Biological Control 144 (2020) 104211

Contents lists available at ScienceDirect

Biological Control

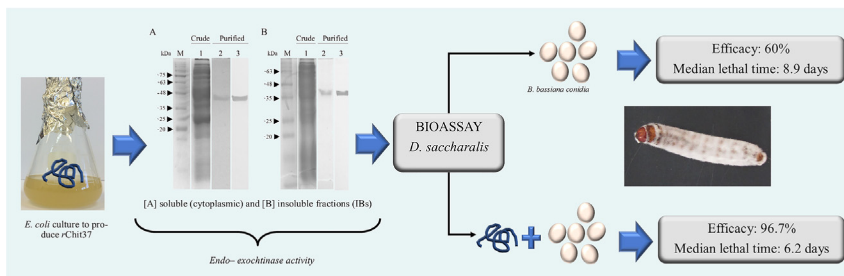
journal homepage: www.elsevier.com/locate/ybcon

Enhanced virulence of *Beauveria bassiana* against *Diatraea saccharalis* using a soluble recombinant enzyme with endo- and exochitinase activity

Andrea Lovera[a,b], Mariano Belaich[c], Laura Villamizar[d], Manuel A. Patarroyo[e,f], Gloria Barrera[a,*]

[a] Corporación Colombiana de Investigación Agropecuaria (AGROSAVIA), Centro de Investigación Tibaitatá, Km 14 vía Mosquera, Bogotá, Colombia
[b] Master's Programme in Sciences – Microbiology, Universidad Nacional de Colombia, Bogotá, Colombia
[c] Laboratorio de Ingeniería Genética y Biología Celular y Molecular, Área Virosis de Insectos, Universidad Nacional de Quilmes, Provincia de Buenos Aires, Argentina
[d] AgResearch Ltd, Lincoln Research Centre, Christchurch 8140, New Zealand
[e] Molecular Biology and Immunology Department, Fundación Instituto de Inmunología de Colombia (FIDIC), Bogotá, Colombia
[f] Basic Sciences Department, Universidad del Rosario, Bogotá, Colombia

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Beauveria bassiana
Chitinase
Virulence
Diatraea saccharalis
Recombinant protein

ABSTRACT

Beauveria bassiana chitinases are involved in degrading the chitin in insects' exoskeletons and internal structures, and thus are important virulence factors as they participate in initial to final steps of infection. In this work, the *B. bassiana* (Bv062 isolate) open reading frame (ORF) encoding a chitinase identified as Chit37 (orthologous Bvchit1) was molecularly cloned and expressed in *Escherichia coli,* and the potential of the recombinant protein (*r*Chit37) to enhance the insecticidal activity of Bv062 conidia against second instar *Diatraea saccharalis* larvae was studied. *r*Chit37 was produced in both soluble and insoluble fractions of an *E. coli* culture. Both fractions expressing endo- (90 mU/μL) and exochitinase (170 mU/μL) enzymatic activity, with optimum conditions for enzyme activity of 45 °C and pH 5.0. His-tag affinity chromatography was used to purify the *r*Chit37 from the soluble fraction. Purified *r*Chit37 was then diluted to 200 and 300 μg/mL for use as Bv062 conidia additive ($1x10^6$con/mL) in a laboratory bioassay against *D. saccharalis* larvae. No significant differences were observed between the efficacy of Bv062 conidia applied alone or mixed with 200 μg/mL purified *r*Chit37. However, 300 μg/mL *r*Chit37 increased BV062 conidia insecticidal activity, achieving 96.7% efficacy (14 days post-infection) and 6.2 days median lethal time (LT_{50}), compared to 60% efficacy and 8.9 days for conidia alone. *r*Chit37 addition did not affect conidial viability in terms of germination (96.6% after 24 h) or vigour estimated as germ-tube elongation rate. This work provides proof of concept about soluble recombinant chitinase as an additive to enhance *B. bassiana* virulence against *D. saccharalis*.

* Corresponding author.
E-mail address: gbarrera@agrosavia.co (G. Barrera).

https://doi.org/10.1016/j.biocontrol.2020.104211
Received 21 October 2019; Received in revised form 6 January 2020; Accepted 27 January 2020
Available online 05 March 2020
1049-9644/ © 2020 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).

1. Introduction

Entomopathogenic fungi play an important role in nature in maintaining dynamic equilibrium in different groups of insect populations (Maina et al., 2018). Many fungal species are currently used as biological control agents for pests because they are environmentally friendly and provide an effective alternative to using chemical products. *Beauveria bassiana* isolate ingredients are most frequently used in bioinsecticide formulations (Butt et al., 2016; Haseeb and Srivastava, 2014) due to their specificity concerning host range, their mode of action by contact, their ability to evade the insect's immune responses and having important attributes facilitating mass production at an industrial level, such as rapid *in vitro* growth in different culture media (Federici, 1999; Haseeb and Srivastava, 2014; Lacey et al., 2001). However, biological control products based on entomopathogenic fungi have poor market share, due to the need for a large amount of propagule for producing an efficient mortality rate. The initial latency period for establishing infection must also be considered; this time can be lengthy and susceptible insects can stay active and cause serious damage to crops until the fungus compromises affected individuals' welfare (Fan et al., 2007a; Federici, 1999; St Leger and Screen, 2001).

Strategies for improving and expanding the use of biological control agents in agriculture have thus been proposed and evaluated, such as the use of genetically-modified organisms (Zhao et al., 2016), mixtures of entomopathogenic agents having synergistic activity (Cuartas et al., n.d.; Salvador, 2010) and using secondary microbial metabolites and/or virulence factors as additives for biological control agents (Shternshis, 2004; Zhang et al., 2008). The biological stages of infection driven by entomopathogenic agents must thus be analysed for identifying key factors which can be added as enhancers. For example, entomopathogenic fungi require the action of the hydrolytic enzymes known as chitinases. These proteins play a fundamental role in degrading the chitin that makes up insects' cuticle, thereby enabling fungal entry to the haemocoel and enabling the subsequent colonisation of other tissues (Butt et al., 2016; Ortiz and Keyhani, 2016; Valero et al., 2016; Xiao et al., 2012; Zhao et al., 2016).

Fungal strains have therefore been genetically modified to overexpress this type of enzyme, achieving increased virulence against a particular host (Boldo et al., 2009; Fan et al., 2007a; Fang et al., 2005); however, their release concerning the field of genetically-modified microorganisms raises controversy over possible environmental risks (Arora and Shera, 2014). Their use in formulating biological control products is restricted in Colombia (ICA, 2011a) and, although genetically-modified microorganisms are not prohibited in European Union countries, in the United States and China, their use is confined and a detailed case-by-case study is carried out at different risk levels before their commercial or environmental release (Ce et al., 2009; Restrictions on Genetically Modified Organisms, 2014). Adding additives acting as enhancers in bioinsecticide formulations to avoid such obstacles seems to be an alternative having a greater future than those based on active ingredients' genomic edition.

Shternshis *et al.*, (Shternshis et al., 2002; Shternshis, 2004) have carried out studies demonstrating that exogenous chitinases can increase entomopathogenic agents' insecticide activity or reduce required application doses when used together with *Bacillus thuringiensis*, the *Mamestra brassicae* nucleopolyhedrovirus (MabrNPV) and/or *Cydia pomonella* granuloviruses (CpGV). Biocontrol agents' endogenous virulence factors can also be used for enhancing infection when added to formulations.

To the best of our knowledge, endogenous chitinase used as additives for enhancing entomopathogenic fungi has not been reported previously. This work was thus aimed at producing a soluble recombinant chitinase from a gene from the Colombian *B. bassiana* BV062 isolate using an *Escherichia coli* expression system and evaluating its potential for enhancing the insecticidal activity of conidia from the same fungus. The expression and effectiveness of fungal formulations containing this additive evaluated on *Diatraea saccharalis* larvae are presented and discussed.

2. Materials and methods

2.1. Maintaining Beauveria bassiana and rearing Diatraea saccharalis

The Colombian isolate of *Beauveria bassiana* Bv062 was provided by the Germplasm Bank of Microorganisms of Interest in Biological Control from the Corporación Colombiana de Investigación Agropecuaria (Agrosavia) (Tibaitatá Research Centre, Mosquera, Colombia). The fungus was reactivated in potato dextrose agar (PDA) supplemented with 0.1% chloramphenicol at 25 °C for 7 days (García et al., 2018). Second instar *D. saccharalis* larvae maintained in Agrosavia's entomology laboratory were used in controlled temperature (25 °C), relative humidity (60%) and photoperiod conditions (16: 8 h of light: dark) and fed with artificial diet (Lastra et al., 2006).

2.2. Bioinformatics analysis

PacBio and Illumina Technologies (Macrogen Services, South Korea; own data, unpublished) obtained and provided the nucleotide and amino acid (aa) sequence of a chitinase encoding gene from the fully sequenced *B. bassiana* BV062 genome. From now on, this protein will be called Chit37 (GenBank ID: MN871410), according to the activity it would have (chitinase; "Chit") and its theoretical molecular weight (approximately 37 kDa; "37"). Homologous sequences were obtained from GenBank (https://www.ncbi.nlm.nih.gov/) and then compared using the BLAST algorithm (Altschul et al., 1990) and MUltiple Sequence Comparison by Log-Expectation (MUSCLE) 3.8 for protein sequence alignment (Altschul et al., 1990). SignalP 4.1 (Petersen et al., 2011), the TMHMM server V 2.0 (Krogh et al., 2001) and Phobius (Kall et al., 2007) were used for identifying and discriminating signal peptides and transmembrane regions; the BaCeIlo prediction server (Pierleoni et al., 2007) was used for predicting subcellular location and the ProtParam tool (Gasteiger et al., 2005) for predicting physicochemical characteristics. InterProScan 5 (Jones et al., 2014) and PrositeScan (De Castro et al., 2006) tools were used for functional annotation.

2.3. Chitinase gene molecular cloning

A ZR Fungal/Bacterial DNA MicroPrep Kit (Zymo Research, Irvine, CA, USA) was used for isolating genomic DNA (gDNA) from the fungus, according to the manufacturer's recommendations. The Bb062_Chit37 open reading frame (ORF) was amplified by PCR, using specific primers (forward: 5′-ATGGCTCCTTTTCTTCAAACCA-3′; reverse: 5′-GCAGTCCCCAAAGTCCCCTTG-3′) and Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The following profile was used: 5 min at 94 °C (1 cycle), 10 sec at 92 °C, 15 sec at 55 °C, 1 min at 72 °C (35 cycles) and 10 min at 72 °C (1 cycle). Amplified fragments were resolved by agarose gel electrophoresis, recovered using Wizard SV gel and a PCR Clean-up system kit (Promega, Madison, WI, USA) and then molecularly cloned in pEXP5-CT/TOPO (Invitrogen, Carlsbad, CA, USA) and *E. coli* Top 10 (chemocompetent cells; Invitrogen, CA, USA), according to the manufacturer's recommendations. The resulting construct (pEXP5-Bb062_Chit37) was isolated using an UltraClean 6-Minute Mini Plasmid Prep kit (Mo Bio, laboratories, Inc.; Qiagen, Hilden, Germany) with Luria-Bertani cultures from selected *E. coli* clones. The Sanger sequencing method (MACROGEN, Seoul, South Korea) was used for verifying their identity.

2.4. Protein expression and purification

pEXP5-Bb062_Chit37 was transformed in *E. coli* BL21-DE3 cells (Invitrogen, CA, USA), following the manufacturer's recommendations.

Protein expression tests were carried out in 5 mL LB broth cultures supplemented with 0.1 mg/mL ampicillin (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37 °C with shaking at ∼200× rpm. 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, St. Louis, MO, USA) was added when the cultures reached 0.5 optical density (OD600). After 4 h incubation in induced conditions, cells were recovered by spinning at 4000 × *g* for 10 min at 4 °C and stored at −20 °C until use. Protein expression was scaled-up to 200 mL using an inoculum grown as mentioned above. 0.5 mM IPTG was used to induce expression by incubation for 4 h at 30 °C with shaking at ∼250 rpm. The culture was then spun at 4000 × *g* for 150 min and the pellet was collected for recombinant protein extraction.

Chitinase recombinant protein (*r*Chit37) was extracted from soluble (S) and insoluble (I) fractions by adapting reported methods (Fan et al., 2007b). Briefly, cell pellets were suspended in lysis buffer (50 mM Tris HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 1 mM PMSF, 10 μg/mL lysozyme -Sigma-Aldrich- and 10 μg/mL DNAse I -Invitrogen-) with three freezing cycles at −80 °C/15 min, followed by 30 min in an ice bath. The mixture was spun for 30 min at 4 °C at 4000xg and the supernatant was used for purification as soluble fraction and the pellet as insoluble fraction. Part of the supernatant was reserved and used as crude extract in bioassays; the remainder was used to purify the recombinant protein. The insoluble fractions were washed 2x with washing buffer (0.5% v/v Triton X-100, 10 mM EDTA and 1 mM PMSF) followed by overnight incubation at 4 °C with shaking at 10 rpm with denaturing extraction buffer (8 M Urea, 1 mM EDTA, 1 mM iodoacetamide, 1 mM PMSF and 1μg/mL leupeptin) to solubilise inclusion bodies (IB). The supernatant was recovered by ultracentrifugation for 2 h at 40,000 × *g* at 4 °C and dialysed in 20 mM Tris HCl buffer (pH 8.5) to eliminate urea and refold the protein. Affinity chromatography was used for protein purification of soluble (S) and insoluble (I) fractions.

Ni + 2-NTA resin (Qiagen, Hilden, Germany) was incubated with each fraction overnight at 4 °C. The protein resin mixture was placed on a column and weakly bound proteins were eluted by washing with 100 mL buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole (three times), including 0.1% Triton 114 in the second wash. The resin was washed five times with PBS-urea solution in descendent concentrations (3 M, 1.5 M 0.75 M, 0.3 M, 0 M) just for insoluble fractions. Bound proteins were eluted with 1X native purification buffer containing ascending concentrations of imidazole (50 mM, 75 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM and 500 mM) in 2 mL fractions. The fractions containing the recombinant protein were pooled and dialysed in Tris-HCl buffer, pH 8.5. The protein was concentrated on an Amicon Ultra-4 centrifugal filter device (Merck, Darmstadt, Germany) and the resulting concentration was quantified by Micro BCA Protein Assay (Thermo scientific, Waltham MA, USA), using the bovine serum albumin (BSA) curve as reference.

SDS-PAGE polyacrylamide gel electrophoresis was used for analysing expression and purification (Laemmli, 1970), using a commercial molecular weight marker (XL-OptiProtein, New England-Biolabs, Ipswich, MA, USA) as reference. Western blot assays involved using an anti-histidine monoclonal antibody (A7058, Sigma-Aldrich, St. Louis MO, USA), nitrocellulose membrane (Trans-Blot SD-semi-dry electrophoretic transfer cell) and revealed using a peroxidase substrate kit (Vector Laboratories, Burlingame, Canada), following the manufacturer's recommendations.

2.5. Enzyme activity assays

A Chitinase Assay kit (Sigma-Aldrich, St. Louis MO, USA) was used for evaluating *r*Chit37 activity; it included three soluble substrates: 4-Nitrophenyl N-acetyl-β-D-glucosamine and 4-Nitrophenyl N, N'-diacetyl-β-D -chitobioside to detect exo-chitinase activity and 4-Nitrophenyl β -DN, N ', N – triacetylchitotriose to detect endo-chitinase activity; *Trichoderma viride* chitinase was used as positive control. One unit of chitinase activity was defined as the amount of enzyme capable of releasing 1 μmol of p-nitrophenol from the appropriate substrate per minute, in test conditions. All quantifications were made in triplicate. Optimum pH and temperature for recombinant chitinase activity were ascertained using 4-Nitrophenyl N, N'-diacetyl-β-D-chitobioside, different buffer solutions (0.1 M acetate buffer (pH 4.0); 0.1 M trisodium citrate buffer (pH 5.0); 0.1 M potassium phosphate buffer (pH 6.0–7.0) and different incubation conditions (25, 35, 45, 55 and 65 °C). *r*Chit37 purification performance obtained from the soluble fraction was calculated from chitobiosidase activity and the concentration of crude and purified protein. The purification factor was determined as the ratio of specific purified and crude protein activities, and percentage yield was calculated from the ratio of total purified and crude protein activities.

2.6. Conidial vigour and viability

Germination and germ-tube growth percentages were determined to test Bv062 conidial viability. A suspension of conidia (con) in 0.1% Tween 80 was prepared from an 8-day-old solid culture and adjusted to 1×10^8 con/mL concentration as stock suspension which was used for preparing 3 treatments using a mixture of an equal volume of Tween 80 (0.1%), purified chitinase (*r*Chit37) and crude *r*Chit37 extract (CE*r*Chit37). The final protein concentrations for *r*Chit37 and CE*r*Chit37 were 300 μg/mL. The treatments were incubated for 1 h at room temperature. Conidial viability was determined as germination percentage by adapting the method reported by Santos [28]; 100 μL samples of the 10^{-1} dilution of each treatment were inoculated on plates with malt extract agar (0.1%) supplemented with chloramphenicol (0.1%) and benomyl (0.00015%). The percentage of germinated conidia was evaluated under a light microscopy after 24 h' incubation at 25 °C. Elongation speed of germ-tubes was assessed to determine conidia vigour. This involved inoculating all treatments into plates containing the same agar, but without benomyl. Around 1 cm^2 agar samples were taken after 4, 8- and 12-hours incubation, stained with lactophenol blue solution and observed under a Vision DX41 microscope, adapted with a UCMOS14000KPA camera. The germ-tubes' net length without spore diameter (Nguyen et al., 2010) was estimated by ImageJ 1.52a image processing program (Schneider et al., 2012).

2.7. *Diatraea saccharalis* bioassays

The methodology described by Garcia was used for the bioassays (García et al., 2018); Table 1 describes the nine treatments evaluated here. Briefly, 2 μL of each treatment was inoculated topically on the back of second instar larvae of *D. saccharalis*. The larvae were individually arranged in a half-ounce container and fed on a natural diet of sweet corn grains. They were placed in 1774.41 mL SelloPlus boxes, in groups of 15 units, in triplicate, and kept at 25 °C. An absolute control involved larvae which did not receive any treatment. Mortality was evaluated daily from the second day onwards, until day 14 post-

Table 1
Treatments used for evaluating the effect of *r*Chit37 regarding *B. bassiana* conidia insecticidal activity against *D. saccharalis* larva.

Treatment		Conidia concentration (con/mL)	Protein concentration (μg/mL)
1	Bv062 (Bv)	$1x10^6$	0
2	*r*Chit37*	0	200
3	*r*Chit37*	0	300
4	CE*r*Chit37**	0	200
5	CE*r*Chit37**	0	300
6	Bv + *r*Chit37*	$1x10^6$	200
7	Bv + *r*Chit37*	$1x10^6$	300
8	Bv + CE*r*Chit37**	$1x10^6$	200
9	Bv + CE*r*Chit37**	$1x10^6$	300

*Purified recombinant chitinase recovered from soluble fractions.
**Crude extract (CE) recombinant chitinase recovered from soluble fractions.

infection. The Schneider-Orelli formula was used for calculating efficacy (Zar, 1999).

$$Efficacy\ (\%) = \frac{(100 - A)}{(100 - C)} \times 100$$

where

A = Mortality in the treatment
C = Mortality in the control treatment

2.8. Statistical analysis

The Shapiro Wilk test was used for determining data normality and the Barlett test for homoscedasticity (95% confidence level). Once such assumptions had been confirmed, analysis of variance (ANOVA) was used for determining the differences between treatments and means were compared by Tukey test (95%) and the Statistix statistical analysis program (8.0) (Analytical Software, FL, USA). Probit analysis with BioStat software (version 5.8.1) was used to analyse daily mortality and estimate lethal times (LT_{50} and LT_{90}).

3. Results and discussion

3.1. rChit37 production

The chitinase Chit37 of *Beauveria bassiana* (strain Bv062) was highly similar (99% identity) to previously reported Bbchit1 (Fan et al., 2007b), being 348 aa-long, encoded by an ORF without introns, having a 36.7 kDa theoretical molecular weight (MW) and 5.94 isoelectric point (PI). This protein was predicted to have a signal peptide (PS) located in positions 1–22, with a predicted cleavage site between residues 22 and 23, suggesting that it may be a secreted protein. Functional prediction showed that the protein would have a glycoside hydrolase catalytic domain belonging to chitinase family 18 (GH18), having SxGG and DxxDxDxE motifs characteristic of the active site (Terwisscha van Scheltinga et al., 1994); however, a chitin binding domain (CBD) could not be predicted. The chit37 ORF was molecularly cloned into an *E. coli* BL21 DE3 cell expression vector, not including the previously predicted putative signal peptide sequence. The expression assays tested the effect of inductor concentration (0.2, 0.5 and 1 mM IPTG) and exposure time in standard conditions, the best results occurring with 1 mM IPTG and 4 h induction (Fig. 1A).

Western blot revealed notorious differences regarding recovered soluble and insoluble fractions; thus, most *r*Chi37 was found as IBs (Inclusion bodies) in the insoluble fraction (Fig. 1B), being similar to that previously reported in recombinant chitinases (Boer et al., 2007; Fan et al., 2007b; Rao et al., 2004). Given this evidence, an expression assay was conducted at 30 °C, using 0.2 mM IPTG; these conditions promoted a greater amount of *r*Chit37 in the soluble fraction (Fig. 1B).

Soluble fractions obtained from bacterial cell lysates in recombinant protein production can be used as crude extracts or purified proteins, whilst IB proteins require additional steps to solubilise and promote refolding (Vallejo and Rinas, 2004). Fermentation temperature and inductor concentration were reduced in this study to increase the amount of protein in the soluble fraction. It has been reported that changing growth conditions, such as growth temperature, inducer concentration and induction time, can often help decrease IB formation (Yamaguchi and Miyazaki, 2014), minimising physicochemical conditions favouring conformational stress and aggregation (Ventura and Villaverde, 2006).

*r*Chit37 was purified to homogeneity (SDS-PAGE criteria), in a single step (affinity chromatography), from soluble (cytoplasmic) and insoluble fractions (*r*Chit37 IBs denatured and later renatured by dialysis) (Fig. 2).

3.2. Enzyme activity

Purified *r*Chit37 from the soluble and insoluble fractions showed endo- and exo-mode of chitin hydrolysis by using different substrates. The *r*Chit37 presented 170 mU/mL and 220 mU/mL exo-chitinase activity (chitobiosidase) for soluble and insoluble fractions, respectively. β-N-acetylglucosaminidase activity was not detected in neither fraction. This contrasted with that shown by Fan et al., (Fan et al., 2007b) who failed to obtain functionally active Bbchit1 for endo-mode chitin hydrolysis from a bacterial cytoplasmic fraction, suggesting incorrect folding or modification of the protein in the *E. coli* cytoplasmic space.

Total chitobiosidase activities were 1.584 U for crude extract and 0.85 U for purified protein, i.e. 17.86 purification (fold) and 53.6% recovery yield. Purified *r*Chit37 yield was 2.53 mg/L, which was similar to that reported for Chit42 and ChiA (Boer et al., 2007; Rao et al., 2004), but lower than the yield obtained for Chit33 and Bbchit1 (Boer et al., 2007; Fan et al., 2007b). Related with the endo-chitinase activity, the soluble fraction presented 90 mU/mL, being lower than reported by Fan et al., (Fan et al., 2007b) for the protein purified from IBs.

*r*Chit37 from IB and purified in denaturing conditions was an inactive protein. Simple dialysis was thus carried out for increasing the amount of active protein, after solubilisation and prior to purification in soluble conditions. This was enough to convert *r*Chit37 from IB inactive state to a functionally active polypeptide having chitobiosidase activity (0.22 U/mL), as also achieved for *Trichoderma harzianum* Chit33 and Chit42 chitinases (Boer et al., 2007) and *B. bassiana* Bbchit1 (Fan et al., 2007b). The successful recovery of functional protein from IB could have been due to insoluble protein aggregate formation which might have been a reservoir for alternative conformational states of the protein conserving correctly-folded functional domains (Ventura and Villaverde, 2006). Purified proteins from IB could thus have had enzymatic activity similar or higher than that from soluble fractions, highlighting no relationship between solubility and correct folding (García-fruitós et al., 2005).

Dual activity has also been reported for other chitinases related to biological control agents, such as ChiA from Autographa californica nucleopolyhedrovirus (AcMNPV) (Rao et al., 2004; Thomas et al., 2000), VChiA from Cydia pomonella granulovirus (CpGV) (Daimon et al., 2006; Salvador and Ferrelli, 2014), Bbchit1 from *B. bassiana* and CHI60 from *Serratia* sp. (Fan et al., 2007b; Kuttiyawong et al., 2008), and could be advantageous regarding the processing of different types of substrate, depending on their molecular organisation. Some researchers have proposed that this group of proteins use their endochitinase function involving non-processive action on substrates having tightly bound chains, such as colloidal chitin, due to the limitation of enzyme movement (Kuttiyawong et al., 2008). Similarly, the α chitin prevalent in insect cuticle has tightly packed microfibrils (Merzendorfer, 2003); initial *r*Chit37 action on insect cuticle could thus be of non-processive endochitinase type and processive exochitinase activity (Kuttiyawong et al., 2008) could therefore act on substrates having less rigidity amongst their microfibers, possibly triggered by endochitinase action.

Analysing Bv062 conidia chitinases produced in semisolid-state fermentation revealed B-N-acetylglucosaminidase activity, whilst endochitinase activity was very low (own data, unpublished), indicating that such enzymatic activities could not be induced in the fermentation conditions evaluated here. The foregoing suggests that *r*Chit37 could improve endo- and exo-chitinase activity that is not easily inducible in fermentation conditions and whose dual activity has been shown to influence other biological control agents' virulence (Fang et al., 2005; Rao et al., 2004).

3.3. The effect of temperature and pH on rChit37 enzymatic activity

*r*Chit37 enzymatic activity becomes reduced as pH increases, having maximum activity at pH 5.0 (Fig. 3A). Such results agreed with

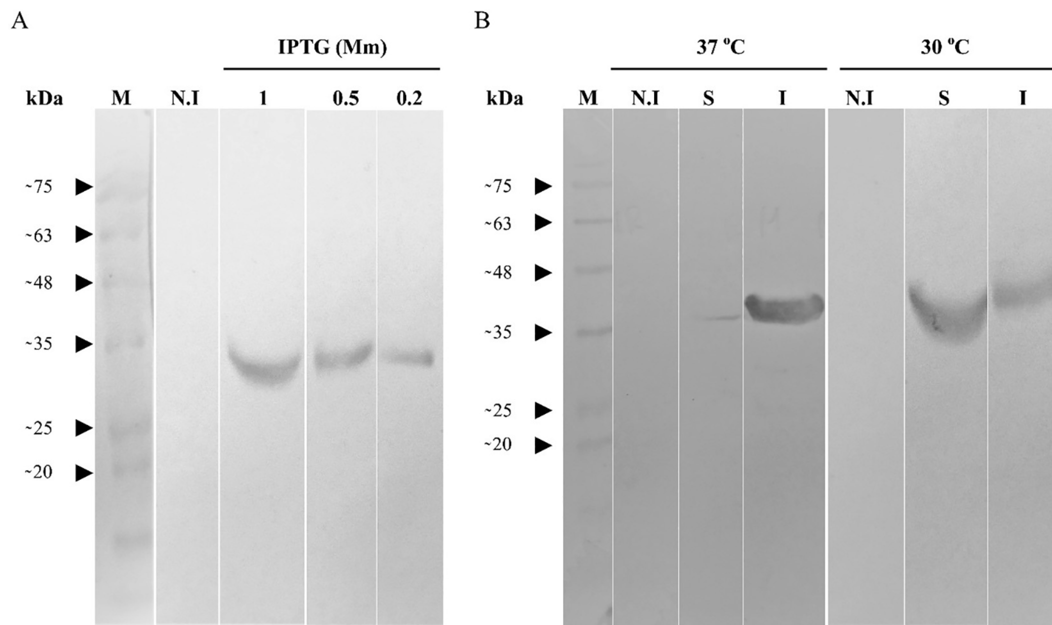


Fig. 1. Western blot using anti-histidine monoclonal antibody for standardising optimal *rChit37* expression conditions [A]. IPTG concentration effect on 4 h post-induction expression level. [B] The effect of temperature on protein expression and solubility. Molecular weight protein standards (M), not induced (N.I.) soluble or cytoplasmic fraction (S), insoluble fraction (I).

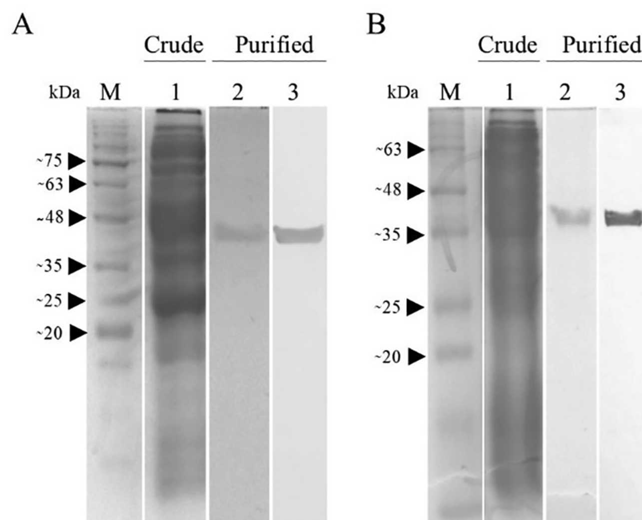


Fig. 2. *r*Chit37 purification from [A] soluble (cytoplasmic) and [B] insoluble fractions (IBs). Lane 1: Crude extract stained with Coomassie blue; Lane 2: purified protein verification by Coomassie blue staining; Lane 3: Western blot using anti-histidine monoclonal antibody.

optimum pH ranges (4.0–7.0) described for fungal chitinases, particularly having values described for GH18 entomopathogenic fungi, having 5.0 to 6.0 optimum pH (Bidochka et al., 1993; Fan et al., 2007b; Han et al., 2016; Ike et al., 2006; St. Leger et al., 1991; Takaya et al., 1998; Yang et al., 2014). However, they differed from the optimum pH range (5.0–9.0) reported for bacterial and viral chitinases having insecticidal activity (Busby et al., 2012; Daimon et al., 2007, 2006; Danişmazoğlu et al., 2015; Liu et al., 2010; Ni et al., 2015).

Fig. 3B shows that *r*Chit37 chitinase activity gradually increased with a rise in temperature, reaching maximum activity at 45 °C and starting to become reduced from 55 °C. However, it is worth noting that the enzyme remained functionally active over a wide range of temperatures, since it had higher than 187 mU/mL chitobiosidase activity in all conditions evaluated here. Recombinant enzyme optimum temperature was higher than that for chitinases from entomopathogenic bacteria (33–37 °C) (Danişmazoğlu et al., 2015; Ni et al., 2015). *r*Chit37 optimum temperature did not differ considerably from that reported for most chitinolytic enzymes from other entomopathogenic fungi, being equal to that determined for *Metarhizium anisopliae* chitinases (St. Leger et al., 1991) and similar to the temperature for its homologue Bbchit1 (55 °C) (Fan et al., 2007b), but higher than that for *B. bassiana* NAGAse2 (37 °C) (Bidochka et al., 1993). It is also worth noting that *r*Chit37 had activity at 65 °C, the temperature at which native and recombinant Bbchit1 produced in *P. pastoris* and *E. coli* is inactive (Fan et al., 2007b).

3.4. The effect of rChit37 on B. bassiana Bv062 conidial viability and vigour

Whether conidia were treated or not with enzyme they had a development pattern consistent with the transition of spores to hyphae described for *B. bassiana* (Liu et al., 2015). A large amount of germinated conidia were observed after 12 h' incubation and germ-tube elongation became evident after 16 h, hyphae branching was observed 20 to 24 h later (Fig. 4).

Conidial germination was similar for the three treatments at all evaluated times (55 to 63% values after 16 h, 87 to 93% after 20 h and 93 to 100% after 24 h) (Fig. 5A). The germination percentage reached in this study for all treatments after 24 h' incubation was the minimum (85%) recommended as suitable for ensuring the quality of *B. bassiana*-based commercial formulations (Marín et al., 2000). Linear regression was used for analysing germination *vs* time percentages and correlation coefficients (R^2) higher than 0.91 suggested a suitable fit with a zero-order kinetics model. The equation was used for estimating the slope of the line for conidial germination speed regarding each treatment; values which were not statistically different ($F_{2,6} = 0.80$; $p = 0.4903$), indicated that the enzymes being evaluated did not affect germination rate. Germination speed is a critical parameter determining the efficacy of entomopathogenic fungal conidia from regarding their insecticidal activity, as reported by Faria et al., (Faria et al., 2015) for *B. bassiana.* They demonstrated that conidia could germinate after 16 h incubation (considered vigorous) were more virulent, causing greater *Spodoptera frugiperda* larvae mortality than those having a maximum germination

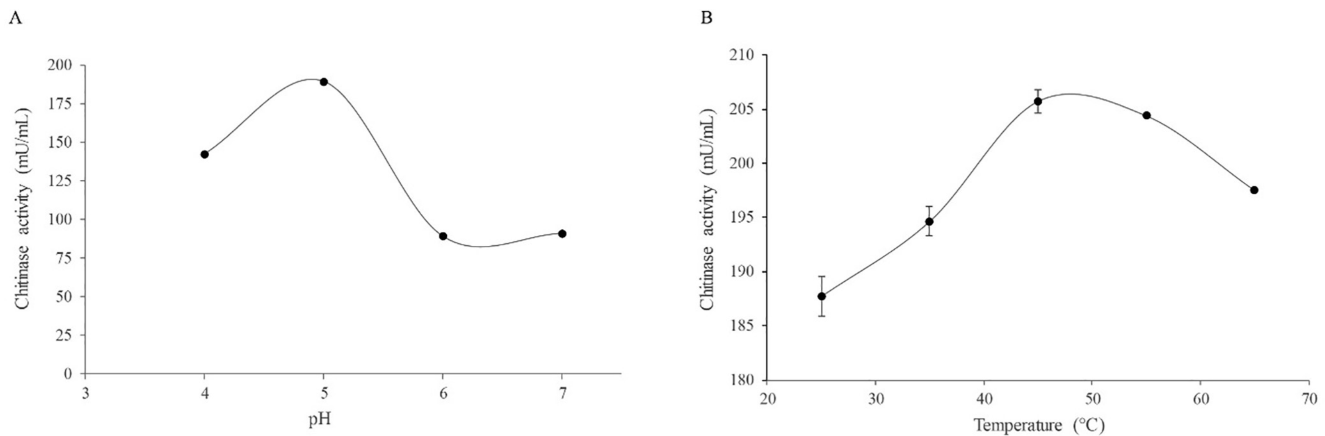


Fig. 3. The effect of pH and temperature on rChit37 chitinase activity. Purified rChit37 endochitinase activity in soluble conditions at different pH [A] and temperatures [B]. The values represent the average of three repeats and the error bars the standard deviation.

rate after 48 h. The conidia in the three treatments in the present study were equally vigorous in the absence and presence of *r*Chit37 and CE*r*Chit37, more than 90% of them germinating before 24 h' incubation.

Fig. 5B shows conidial germ-tube development in the absence and presence of *r*Chit37 and CE*r*Chit37 between 12- and 24-hours incubation. The data were logarithmically transformed, and lineal regression was used for calculating germ-tube elongation speed, obtaining a suitable fit with the mathematical model ($R^2 > 0.99$ for all treatments) for first order kinetics. The slopes of the lines obtained (Fig. 5B) were assumed as an expression of elongation speed and compared by ANOVA which did not detect significant differences between treatments ($F_{2,6} = 0.26$, $p = 0.7791$), suggesting that the enzymes did not affect conidial germinal tube development.

Germination and germ-tube development of *B. bassiana* conidia occur normally in the first 36 h after binding to insect cuticle (Lai et al., 2017). This time is longer than that required to germinate when conidia are inoculated on agar (*in vitro*), possibly because the fungus requires more time in *in vivo* conditions to become adapted to insect's epicuticle environment and also because certain types of hydrocarbons and lipids can inhibit the germination (Pedrini et al., 2013).

The foregoing results led to conclude that crude and purified recombinant chitinase did not affect Bv062 conidial viability or the normal development of germ-tube emergence and elongation (i.e. positively or negatively). Although *B. bassiana* chitinases are involved in cell remodelling and growth (Seidl, 2008), *r*Chit37 may be playing a more important role in fungal virulence as was demonstrated for some entomopathogenic and mycoparasite fungi when their chitinase genes were mutated, negatively altering the virulence but without effects on fungal morphology and growth, regarding spore, appressoria and hyphae formation (Boldo et al., 2009; Mamarabadi et al., 2008).

*r*Chit37's did not affect *in vitro* germination of conidia in the present study but this enzyme could indirectly stimulate germination during host interaction since its chitin's catalytic action on the cuticle could release assimilable compounds as carbon source (Hamid et al., 2013). This effect has been observed for CDEP-1, a (recombinant) cuticle-degrading protease which, when used as *B. bassiana* additive in a culture with exuviae as sole carbon source, accelerated germination; this was correlated later on with the fungi's increased insecticidal activity against aphids (Zhang et al., 2008).

3.5. The effect of rChit37 on B. bassiana Bv062 effectiveness

Efficacy results, evaluated 14 days after inoculating the treatments, led to clearly establish three significantly different groups ($F_{8,18} = 22.4$, $p = 0.0$) (Fig. 6). Topical inoculations of crude (obtained from a *E. coli* soluble fraction) and purified *r*Chit37 did not induce an important insecticidal effect on *D. saccharalis* larvae (less than 10% efficacy) when the enzymes were used in the absence of the fungi. Previous papers present contrasting results regarding the effect of using purified and crude enzyme extracts in the absence of propagules of entomopathogenic fungi. For example, a topical application of partially-purified N-acetylglucosaminidase (NAGase) from *T. konigiopsis* on *D. saccharalis* larvae (Mejia, 2018), CDEP-1 and CDEP:BmChBD recombinant proteases (a hybrid protease having a chitin binding domain) evaluated on *Mysus persicae* adults and nymphaea (Fan et al., 2010; Zhang et al., 2008), did not lead to insect mortality. By contrast, oral administration of partially-purified bacterial and fungal chitinases could cause delays during insects' different development stages, thereby affecting digestive process due to alterations in peritrophic membrane formation and even induce death (Berini et al., 2016; Okongo et al., 2018; Regev et al., 1996). The foregoing indicates that hydrolytic enzymes' effect on insect mortality could vary, depending on the source, administration route, structure or target species. This could be explained regarding fungal chitinases due to broad diversity and specialisation of functions, according to their role played in living systems (Seidl, 2008; Seidl et al., 2005).

Treatments involving just conidia, or when combined with crude and purified *r*Chit37, had an insecticidal effect in all cases (higher than 50% efficacy values). The mortality of larvae inoculated with the mixture of Bv062 conidia and purified enzyme at 200 µg/mL concentration and crude extract at 200 and 300 µg/mL was similar to that obtained with an individual application of conidia at all times evaluated here (Fig. 6), having 50 to 60% efficacy values (which were not statistically different). However, the insecticidal activity of conidia applied with 300 µg/mL purified *r*Chit37 was significantly greater ($F_{8,18} = 5.49$, $p = 0.0013$) from the fourth day of the assay onwards (Fig. 6). This demonstrated a progressive, synergic effect, resulting in 96.7% efficacy after 14 days (Fig. 6), with 61% increase, compared to the fungal application without enzyme.

This effect could have been attributed to *r*Chit37-mediated, faster and facilitated penetration of insect procuticle. The procuticle is the only insect cuticle layer containing chitin, consisting of a protein matrix with chitin fibres embedded within it (Andersen, 2009); it is located under the epicuticle, a complex lipid-rich structure covering an insect's entire external surface (Prakash, 2008). Integument digestion follows a lipase–protease–chitinase sequence due to the cuticle structure that has three sections: the outermost layer called the envelope (lipids), the epicuticle (proteins) and the procuticle (chitin and proteins) (Tanada and Kaya, 1993). In this context, *r*Chit37 added to the conidia suspension could have penetrated the epicuticle together with the appressoria and the germ tube and developed its catalytic activity upon

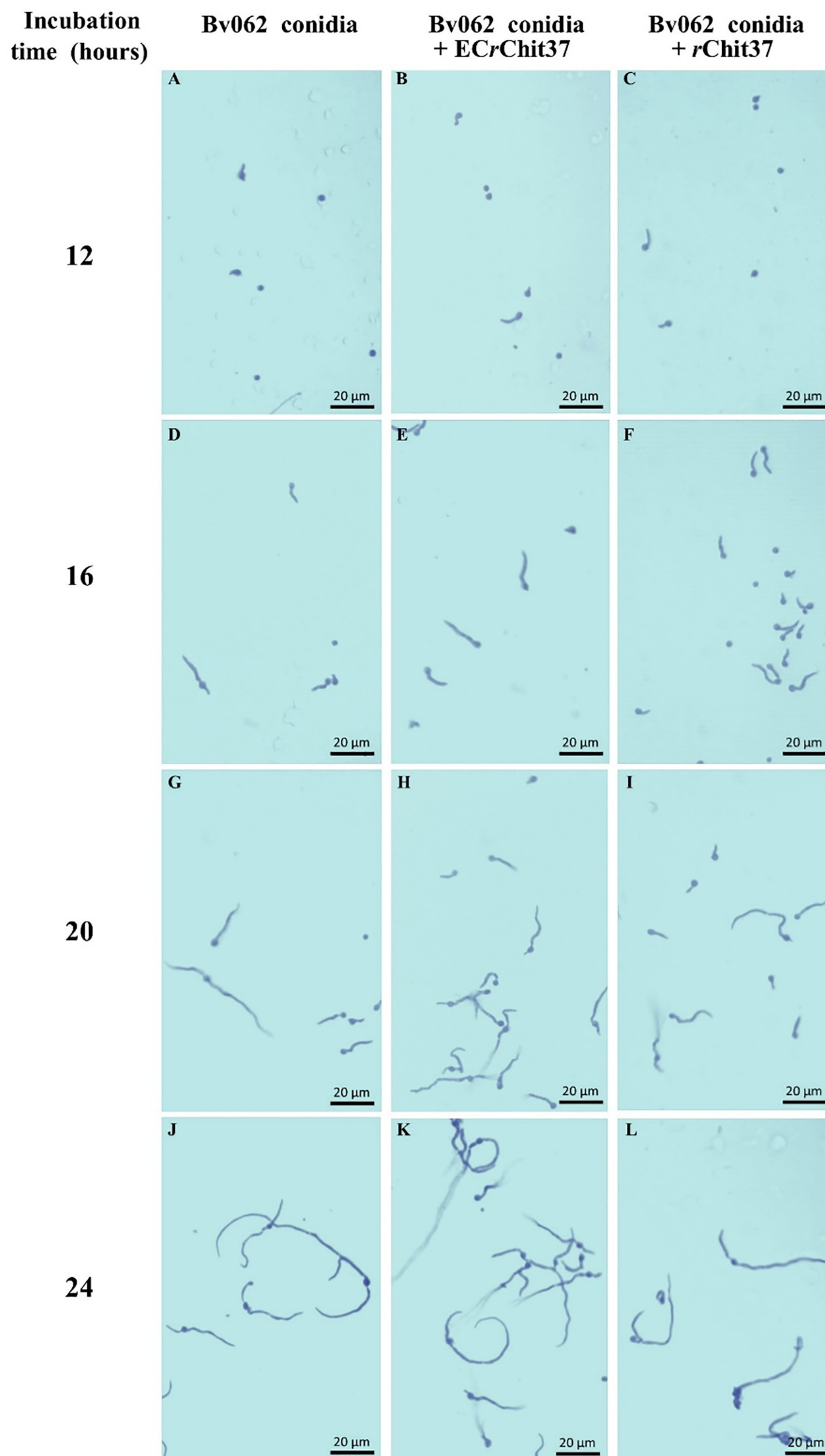


Fig. 4. Development of germinal tube of Bv062 conidia alone and when mixed with *r*Chit37. All images were recorded at 40X magnitude.

reaching the chitin fibres in the procuticle, synergistically with the enzymes naturally expressed and excreted by the fungus. An alternative hypothesis is that the *r*Chit37 added to the conidia suspension produced stress in the germinated conidia and the appressoria, due to an effect on structural chitin resulting in a higher hydrolytic enzyme expression and production where the fungus reached the insect procuticle, thereby facilitating penetration.

The synergic effect of other hydrolytic enzymes used as *B. bassiana* conidia additive has already been reported (Mejia, 2018; Zhang et al., 2008). For example, using 100 µg/mL CDEP-1 improved the mortality

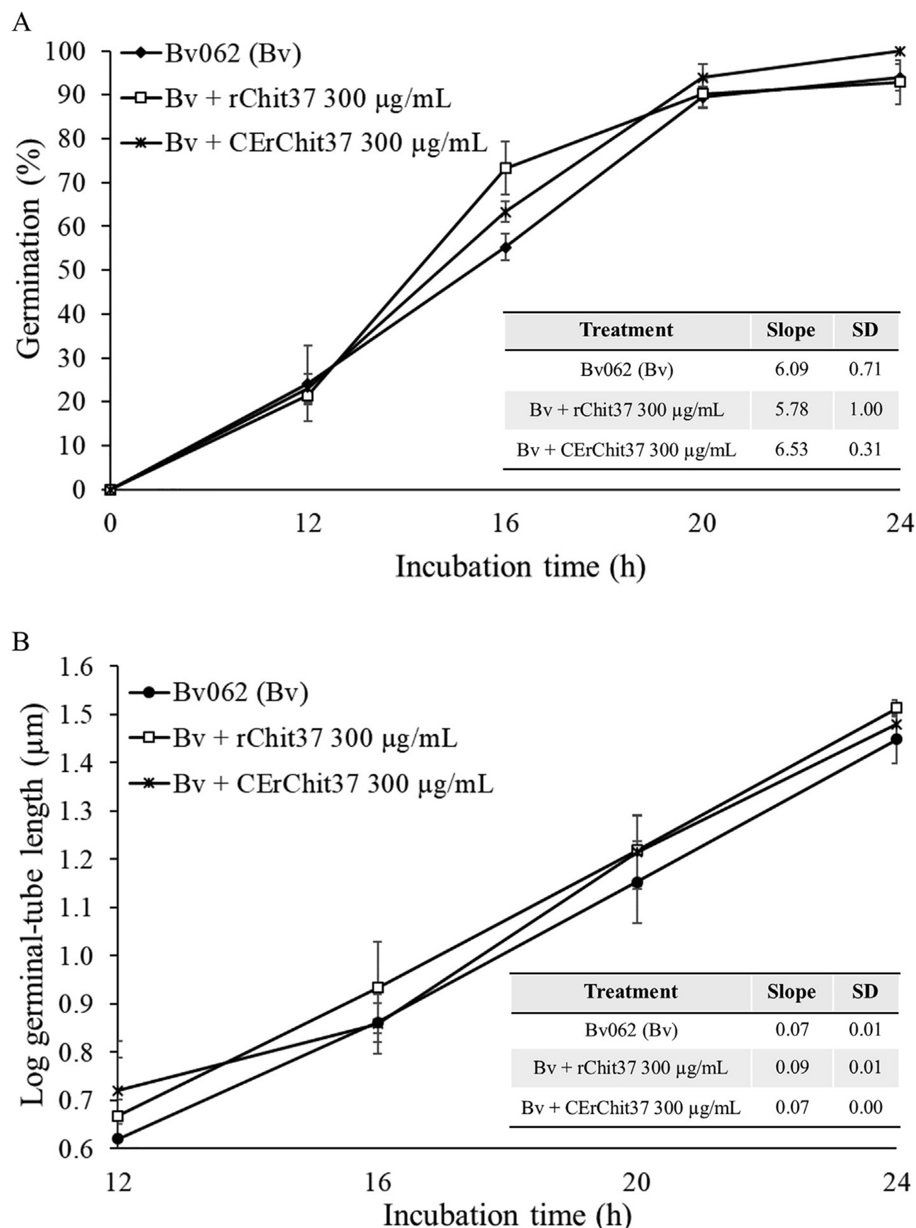


Fig. 5. The effect of *r*Chit37 on Bv062 conidial viability. A. The effect of *r*Chit37 on conidial germination. B. The effect of *r*Chit37 on germinal tube elongation.

caused by the fungi on *Mysus persicae* aphids when being used as additive at 20% at 1x10^6 and 25% at 5x10^7 con/mL (Zhang et al., 2008), this being a lower enhancer effect than that found in the present study with *r*Chit37. Such difference could have been due to chitinase activity having a greater effect on insect mortality than protease action, as demonstrated experimentally in enzyme inhibition assays with *B. bassiana* extracts (Kim et al., 2010). It is worth mentioning that García *et al.*, (García et al., 2018) evaluated the same isolation regarding second instar *D. saccharalis* larvae, achieving 73.3% efficacy when using a concentration 10 times greater than that evaluated regarding the mixture with *r*Chit37 in the present study (i.e. 96.6% efficacy). These results suggested interesting potential for using the recombinant chitinase developed in this work (300 µg/mL) as additive when developing and using entomopathogenic fungi for pest control.

3.6. The effect of rChit37 on B. bassiana Bv062 lethal times

In general, the lethal times varied between 5.4 and 12.6 days (LT_{50}) and 11.8 to 47.2 days (LT_{90}) (Table 2). Fungal conidia combined with 300 µg *r*Chit37 presented significantly inferior values to those obtained with the other treatments (without overlapping confidence limits).

The lethal times obtained by combining *B. bassiana* conidia with *r*Chit37 (300 µg/mL) were lower than those determined previously using conidial suspensions from different entomopathogenic fungal species on *D. saccharalis* larvae. For example, LT_{50} was 6.0 days and LT_{90} 12.2 days in a study using the same Bv062 strain at 100-fold higher concentration than that evaluated in this work (García et al., 2018). LT_{50} was 15.14 days for *B. bassiana* Bb-HN1 strain first instar larvae when used at 2×10^8con/mL concentration (Zúñiga-oviedo et al., 2016) and 7.31 to 12.41 days at 1×10^8 con/mL concentrations evaluated on third instar larvae (Diaz and Lecuona, 1995; Leucona and Alves, 1988). Some studies have reported lower LT_{50} than those determined in this research, i.e. 2.09 to 5.02 days for *B. bassiana* and *M. anisopliae* strains, but using concentrations up to 100-fold higher than those used in this study (Acevedo et al., 2007; Arcas et al., 1999; Diaz and Lecuona, 1995; Svedese et al., 2013).

A 46% reduction in LT_{50} and 68% in LT_{90} was obtained when the Bv062 conidia suspension (1x10^6 con/mL) was supplemented with

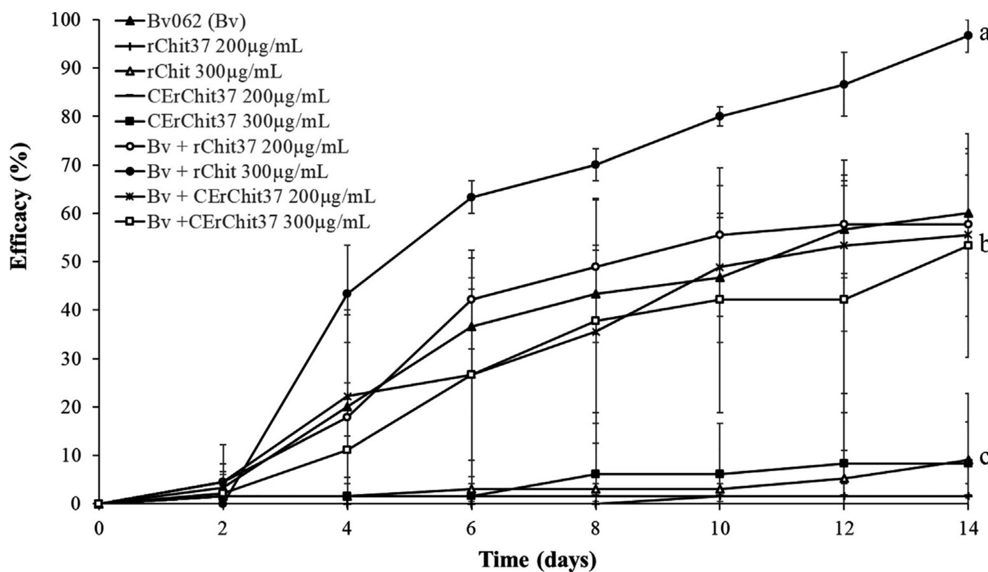


Fig. 6. Effect of *r*Chit37 on Bv062 insecticidal activity against *D. saccharalis* larvae. Cumulative efficacy. The data represent the average of three repeats, the bars indicate the standard deviation calculated on the repeats. Different letters indicate significant differences between treatments when using Tukey's range (honestly significant difference – HSD) test (95%).

Table 2
LT_{50} and LT_{90} for *B. bassiana* Bv062 conidia (1×10^6 con/mL) used by themselves and when mixed with crude and purified *rChit37* on second instar *D. saccharalis* larvae.

Treatment	LT_{50}	Confidence limits (95%)		LT_{90}	Confidence limits (95%)		X^2	df	n	Slope
		Lower	Upper		Lower	Upper				
Bv062 (Bv)	10.0	9.0	11.4	37.3	28.4	55.5	4	5	45	2.245 ± 0.225
Bv + *r*Chit37 200 µg/mL	9.3	7.8	11.6	34.5	23.0	75.3	9	5	45	2.245 ± 0.220
Bv + *r*Chit37 300 µg/mL	5.4	4.2	6.4	11.8	9.6	16.4	18	5	45	3.731 ± 0.257
Bv + CE*r*Chit37 200 µg/mL	11.4	10.0	13.3	47.2	34.0	77.6	2	5	45	2.073 ± 0.223
Bv + CE*r*Chit37 300 µg/mL	12.6	11.2	14.8	45.8	33.4	73.5	5	5	45	2.290 ± 0.247

300 µg/mL recombinant chitinase, compared to using just fungal conidia. This result suggested that *r*Chit37 improved Bv062 conidia virulence against second instar larvae of *D. saccharalis*, reducing doses and lethal times. Such reduction could have been attributed to *r*Chit37 chitobiosidase and endochitinase activity considering that it has been demonstrated that the chitinolytic activity of entomopathogenic fungi such as *B. bassiana* and *M. anisopliae* is directly related to their virulence (Boldo et al., 2009; Fan et al., 2007a; Fang et al., 2005).

The recombinant chitinases studied in this work could provide a promising alternative for potentiating bio-controller microorganisms and developing new-generation biopesticides in the light of the above and that using genetically-modified microorganisms as biological control agents is restricted in Colombia (ICA, 2011b) and other countries worldwide.

4. Conclusions

*r*Chit37 chitinase was expressed in *E. coli* into soluble and insoluble fractions, being functionally active in both forms. *r*Chit37 presented the endochitinase activity previously reported for the similar enzyme Bbchit. In addition, exochitinase activity that has not been previously reported for this enzyme, was also found in *r*Chit37. The purified, soluble, recombinant protein did not have any inhibitory or stimulating effects on conidial viability in terms of germination and germ-tube elongation when used as additive. However, *r*Chit37 improved *B. bassiana* Bv062 insecticidal activity on *D. saccharalis* larvae in laboratory conditions, increasing efficacy by 61% and reducing LT_{50} by 46%. The results of this work constitute an initial proof-of-concept for the development of a biopesticide based on *B. bassiana* enhanced with a recombinant enzyme with endo- and exochitinase activity to control *D. saccharalis*.

Acknowledgements

This work was supported by grants from the Colombian Ministry of Agriculture and Rural Development (MADR). This work was carried out under the RGE0229-2 contract for access to genetic resources and their derived products in Colombia.

We are grateful to the Corporación Colombiana de Investigación Agropecuaria (AGROSAVIA) and the Fundación Instituto de Inmunología de Colombia (FIDIC) for providing physical and human resources. The authors thank Luis Alfredo Baquero, Gustavo Araque, Cindy Mejía and Andrea Estefania Ramos for the technical support in laboratory assays and Jason Garry for English corrections. The current project was funded through the Colombian Royalties System by the Gobernación de Nariño, grant 1865.

References

Acevedo, J.P.M., Samuels, R.I., Machado, I.R., Dolinski, C., 2007. Interactions between isolates of the entomopathogenic fungus *Metarhizium anisopliae* and the entomopathogenic nematode *Heterorhabditis bacteriophora* JPM4 during infection of the sugar cane borer *Diatraea saccharalis* (Lepidoptera: Pyralidae). J. Invertebr. Pathol. 96, 187–192. https://doi.org/10.1016/j.jip.2007.04.003.

Altschul, S., Gish, W., Miller, W., Lipman, D., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Andersen, S.O., 2009. Cuticle. In: Vincent, H.R., Ring, T.C., (Eds.), Enciylopedia of Insects. Academic Press, pp. 245–246. doi: 10.1016/B978-0-12-374144-8.00073-4.

Arcas, J., Dıaz, B., Lecuona, R., 1999. Bioinsecticidal activity of conidia and dry mycelium preparations of two isolates of *Beauveria bassiana* against the sugarcane borer *Diatraea saccharalis*. J. Biotechnol. 67, 151–158.

Arora, R., Shera, P.S., 2014. Genetic Improvement of Biocontrol Agents for Sustainable Pest Management. doi: 10.1007/978-81-322-1877-7.

Berini, F., Caccia, S., Franzetti, E., Congiu, T., Marinelli, F., Tettamanti, G., 2016. Effects of *Trichoderma viride* chitinases on the peritrophic matrix of Lepidoptera. Pest Manage. Sci. 72, 980–989. https://doi.org/10.1002/ps.4078.

Bidochka, M.J., Tong, K.I., Khachatourians, G.G., 1993. Partial purification and

characterization of two extracellular N-acetyl-D-glucosaminidases produced by the entomopathogenic fungus *Beauveria bassiana*. Can. J. Microbiol. 39, 40–45. https://doi.org/10.1139/m93-006.

Boer, H., Simolin, H., Cottaz, S., Söderlund, H., Koivula, A., 2007. Heterologous expression and site-directed mutagenesis studies of two *Trichoderma harzianum* chitinases, Chit33 and Chit42, in Escherichia coli. Protein Expr. Purif. 51, 216–226. https://doi.org/10.1016/j.pep.2006.07.020.

Boldo, J.T., Junges, A., do Amaral, K.B., Staats, C.C., Vainstein, M.H., Schrank, A., 2009. Endochitinase CHI2 of the biocontrol fungus Metarhizium anisopliae affects its virulence toward the cotton stainer bug Dysdercus peruvianus. Curr. Genet. 55, 551–560. doi: 10.1007/s00294-009-0267-5.

Busby, J.N., Landsberg, M.J., Simpson, R.M., Jones, S.A., Hankamer, B., Hurst, M.R.H., Lott, J.S., 2012. Structural analysis of Chi1 chitinase from Yen-Tc: the multisubunit insecticidal ABC toxin complex of *Yersinia entomophaga*. J. Mol. Biol. 415, 359–371. https://doi.org/10.1016/j.jmb.2011.11.018.

Butt, T.M., Coates, C.J., Dubovskiy, I.M., Ratcliffe, N.A., 2016. Entomopathogenic fungi: new insights into host-pathogen interactions. Adv. Genet. 94, 307–364. https://doi.org/10.1016/bs.adgen.2016.01.006.

Ce, D., Parlamento, D.E.L., Del, E.Y., 2009. DIRECTIVA 2009/41/CE DEL PARLAMENTO EUROPEO Y DEL CONSEJO de 6 de mayo de 2009 relativa a la utilización confinada de microorganismos modificados genéticamente. Diario oficial de la Unión europea, Unión europea.

Cuartas, P., Barrera, G., Gómez, J., Barreto, E., Villamizar, L., n.d. Synergistic effect of nucleopolyhedrovirus and granulovirus mixtures for controlling Spodoptera frugiperda (Lepidoptera: Noctuidae) larvae. Pest Manage. Sci.

Daimon, T., Katsuma, S., Kang, W.K., Shimada, T., 2006. Comparative studies of Bombyx mori nucleopolyhedrovirus chitinase and its host ortholog. BmChi-h. Biochem. Biophys. Res. Commun. 345, 825–833. https://doi.org/10.1016/j.bbrc.2006.04.112.

Daimon, T., Katsuma, S., Kang, W.K., Shimada, T., 2007. Functional characterization of chitinase from Cydia pomonella granulovirus. Arch. Virol. 152, 1655–1664. https://doi.org/10.1007/s00705-007-1000-7.

Danişmazoğlu, M., Demir, İ., Sezen, K., Muratoğlu, H., Nalçacioğlu, R., 2015. Cloning and expression of chitinase A, B, and C (chiA, chiB, chiC) genes from *Serratia marcescens* originating from *Helicoverpa armigera* and determining their activities. Turkish J. Biol. 39, 78–87. https://doi.org/10.3906/biy-1404-31.

De Castro, E., Sigrist, C., Gattiker, A., Bulliard, V., Langendijk-Genevaux, P., Gasteiger, E., Bairoch, A., Hulo, N., 2006. ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. Nucl. Acids Res. 1, 362–365.

Diaz, B., Lecuona, R., 1995. Evaluación de cepas nativas del hongo entomopatógeno Beauveria bassiana Bals. (Vuill.) (Deuteromicotina) como base para la selección de bioinsecticidas contra el barrenador Diatraea saccharalis (F.). Agriscientia XII, 33–38.

Fan, Y., Fang, W., Guo, S., Pei, X., Zhang, Y., Xiao, Y., Li, D., Jin, K., Bidochka, M.J., Pei, Y., 2007a. Increased insect virulence in *Beauveria bassiana* strains overexpressing an engineered chitinase. Appl. Environ. Microbiol. 73, 295–302. https://doi.org/10.1128/AEM.01974-06.

Fan, Y., Zhang, Y., Yang, X., Pei, X., Guo, S., Pei, Y., 2007b. Expression of a *Beauveria bassiana* chitinase (Bbchit1) in *Escherichia coli* and *Pichia pastoris*. Protein Expr. Purif. 56, 93–99. https://doi.org/10.1016/j.pep.2007.06.012.

Fan, Y., Pei, X., Guo, S., Zhang, Y., Luo, Z., Liao, X., Pei, Y., 2010. Increased virulence using engineered protease-chitin binding domain hybrid expressed in the entomopathogenic fungus *Beauveria bassiana*. Microb. Pathog. 49, 376–380. https://doi.org/10.1016/j.micpath.2010.06.013.

Fang, W., Leng, B., Xiao, Y., Jin, K.M., Fan, Y., Feng, J., Yang, X., Zhang, Y., Pei, Y., 2005. Cloning of *Beauveria bassiana* chitinase gene Bbchit1 and its application to improve fungal strain virulence. Appl. Environ. Microbiol. 71, 363–370. https://doi.org/10.1128/AEM.71.1.363.

Faria, M., Lopes, R.B., Souza, D.A., Wraight, S.P., 2015. Conidial vigor vs. viability as predictors of virulence of entomopathogenic fungi. J. Invertebr. Pathol. 125, 68–72. https://doi.org/10.1016/j.jip.2014.12.012.

Federici, B.A., 1999. A perspective on pathogens as biological control agents for insect pests. In: Bellows, T., Fisher, T. (Eds.), Handbook of Biological Control Principles and Applications of Biological Control. Academic Press, San Diego, pp. 517–548. doi: 10.1016/B978-012257305-7/50065-5.

García, J., Sotelo, P., Monroy, D., Barrera, G., Gómez-Valderrama, J., Espinel, C., Barreto, E., Villamizar, L.F., 2018. Identification and characterization of a *Beauveria bassiana* (Bals.) Vuill. isolate having a high potential for the control of the Diatraea sp. sugarcane stem borer. BiotecnolApl 35, 1201–1207.

García-fruitós, E., González-montalbán, N., Morell, M., Vera, A., Ferraz, R.M., Arís, A., Ventura, S., Villaverde, A., 2005. Aggregation as bacterial inclusion bodies does not imply inactivation of enzymes and fluorescent proteins. Microb. Cell Fact. 4, 1–6. https://doi.org/10.1186/1475-2859-4-27.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M., Appel, R., Bairoch, A., 2005. Protein identification and analysis tools on the ExPASy Server. In: Walker, J.M. (Ed.), The Proteomics Protocols Handbook. Humana Press, pp. 571–607.

Hamid, R., Khan, M.A., Ahmad, M., Ahmad, M.M., Abdin, M.Z., Musarrat, J., Javed, S., 2013. Chitinases: an update. J. Pharm. BioAllied Sci. 5, 21–29. https://doi.org/10.4103/0975-7406.106559.

Han, B., Zhou, K., Li, Z., Sun, B., Ni, Q., Meng, X., Pan, G., Li, C., Long, M., Li, T., Zhou, C., Li, W., Zhou, Z., 2016. Characterization of the first fungal glycosyl hydrolase family 19 chitinase (NbchiA) from *Nosema bombycis* (Nb). J. Eukaryot. Microbiol. 63, 37–45. https://doi.org/10.1111/jeu.12246.

Haseeb, M., Srivastava, R., 2014. Potencial of entomopathogenic fungi in bio-management insect pest. In: Sharma, N. (Ed.), Biological controls for preventing food deterioration: estrategies for pre - and postharvest manangement. John Wiley y Sons Ltd., pp. 163–182.

ICA, 2011a. Silenciosa batalla contra el barrenador de la caña Diatraea saccharalis [WWW Document]. URL http://www.ica.gov.co/Noticias/Agricola/2011/Silenciosa-batalla-contra-el-barrenador-de-la-cana.aspx (accessed 9.14.16).

ICA, 2011b. Resolución 00698 de 2011. ICA, Colombia.

Ike, M., Nagamatsu, K., Shioya, A., Nogawa, M., Ogasawara, W., Okada, H., Morikawa, Y., 2006. Purification, characterization, and gene cloning of 46 kDa chitinase (Chi46) from *Trichoderma reesei* PC-3-7 and its expression in *Escherichia coli*. Appl. Microbiol. Biotechnol. 71, 294–303. https://doi.org/10.1007/s00253-005-0171-y.

Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A.F., Sangrador-Vegas, Amaia Scheremetjew, M., Yong, S.-Y., Lopez, R., Hunter, S., 2014. InterProScan 5: genome-scale protein function classification. Bioinformatics. doi: 10.1093/bioinformatics/btu031.

Kall, L., Krogh, A., EL, S., 2007. Advantages of combined transmembrane topology and signal peptide prediction–the Phobius web server. Nucl. Acids Res. Jul, W429-32.

Kim, J., Roh, J., Choi, J., Wang, Y., Shim, H., Je, Y., 2010. Correlation of the aphicidal activity of *Beauveria bassiana* SFB-205 supernatant with enzymes. Fungal Biol. 114, 120–128. https://doi.org/10.1016/j.mycres.2009.10.011.

Krogh, A., Larsson, È., Heijne, G. Von, Sonnhammer, E.L.L., 2001. Predicting transmembrane protein topology with a hidden markov model: application to complete genomes. J. Mol. Biol. 305, 567–580. https://doi.org/10.1006/jmbi.2000.4315.

Kuttiyawong, K., Nakapong, S., Pichyangkura, R., 2008. The dual exo/endo-type mode and the effect of ionic strength on the mode of catalysis of chitinase 60 (CHI60) from *Serratia* sp. TU09 and its mutants. Carbohydr. Res. 343, 2754–2762. https://doi.org/10.1016/j.carres.2008.05.020.

Lacey, L.A., Frutos, R., Kaya, H.K., Vail, P., 2001. Insect pathogens as biological control agents: do they have a future? Biol. Control 21, 230–248. https://doi.org/10.1006/bcon.2001.0938.

Laemmli, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lai, Y., Chen, H., Wei, G., Wang, G., Li, F., Wang, S., 2017. *In vivo* gene expression profiling of the entomopathogenic fungus *Beauveria bassiana* elucidates its infection stratagems in *Anopheles* mosquito. Sci. China Life Sci. 60, 839–851. https://doi.org/10.1007/s11427-017-9101-3.

Lastra, B., Gómez, L.a., 2006. La cría de *Diatraea saccharalis* (F.) para la producción masiva de sus enemigos naturales. Ser. Técnica 36, 30.

Liu, D., Cai, J., Xie, C.chu, Liu, C., Chen, Y.Hua, 2010. Purification and partial characterization of a 36-kDa chitinase from *Bacillus thuringiensis* subsp. *colmeri,* and its biocontrol potential. Enzyme Microb. Technol. 46, 252–256. https://doi.org/10.1016/j.enzmictec.2009.10.007.

Liu, Hongxia, Zhao, X., Guo, M., Liu, Hui, Zheng, Z., 2015. Growth and metabolism of *Beauveria bassiana* spores and mycelia. BioMedCentral Microbiol. 15, 267. https://doi.org/10.1186/s12866-015-0592-4.

Maina, U.M., Galadima, I.B., Gambo, F.M., Zakaria, D., 2018. A review on the use of entomopathogenic fungi in the management of insect pests of field crops. J. Entomol. Zool. Stud. 6, 27–32.

Mamarabadi, M., Jensen, B., Lübeck, M., 2008. Three endochitinase-encoding genes identified in the biocontrol fungus *Clonostachys rosea* are differentially expressed. Curr. Genet. 54, 57–70. https://doi.org/10.1007/s00294-008-0199-5.

Marín, P., Posada, F., Gonzalez, M.teresa, Bustillo, A.E., 2000. Calidad biológica de formulaciones de Beauveria bassiana usadas en el control de la broca del café. Rev. Colomb. Entomol. 26, 17–23.

Mejia, C., 2018. Potenciación de conidios de Beauveria bassiana como principio activo de un bioplaguicida para el control de larvas de Diataraea spp. Universidad Nacional de Colombia.

Merzendorfer, H., 2003. Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. J. Exp. Biol. 206, 4393–4412. https://doi.org/10.1242/jeb.00709.

Nguyen, L., Bodiroga, D., Kelemen, R., Joo, J., Gwinn, K.D., 2010. Modeling the effects of cymene on the distribution of germination and growth of Beauveria bassiana.

Ni, H., Zeng, S., Qin, X., Sun, X., Zhang, S., Zhao, X., Yu, Z., Li, L., 2015. Molecular docking and site-directed mutagenesis of a Bacillus thuringiensis chitinase to improve chitinolytic, synergistic lepidopteran-larvicidal and nematicidal activities. Int. J. Biol. Sci. 11, 304–315. https://doi.org/10.7150/ijbs.10632.

Okongo, R.N., Puri, A.K., Wang, Z., Singh, S., Permaul, K., 2018. Comparative biocontrol ability of chitinases from bacteria and recombinant chitinases from the thermophilic fungus *Thermomyces lanuginosus*. J. Biosci. Bioeng. https://doi.org/10.1016/j.jbiosc.2018.11.007.

Ortiz, A., Keyhani, N.O., 2016. Molecular genetics of *Beauveria bassiana* infection of insects. Adv. Genet. 94, 165–249. https://doi.org/10.1016/bs.adgen.2015.11.003.

Pedrini, N., Ortiz-urquiza, A., Huarte-bonnet, C., Zhang, S., Dispirito, A.A., State, O., 2013. Targeting of insect epicuticular lipids by the entomopathogenic fungus *Beauveria bassiana*: hydrocarbon oxidation within the context of a host-pathogen interaction. Front. Microbiol. 4, 1–18. https://doi.org/10.3389/fmicb.2013.00024.

Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786.

Pierleoni, A., Martelli, P.L., Fariselli, P., Casadio, R., 2007. BaCelLo: a Balanced subCellular Localization predictor. Protoc. Exch.

Prakash, M., 2008. Insect biochemistry, in: Encyclopedia of Entomology Vol 1. Discovery publishing house PVT. Ltd., New Delhi, pp. 36–37.

Rao, R., Fiandra, L., Giordana, B., De Eguileor, M., Congiu, T., Burlini, N., Arciello, S., Corrado, G., Pennacchio, F., 2004. AcMNPV ChiA protein disrupts the peritrophic membrane and alters midgut physiology of *Bombyx mori* larvae. Insect Biochem. Mol. Biol. 34, 1205–1213. https://doi.org/10.1016/j.ibmb.2004.08.002.

Regev, A., Keller, M., Strizhov, N., Sneh, B., Prudovsky, E., Chet, I., Ginzberg, I., Koncz-kalman, Z., Koncz, C., Schell, J., Zilberstein, A., Planck, M., 1996. Synergistic activity

of a *Bacillus thuringiensis* δ-endotoxin and a bacterial endochitinase against spodoptera littoralis larvae. Appl. Environ. Microbiol. 62, 3581–3586.
Restrictions on Genetically Modified Organisms, 2014.
Salvador, R., Ferrelli, M.L., 2014. Analysis of a chitinase from EpapGV, a fast killing betabaculovirus. Virus Genes 48, 406–409. https://doi.org/10.1007/s11262-013-1019-7.
Salvador, R., 2010. Caracterización biológica y molecular de genes involucrados en la virulencia de baculovirus de importancia agronómica. Universidad Nacional de la Plata.
Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9 (7), 671–675.
Seidl, V., 2008. Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions. Fungal Biol. Rev. 22, 36–42. https://doi.org/10.1016/j.fbr.2008.03.002.
Seidl, V., Huemer, B., Seiboth, B., Kubicek, C.P., 2005. A complete survey of *Trichoderma* chitinases reveals three distinct subgroups of family 18 chitinases. FEBS J. 272, 5923–5939. https://doi.org/10.1111/j.1742-4658.2005.04994.x.
Shternshis, M., Ovchinnikova, L., Duzhak, A., Tomilova, O., 2002. The efficiency of viral and bacterial entomopathogens formulated with chitinase for biocontrol of lepidopteran cabbage pests. Arch. Phytopathol. Plant Prot. 35, 161–169. https://doi.org/10.1080/03235400214209.
Shternshis, M.V, 2004. Ecologically safe control of insect pest : The past, the present and the future, in: Lartey, R.T., Caesar, A. (Eds.), Emerging Concepts in Plant Health Management. Research Signpost, Kerala, India. doi: 10.13140/2.1.3354.4648.
St Leger, R.J., Screen, S., 2001. Prospects for strain improvement of fungal pathogens of insects and weeds. In: Butt, T. M., Jackson, C., Magan, N. (Ed.), Fungi as Biocontrol Agents: Progress, Problems and Potential. CABI Publishing, New York, pp. 219–238. doi: 10.1079/9780851993560.0219.
St. Leger, R.J., Cooper, R.M., Charnley, A.K., 1991. Characterization of chitinase and chitobiase produced by the entomopathogenic fungus Metarhizium anisopliae. J. Invertebr. Pathol. 58, 415–426. doi: 10.1016/0022-2011(91)90188-V.
Svedese, V.M., de Lima, E.Á. de L.A., Porto, A.L.F., 2013. Horizontal transmission and effect of the temperature in pathogenicity of *Beauveria bassiana* against *Diatraea saccharalis* (Lepidoptera: Crambidae). Brazilian Arch. Biol. Technol. 56, 413–419. https://doi.org/10.1590/S1516-89132013000300009.
Takaya, N., Yamazaki, D., Horiuchi, H., Ohta, A., Takagi, M., 1998. Oligosporus: molecular cloning and characterization. Mycrobiology 144, 2647–2654.
Tanada, Y., Kaya, H.K., 1993. Insect Pathology. Academic Press, San Diego.
Terwisscha van Scheltinga, A.C., Kalk, K.H., Beintema, J.J., Dijkstra, B.W., 1994. Crystal structures of hevamine, a plant defence protein with chitinase and lysozyme activity, and its complex with an inhibitor. Structure 2, 1181–1189. https://doi.org/10.1016/S0969-2126(94)00120-0.
Thomas, C.J., Gooday, G.W., King, L.A., Possee, R.D., 2000. Mutagenesis of the active site coding region of the *Autographa californica* nucleopolyhedrovirus chiA gene. J. Gen. Virol. 81, 1403–1411. https://doi.org/10.1099/0022-1317-81-5-1403.
Valero, C.A., Wiegers, H., Zwaan, B.J., Koenraadt, C.J.M., van Kan, J.A.L., 2016. Genes involved in virulence of the entomopathogenic fungus *Beauveria bassiana*. J. Invertebr. Pathol. 133, 41–49. https://doi.org/10.1016/j.jip.2015.11.011.
Vallejo, L.F., Rinas, U., 2004. Strategies for the recovery of active proteins through refolding of bacterial inclusion body proteins. Microb. Cell Fact. 3, 1–12. https://doi.org/10.1186/1475-2859-3-11.
Ventura, S., Villaverde, A., 2006. Protein quality in bacterial inclusion bodies. TRENDS Biotechnol. 24. https://doi.org/10.1016/j.tibtech.2006.02.007.
Xiao, G., Ying, S.-H., Zheng, P., Wang, Z.-L., Zhang, S., Xie, X.-Q., Shang, Y., St Leger, R.J., Zhao, G.-P., Wang, C., Feng, M.-G., 2012. Genomic perspectives on the evolution of fungal entomopathogenicity in *Beauveria bassiana*. Sci. Rep. 2, 483. https://doi.org/10.1038/srep00483.
Yamaguchi, H., Miyazaki, M., 2014. Refolding Techniques for recovering biologically active recombinant proteins from inclusion bodies 235–251. https://doi.org/10.3390/biom4010235.
Yang, S., Song, S., Yan, Q., Fu, X., Jiang, Z., Yang, X., 2014. Biochemical characterization of the first fungal glycoside hydrolyase family 3 β-N-acetylglucosaminidase from *Rhizomucor miehei*. J. Agric. Food Chem. 62, 5181–5190. https://doi.org/10.1021/jf500912b.
Zar, J., 1999. Biostatistical analysis, cuarta Ed. ed. New Jersey.
Zhang, Y.-J., Feng, M.-G., Fan, Y.-H., Luo, Z.-B., Yang, X.-Y., Wu, D., Pei, Y., 2008. A cuticle-degrading protease (CDEP-1) of *Beauveria bassiana* enhances virulence. Biocontrol Sci. Technol. 18, 551–563. https://doi.org/10.1080/09583150802082239.
Zhao, H., Lovett, B., Fang, W., Microbiolog, C.D.E., Hubbard, M., Hynes, R.K., Erlandson, M., Bailey, K.L., Federici, B.A., 2016. Genetically engineering entomopathogenic fungi. Handb. Biol. Control Princ. Appl. Biol. Control 94, 137–163. doi: 10.1016/B978-012257305-7/50065-5.
Zúñiga-oviedo, M.A., Soto-Giraldo, A., Cruz-Ceron, G., 2016. Actividad biológica de hongos y bacterias entomopatógenas sobre *Diatraea saccharalis* Fabricius (Lepidoptera: crambidae). Bol. científico Mus. Hist. Nat. 20, 82–92.