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EVALUATION OF THE TOXICITY OF THE Cry2Aa AND Cry2Ab RECOMBINANT PROTEINS AGAINST *Tecia solanivora* (Lepidoptera: Gelechiidae)

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ABSTRACT

Potatoes can be attacked by several pests such as *Tecia solanivora*. The *cry* genes of *Bacillus thuringiensis* have been used to generate transgenic plants. The objective of this study was to determine the toxicity of the Cry2Aa and Cry2Ab recombinant proteins against larvae of *T. solanivora*. By PCR, the *cry*2Aa and *cry*2Ab genes were amplified using DNA from *B. thuringiensis* subsp. *kurstaki* HD-1. Each amplified product was ligated into the pET151/D-TOPO® vector, and subsequently transformed and expressed in *Escherichia coli* strains BL21. The recombinant clones were confirmed by PCR, restriction enzyme cutting and sequencing analysis. Recombinant proteins were detected by 8% SDS PAGE gels and immunodetection strips. For bioassays, three replicates of Parda Pastusa potato cultivar were nebulized with *E. coli* BL21-Cry2Aa or *E. coli* BL1-Cry2Ab recombinant strains and then infected with *T. solanivora* larvae, reaching levels of efficacy of 71.3 and 36.8 %, respectively. Biostat® 2007 was used to determine lethal concentrations, obtaining LC50 values of 167.7 and 554.6 µg/ml for BL21 Cry2Aa and Cry2Ab, respectively. The results allow concluding that the Cry2Aa protein can be a promising alternative for the biological control of *Tecia solanivora* .

Key words: *cry*2 genes, *Bacillus thuringiensis,* insect control, bioassay.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the fifth largest food crop in the world after sugar cane, maize, wheat and rice (FAO 2019). It is a globally important crop plant due to its quick growth and high nutritional content, with vitamins, proteins, and carbohydrates in the tubers (Díaz, 2020). In Colombia, potato is one of the most commonly consumed food crops and its cultivation is a large source of employment (López et al., 2013). In 2020, the total potato production reached 2.625.272 tons and an estimated planting area of 125.425 hectares, being the largest tuber crop at the country's level (Fedepapa, 2020).

Potatoes are attacked by several insect pests and diseases that damage the tuber, reducing the marketability of the final product. The white

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worm (*Premnotrypes vorax*) and the Guatemalan moth (*Tecia solanivora*) are among the most important insect pests with economic importance (López and Espitia, 2000; López et al., 2013). In the larval stage, *Tecia solanivora* resides within the tuber and feeds from it, consuming the flesh and leaving waste, which reduces the quality of the tuber (López and Espitia, 2000; López et al., 2013). Since its presence was detected in the country, it has had a strong economic impact on every production region. To control and limit the impact of this pest, farmers use several different methods, including cultural, ethological, biological and chemical control (López Espitia, 2000); Ordoñez et al., 2012; López et al., 2013). In fact, the crop has the highest demand for fungicides and insecticides, and the second highest demand for chemical fertilizers after coffee. This does not only generates high production costs, but it also poses risks to the environment and human health due to the toxic effects and mutagenic characteristics of these chemical agents (Villanueva and Saldamando, 2013).

It has been reported that genotypes from genetically engineered potato varieties expressing the *cry*1Ac gene of *Bacillus thuringiensis* have recorded 100% mortality of *T. solanivora* larvae (Valderrama et al., 2007; Villanueva et al., 2014), showing the potential that Cry proteins have to control *T. solanivora* (Martínez et al., 2003; Pitre et al., 2008). Cry proteins are produced by *B. thuringiensis* and have been widely known for presenting toxic activity against different invertebrates, particularly insect larvae (Schnepf et al., 1998). In fact, they are highly effective for insect control, being safe for other animals, and showing little environmental impact. Due to this, *B. thuringiensis* is currently considered as the most successful biocontroller in the world (Gómez et al., 2000; Sauka and Benintende, 2008; Sevim et al., 2012).

However, target insects have developed resistance mainly due to the continuous exposure to a single toxin (Tabashnik et al., 2002; Mahon et al., 2007). In this sense, gene pyramiding, which is based on the simultaneous expression of more than one toxin in a transgenic plant, is an efficient approach to delay resistance development in target insects (Shelton et al., 2002). Hence, it is necessary to search for new Cry proteins with toxic potential against *T. solanivora.* The Cry2A family has demonstrated insecticidal activity against other Lepidoptera considered as pests (Kumar et al., 2004). However, there are no studies on the biological activity of the Cry2Aa and Cry2Ab proteins of *B. thuringiensis* subsp. *kurstaki* against *T. solanivora*. Therefore, the objective of this study was to evaluate the toxicity of the Cry2Aa and Cry2Ab recombinant proteins from *B. thuringiensis* subsp. *kurstaki* expressed in *E. coli* strain BL21 against first instar larvae of *T. solanivora.*

MATERIALS AND METHODS

Microbiological and entomological material

The reference strain of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 was used as a source of genetic material for the isolation of the *cry*2Aa and *cry*2Ab genes. The bacteria were cultured and maintained in Luria-Bertani (LB) solid medium and incubated at 28 ° C for 7 days to obtain the spore-crystal complex. For the bioassays, first instar larvae of *T. solanivora* were used. The eggs were provided by the Biological Control Laboratory of the Tibaitatá Research Center of AGROSAVIA, and then incubated at room temperature until hatching. The most active larvae were selected for the bioassays.

Amplification, cloning and analysis of the *cry*2Aa and *cry*2Ab genes

The plasmid DNA of *B. thuringiensis* subsp. *kurstaki* was extracted following the mini-prep alkaline lysis (Sambrook and Russell, 2001). Primers were used for amplification of cry cry2Aa and cry2Ab genes (Gómez, 2011) and 100 ng / μL of plasmid DNA as a template, in a reaction mixture containing 4 μM of each primer, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 1X of PCR buffer and 1 U of high-fidelity DNA polymerase (Invitrogen ®).

PCR cycling conditions were adjusted as follows: one cycle at 94 °C for 2 minutes, followed by 35 cycles at 94 °C for 30 seconds, annealing at 49 °C for 30 seconds and extension at 68 °C for 2 minutes, with a final extension at 68 °C for 7 min. For the *cry*2Ab gene, the annealing temperature was adjusted to 47 °C. The amplification products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega®).

The plasmid pET151 / D-TOPO® (Invitrogen) was used as cloning and expression vector. Ligation was conducted according to the manufacturer's guidelines and the resulting constructs were named pET151-*cry*2Aa and pET151-*cry*2Ab, which were initially transformed into *Escherichia coli* strain TOP10 for conservation and multiplication of the plasmid. PCR confirmation of the pET151-*cry*2Aa and pET151-*cry*2Ab constructs was performed by amplification of the expected 1902 bp fragment and digestion with *Bgl*II and *Ava*II enzyme, respectively. A second confirmation was performed on the constructs with the single-cutting enzyme *Bste*II, and with multiple-cutting enzymes *Pvu*II and

*Sac*I for pET151-*cry*2Aa. pET151-*cry*2Ab was confirmed using the *Ban*II enzyme.

Once the constructs were confirmed, they were inserted into *E. coli* strain BL21 Star ™ (DE3) (Invitrogen) recommended for expression. Again, recombinant clones of pET151-*cry*2Aa and pET151-*cry*2Ab were identified by PCR amplification and restriction mapping. For pET151-*cry*2Aa, *Bste*II, *Sac*I and *Pvu*II enzymes were used, while the *Ban*II enzyme was used for pET151-cry2Ab. The enzymes were selected using Nebcutter version 2.0 (http://tools.neb.com/NEBcutter2/index.php).

Each gene was sequenced by the Sanger method with the internal primers designed (Gómez, 2011). The sequenced genes were analyzed in the NCBI database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Expression, detection and prediction of the in-silico sequence of recombinant proteins

E. coli Bl21 pET151-*cry*2Aa, pET151-*cry*2Ab and pET151-βgal (expression control) recombinant strains were grown overnight in 10 mL of liquid LB medium supplemented with ampicillin (100 mg/L) at 37 °C at 200 rpm. A 1 mL sample was taken from each culture and inoculated into 4 erlenmeyer flasks containing 10 mL of fresh LB medium supplemented with ampicillin and incubated at 37 °C at 200 rpm until an OD_{600} of 0.6-0.7 was reached. Subsequently, IPTG was added at 1 mM as described by Gómez (2011) and Sevim et al. (2012) and incubated again at 37 °C for 5 hours at 200 rpm. From each culture, a volume of 5 mL was centrifuged, and the pellet was resuspended in 1 mL of lysis buffer (Invitrogen). All the samples were heat shocked three times at 42 °C and then sonicated on ice for 2 min.

After centrifugation for 10 min at 14000 rpm, the pellet was resuspended in 1 mL of 0.1 M sodium carbonate pH 11 and incubated at 4 °C for 20 h for solubilization. Finally, all the samples were spotted on an 8% denaturing polyacrylamide gel as described by Laemmli (1970). The Precision Plus Protein Standards weight marker (Bio Rad) was used.

In addition, the presence of the Cry2Aa and Cry2Ab proteins was verified in the crude extracts of total proteins of *E. coli* pET151-Cry2Aa and pET151-Cry2Ab using cross-flow immune test strips. The detection of the Cry2Aa and Cry2Ab recombinant proteins was carried out using ImmunoStrip® for (Cry2A) developed by Agdia. The test was performed in triplicate in *E. coli* strain Bl21 pET151-*cry*2Aa and pET151-*cry*2Ab induced and solubilized, *E. coli* strain BL21 pET151-*cry*2Aa and pET151-*cry*2Ab were induce and *E. coli* BL21 untransformed, according to the manufacturer's guidelines.

The in-silico amino acid prediction of the Cry2Aa and Cry2Ab proteins was made using BLASTX. To select the best open reading frame of these genes, the data base of the Pfam protein families was used to identify the characteristic domains of this type of protein.

Evaluation of the toxic activity against first instar larvae of *T. solanivora*

Five concentrations were evaluated as treatments: 100, 200, 300, 400 and 500 µg / mL of total protein of *E. coli* strain BL21 pET151-*cry*2Aa and pET151-*cry*2Ab and four controls: absolute control (tuber without nebulizing), 0.1 M sodium carbonate pH 11, positive control (330 µg / mL of *B. thuringiensis* subsp. *kurstaki*) and negative control (330 µg / mL of *E. coli* BL21). All the tubers of the Parda Pastusa potato variety were nebulized on both sides with 2 mL of each treatment, allowed to dry at room temperature for 20 minutes and then placed in plastic jars containing sterile sand used as support. Finally, each tuber was infected with 15 first instar larvae of *T. solanivora.* The jars were covered with plastic caps with small holes to permit ventilation. All the treatments were incubated in the dark at a temperature between 25 and 30 °C. Mortality was measured at 20 days after incubation by counting the number of live larvae and estimating the difference with the number of initial larvae. The concentration of soluble total protein was quantified by the Bradford technique in a Thermo Scientific 1000 nanodrop, using bovine serum albumin as standard (Bradford, 1976).

Experimental design and statistical analysis of the results

A randomized design was used, with three replicates over time, which included absolute, positive and negative controls. An analysis of variance (ANOVA) and a Tukey´s test were performed using the SAS statistical software version 9.1. The percentage of efficacy was determined according to the formula by Schneider Orelli (Zar, 1999) for the treatments: *E. coli* strain BL21 pET151-*cry*2Aa and pET151-*cry*2Ab, a 300 µg / mL concentration and the positive control. To determine lethal doses (LD_{50}), a probit analysis was performed using the Biostat® 2007 program.

RESULTS AND DISCUSSION

Amplification, cloning and analysis of the *cry*2Aa and *cry*2Ab genes

Two fragments of 1902 bp corresponding to the *cry*2Aa and *cry*2Ab genes were amplified from the reference strain *B. thuringiensis* subsp.

kurstaki HD-1 (Fig. 1), matching those reported by Tounsi and Jaocua (2003); Kumar et al. (2004). Subsequently, the ligated and transformed genes in *E. coli* strain TOP10 had a transformation efficiency of 3 X 10^6, which is considered as good according to Sambrook and Russel (2001).

PCR confirmation of the pET151-*cry*2Aa and pET151-*cry*2Ab constructs showed the expected gene size of 1902 bp and, when cut with restriction enzymes, the expected fragments of 1308 and 594 bp were obtained for the *cry*2Aa gene with *Bgl*II, while fragments of 1061 and 841 bp were obtained for the *cry*2Ab gene with *Ava*II (Fig. 2).

For pET151-*cry*2Aa, the single-cutting enzyme *Bste*II was used, obtaining a fragment of 7662 bp; when cut using the *Pvu*II and *Sac*I enzymes, five fragments 3279, 2630, 1502, 158 and 93 bp were generated (Fig. 3 A). Verification of the pET151-*cry*2Ab construct was performed using the *Ban*II enzyme, generating the expected fragments with weights of 4618, 2059, 736,158 and 14 bp (Fig. 3 B). These results were obtained in two strains, *E. coli* TOP10 and *E. coli* BL21.

When comparing the sequences obtained with respect to the genes belonging to related species reported in databases, using the BLAST program, an identity percentage of 100% was obtained. The E value of zero allowed explaining the close relationship that these genes present (Schnepf et al., 1998) as well as the possible homology between them.

Prediction of the structure and function of the putative proteins Cry2A and Cry2Ab

When using the databases of Pfam protein families and comparing the domains reported by Pfam for the Cry2Aa and Cry2Ab recombinant proteins, the three characteristic domains were identified with an identity of 90, 84 and 92%, respectively. It was also observed that the largest difference between amino acid sequences is at domain II. These amino acid changes could explain the differences in toxic activity against *T. solanivora* (75 % and 44% for both proteins, respectively) because domain II participates in the interaction with the receptor, and therefore it is a determinant in the specificity (Villanueva et al., 2014).

The NCBI tblastp tool showed 88% identity between the amino acid sequences of the putative proteins Cry2Aa and Cry2Ab. The study reports the functional analysis through InterProScan of the sequences of the Cry2Aa and Cry2Ab proteins and show that these are related to the biological processes of defense responses and pathogenesis, as well as with a receptor binding at the molecular level (Mitchell et al., 2015).

Expression of the recombinant proteins in *Escherichia coli* strain BL21

The recombinant *E. coli* Bl21 pET151-*cry*2Aa, pET151-*cry*2Ab and pET151-βgal (expression control) strains induced with 1 mM IPTG, when visualized in gels 8% SDS-PAGE showed bands of approximately 66 kDa for the recombinant protein Cry and 120 kDa for the β-galactosidase protein (positive control) (Fig. 4). The presence of the latter protein is an indicator of the proper functioning of the expression system.

The induced and solubilized lysate of *E. coli* BL21 pET151-*cry*2Aa, pET151-*cry*2A and expression control showed expected approximate

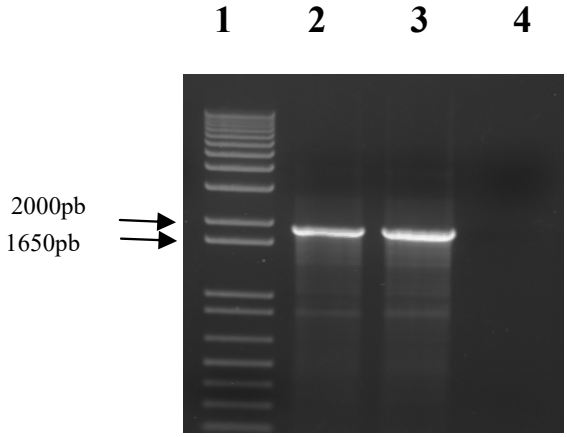

Fig. 1. Electrophoretic profile of the PCR amplification product of the *cry*2Aa and *cry*2Ab genes. Lane 1: 1 Kb plus weight marker. Lane 2: *cry*2Aa gene amplification product. Lane 3: *cry*2Ab gene amplification product. Lane 4: Absolute control (water).

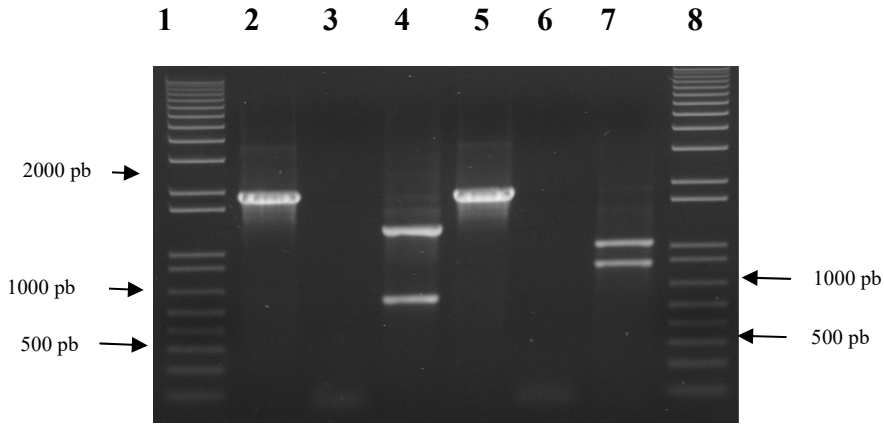

Fig. 2. Electrophoretic profile of the PCR product and restriction analysis of the *cry*2Aa and *cry*2Ab genes. Lanes 1 and 8: 1 Kb plus weight marker. Lane 2: PCR of pET151-*cry*2Aa. Lane 3: Absolute control (water). Lane 4: PCR of pET151-*cry*2Aa digested with the *Bgl*II enzyme. Lane 5: PCR of pET151-*cry*2Ab. Lane 6: Absolute control. Lane 7: PCR of pET151-*cry*2Ab digested with the *Ava*II enzyme.

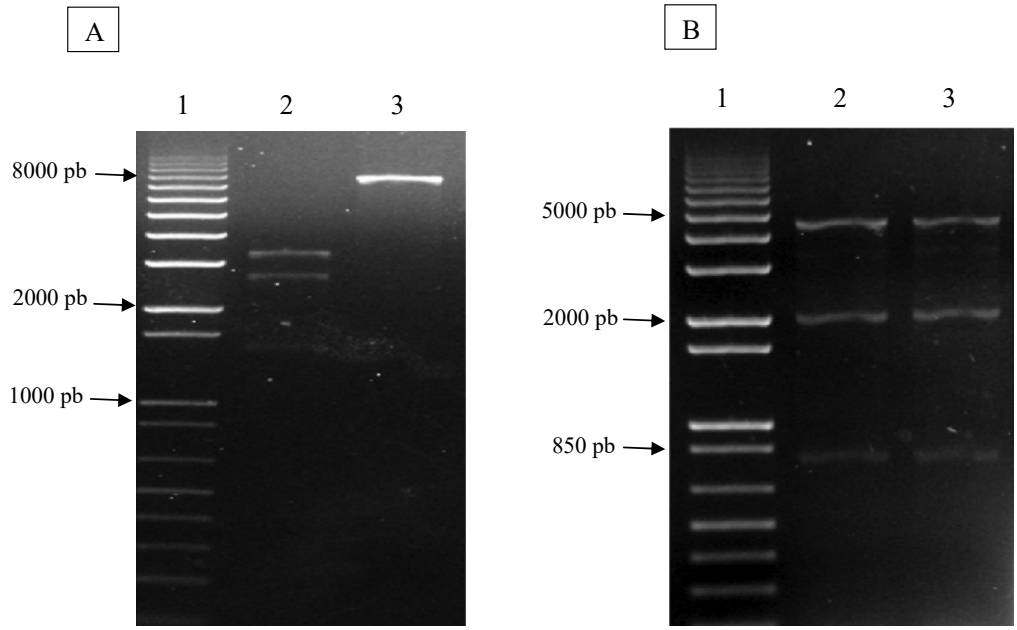

Fig. 3. DNA restriction pattern of the pET151-*cry*2Aa and pET151-*cry*2Ab constructs. A. Lane 1: 1 Kb plus weight marker. Lane 2: pET151-*cry*2Aa digested with the *Pvu*II and *Sac*I enzymes. Lane 3: pET151-*cry*2Aa linearized with the *Bste*II enzyme. B. Lane 1: 1Kb plus weight marker. Lane 2 and 3: pET151-*cry*2Ab digested with the *Ban*II enzyme.

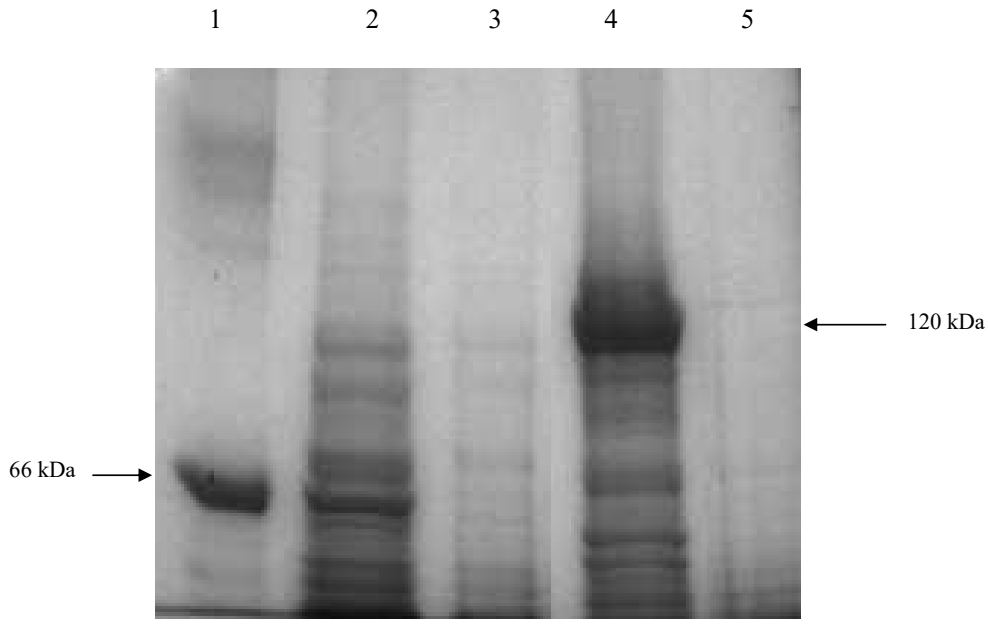

Fig. 4. Electrophoretic profile of crude extract of total proteins of *Escherichia coli* pET151-Cry2Aa and pET151-βgal (expression control) Lane 1: Albumin protein (66 KDa). Lane 2: *E. coli* BL21 pET151-Cry2Aa (IPTG 1mM). Lane 3: *E. coli* BL21 pET151-Cry2Aa (without IPTG). Lane 4: *E. coli* BL21 expression control (IPTG 1mM). Lane 5: *E. coli* BL21 expression control (without IPTG).

weight bands of 66 kDa for recombinant proteins, when visualized in gels 8% SDS-PAGE (Fig. 5). Similar weights for these Cry2A proteins have been found in other studies (Kumar et al., 2004; Lin et al., 2008).

The presence of the Cry2Aa and Cry2Ab proteins was verified in the crude extracts of total proteins of *E. coli* pET151-Cry2Aa and pET151-Cry2Ab solubilized when the presence of two red bands were observed in the immune strips of transverse flow; the first belonged to the control and the second corresponded to the detection of the Cry2Aa and Cry2Ab proteins by the Cry2A antibody contained in these strips (Fig. 6A and 6B). In the untransformed *E. coli* Bl21 strain, only the band belonging to the control was evidenced, demonstrating that the second band observed in *E. coli* pET151-Cry2Aa and pET151-Cry2Ab belongs to the recombinant protein present in these clones.

Biological activity of the Cry2Aa and Cry2Ab recombinant proteins

As it is shown in Fig. 7, using total extracts of *E. coli* proteins pET151-Cry2Aa and pET151-Cry2Ab resulted in mortality rates of 75.5 % and 44.0%, respectively. The control with sodium carbonate recorded the lowest percentage of mortality with 13.0%, while the absolute control (tuber without nebulization) was17.77%, and the negative control (*E. Coli* 330 μg/mL untransformed) recorded the same rate of 17.17% (17.8% eliminar).

Regarding efficacy, the treatments of *E. coli* pET151-Cry2Aa (300 μg / mL), *E. coli* pET151-Cry2Ab (300 μg / mL) and the positive control (*B. thuringiensis* var. *kurstaki* 330 μg / mL) reached values of 71.3, 36.8 and 74.4%, respectively; the values were calculated based on the control with sodium carbonate, which presented the lowest mortality rate (13%). However, significant differences were found with respect to the controls that include the lysate of *E. coli* BL21 without transformation and the absolute control (Fig. 7). The analysis of variance ($P < 0.01$) and Duncan´s test showed that there were no significant differences between the Cry2Aa recombinant extract and the positive control. However, the latter recorded an increase of 3.2% in terms of efficacy, which could be explained by the presence of other Cry proteins (Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab) produced by this bacterium and by the presence of spores in the extract (Fig. 7). Similar results were found by Pitre et al., (2008), who reported that Cry1 proteins have toxic activity against *T. solanivora* and that their presence in the lysate (spore-

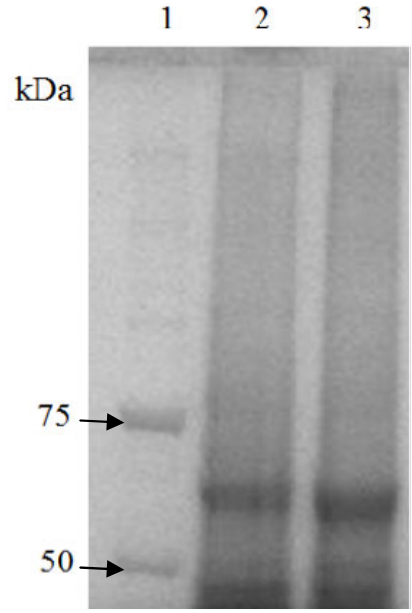

Fig. 5. Electrophoretic profile of crude extract of total proteins of *Escherichia coli* pET151-Cry2Aa and pET151-Cry2Ab solubilized with 0.1mM sodium carbonate pH 11. Lane 1: Precision Plus Protein Standards. Lane 2: *Escherichia coli* pET151-Cry2Aa Lane 3: *Escherichia coli* pET151-Cry2Ab.

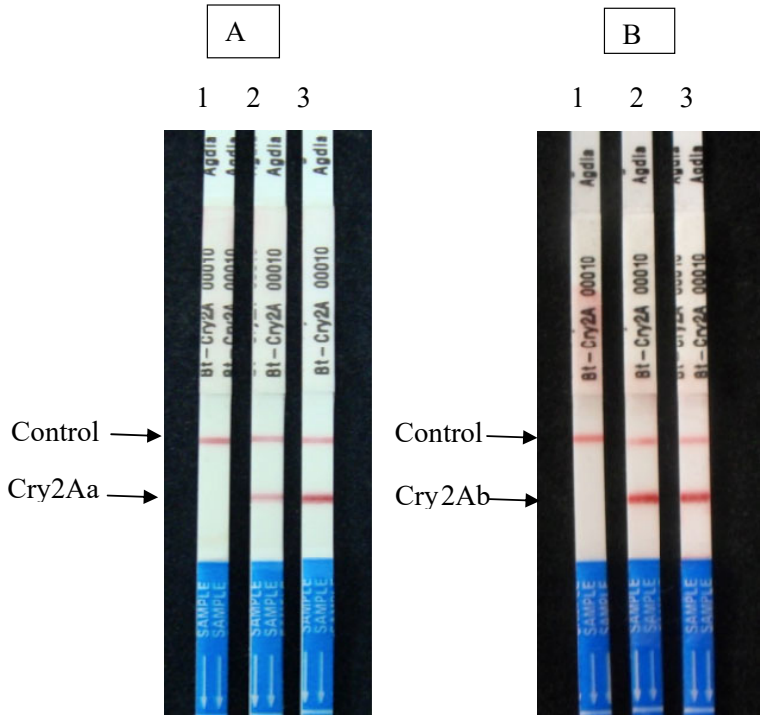

Fig. 6. Detection of the recombinant proteins Cry2Aa and Cry2Ab for immune detection. A. Lane 1: *Escherichia coli* strain BL21 untransformed. Lane 2 and 3: Induced *Escherichia coli* pET151-Cry2Aa. B. Lane 1: *Escherichia coli* strain BL21 untransformed. Lane 2 and 3: Induced *Escherichia. coli* pET151-Cry2Ab.

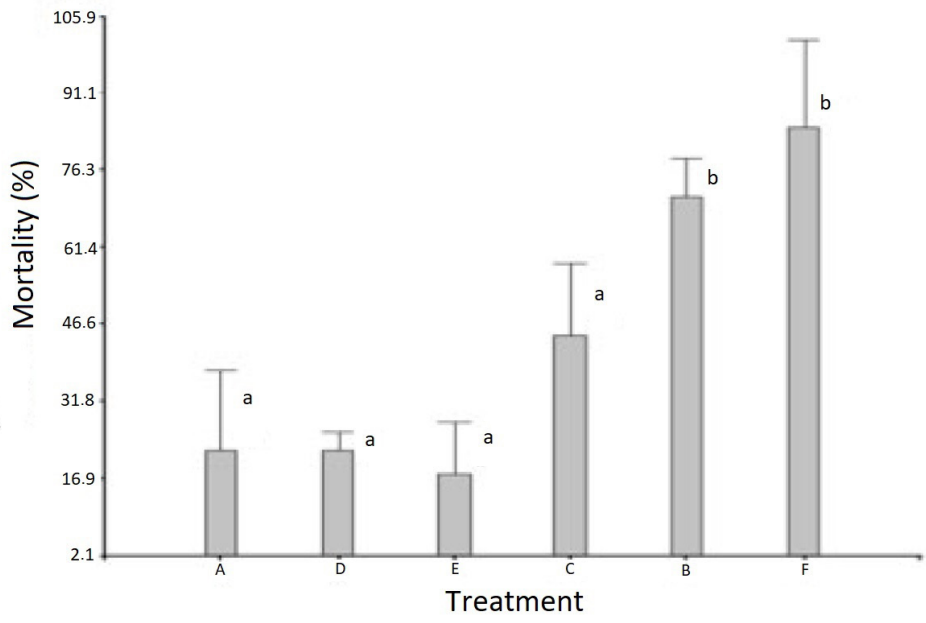

Fig. 7. Mortality rate (%) per treatment, Tukey (P <0.01). A: tuber without nebulization; B: Lysate *Escherichia coli* pET151-Cry2Aa 300 µg/mL; C: Lysate *Escherichia coli* pET151-Cry2Ab 300 µg/mL; D: Control sodium carbonate pH 11; E: Control *Escherichia coli* 330 µg/mL untransformed; F: Control *Bacillus thuringiensis* 330 µg/mL.

crystal complex) of *B. thuringiensis* would help to increase the percentage of efficacy.

LC50 values for the E. coli lysate of pET151-Cry2Aa and pET151-Cry2Ab were 167,744 and 554,633 µg/ml, respectively. Martinez et al., (2003) evaluated the toxic activity of purified recombinant Cry proteins against Tecia solanivora, reporting a mean mortality of purified CrylAa, CrylAb, CrylAc, CrylB, CrylC and CrylD recombinant proteins of 6.24%, 6.76%, 19.26%, 4.16%, 5.72% and 1.56%, respectively, using a concentration of 80µg/mL against *T. solanivora*. A study on the same insect conducted by Pitre et al. (2008) reported LC50 values of 2.06 µg/mL for Cry1Aa , 2.14 µg/mL for Cry1Ac, 1.70 µg/mL for Cry1B and 2.24 µg/mL for Cry1C, using purified and cleaved recombinant proteins. Furthermore, another study using Helicoverpa armigera showed that the Cry2Aa protein is 100% lethal in the larval stage (Gajendra et al., 2002; Kumar et al., 2004). Although these studies differed in the type of bioassay used and in the preparation of the proteins evaluated, they demonstrate that Cry proteins can be used as biological strategies for the control of *T. solanivora* and other lepidoptera.

Considering the continuous use of Cry1 proteins and resistance development in insects (Sivem et al., 2012), other families such as Cry2 become good alternatives for the control of these pests.

CONCLUSIONS

The Cry2Aa and Cry2Ab recombinant proteins expressed and solubilized in *E. coli* strain BL21 showed an efficacy of 71.3 % and 36.8%, respectively, for the biological control of first instar larvae of *T. solanivora.* The LC50 with the *E. coli* lysate pET151-Cry2Aa against first instar larvae of *T. solanivora* was 167.744 µg / mL.

The Cry2Aa recombinant protein from *B. thuringiensis* var. *kurstaki* expressed in *E. coli* strain BL21 can be a promising alternative for the control of *T. solanivora.*

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