Cry1Ac has been reported by Hernández-Rodríguez *et al.* (2009), who - using the same bioassay methodology and purified activated crystal - noted average mortality rates ranging from 63% to 86%, the highest mortality being found for Cry1Ca. The results obtained here with a mixture of spore and crystal solutions indicated that Cry2Aa was the most toxic of the six proteins tested, and thus one of the most active against *P. oleae*. These results are also consistent with previous immunohistochemical research demonstrating cell damage and toxin binding to midgut epithelial cells in *P. oleae* using a mixture of Cry1A and Cry2A toxins obtained from a strain of *B. thuringiensis* (Rouis *et al.*, 2007).

Commercial bio-insecticides widely used for the control of *P. oleae* are based on wild-type strains of *B. thuringiensis* subsp. *kurstaki*, and contain a mixture of Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab (Sauka and Benintende, 2008). Field application of these commercial strains appears to be an effective method of reducing (by over 40%) crop losses caused by *P. oleae* (Varlezt *et al*., 1993; Aldebis *et al.*, 2004). The results obtained here, like those reported by Hernández-Rodríguez *et al*. (2009), suggest higher insecticidal activity for Cry1Ca, Cry1Da and Cry1Ja (not included in the commercial insecticides used to control *P. oleae*) than for Cry1Aa and Cry1Ab produced by wild-type strains of *B. thuringiensis* subsp. *kurstaki*. It may thus be surmised that the effectiveness of field applications could be improved by using a combination of currently-used commercial strains and strains containing Cry1Ca, Cry1Da and Cry1Ja.

Cry2Aa, Cry1Ja, Cry1Da and Cry1Aa have also demonstrated moderate toxicity against *Plutella xylostella* (L.), another major pest belonging to the Yponomeutidae family (Tabashnik *et al.*, 1996; González-Cabrera *et al.*, 2001; Gong *et al.*, 2010). Cry1Ab and Cry1Ca exhibit strong toxic activity against this microlepidopteran pest (Monnerat *et al.*, 1999; González-Cabrera *et al.*, 2001; Liu *et al.*, 2001) although results point to considerable variation among laboratories and among the bioassay methods used.

Mean lethal concentrations of the most toxic proteins (Cry2Aa and Cry1Ja) and Cry1Ca were calculated (table 1). In all cases, χ^2 values generated in goodness-of-fit tests indicated that the probit model was appropriate for each toxin. Based on LC_{50} values and 95% fiducial limits, Cry1Ca displayed the greatest activity (0.12 µg/ml), performing significantly better than Cry1Ja (6.17 µg/ml); neither of these proteins differed significantly from Cry2Aa (1.20 µg/ml), which yielded an intermediate LC_{50} value. It is important to note that, since *P. oleae* cannot be reared in laboratory conditions, the use of field populations entails several constraints: the number of larvae available for bioassays was limited, and treated larvae displayed a highly-variable response, which accounts for the low slope of regression lines and wider fiducial limits.

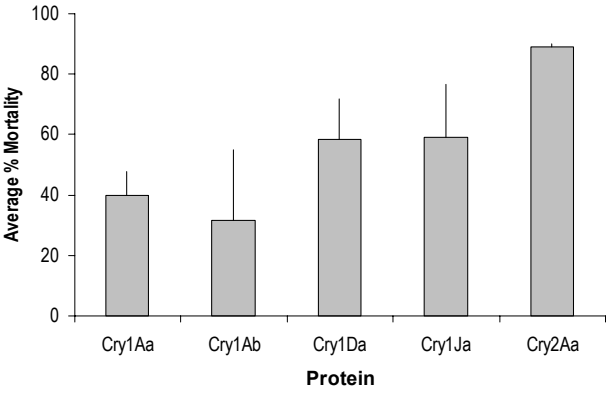

Figure 1. Abbott-corrected mortality caused by active Cry proteins against third-instar larvae of *P. oleae* treated with 16 µg of toxin/ml. Vertical lines show standard error.

Table 1. Activity and relative potencies of the most toxic Cry proteins against third-instar *P. oleae* larvae.

Protein	LC_{50} (μg/ml)	95% fiducial limits Lower	Upper	Slope ± S.E	Intercept ± S.E.	Relative potency*	95% fiducial limits Lower	Upper
Cry1Ca	0.12	0.05	0.23	0.85 ± 0.17	5.90 ± 0.16	44.14	9.61	262.30
Cry2Aa	1.20	0.21	4.41	0.44 ± 0.13	4.96 ± 0.12	3.66	0.79	22.84
Cry1Ja	6.17	1.64	53.11	0.40 ± 0.12	4.69 ± 0.12	1.00	-	-

* Relative potencies refer to the least active protein (Cry1Ja).

Although comparison of bioassay data has frequently been shown to be unreliable, Pérez-Guerrero *et al.* (2011), using a spore and crystal mixture similar to that employed here, also reported strong activity and high LC_{50} values for Cry1Ca, Cry1Ja and Cry2Aa (0.24, 0.29 and 0.43 μg/ml respectively) against the first instar of the cotton pest *Earias insulana* (Boisduval). Strong insecticidal activity has also been observed for Cry2Aa against first instar larvae of *P. xylostella* (González-Cabrera *et al.*, 2001), *Helicoverpa armigera* (Hubner) (Avilla *et al.*, 2005) and *Lobesia botrana* Denis et Schiffermuller (De Escudero *et al.*, 2007); however, since these authors used purified activated crystal solutions for bioassays, LC_{50} values are not directly comparable to those recorded here against *P. oleae*.

Relative potencies of Cry proteins against *P. oleae* were compared (table 1). All regression lines were fitted in parallel, showing that Cry1Ca was 44-fold more toxic than Cry1Ja. There were no significant differences between Cry1Ca and Cry2Aa activity (table 1).

The toxicity data obtained here for the most common lepidopteran-specific Cry proteins active against *P. oleae* larvae suggest that Cry2Aa, and to a lesser extent Cry1Ja, can be used for improved control of this pest. The previously-reported marked toxicity of Cry1Ca against *P. oleae* was confirmed and quantified. Since the rearing and testing of *P. oleae* involves several constraints, it should be stressed that further research is required in order to confirm the degree of toxicity for Cry1Ca, Cry2Aa and Cry1Ja recorded in the present study.

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