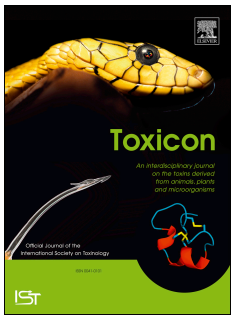
Accepted Manuscript



Expression of an scFv antibody fragment in *Nicotiana benthamiana* and *in vitro* assessment of its neutralizing potential against the snake venom metalloproteinase BaP1 from *Bothrops asper*

Marinna Gomes, Maria Alejandra Alvarez, Leonardo Ramos Quellis, Melina Laguia Becher, Juciane Maria de Andrade Castro, Jacy Gameiro, Maria Cristina Caporrino, Ana Maria Moura-da-Silva, Marcelo de Oliveira Santos

PII: S0041-0101(19)30052-2

DOI: https://doi.org/10.1016/j.toxicon.2019.02.011

Reference: TOXCON 6070

To appear in: *Toxicon*

Received Date: 11 September 2018

Revised Date: 24 January 2019

Accepted Date: 13 February 2019

Please cite this article as: Gomes, M., Alvarez, M.A., Quellis, L.R., Becher, M.L., Maria de Andrade Castro, J., Gameiro, J., Caporrino, M.C., Moura-da-Silva, A.M., de Oliveira Santos, M., Expression of an scFv antibody fragment in *Nicotiana benthamiana* and *in vitro* assessment of its neutralizing potential against the snake venom metalloproteinase BaP1 from *Bothrops asper*, *Toxicon*, https://doi.org/10.1016/j.toxicon.2019.02.011.

Expression of an scFv antibody fragment in *Nicotiana benthamiana* and *in vitro* assessment of its neutralizing potential against the snake venom metalloproteinase BaP1 from *Bothrops asper*

Marinna Gomes[a], Maria Alejandra Alvarez[b], Leonardo Ramos Quellis[a], Melina Laguia Becher[b], Juciane Maria de Andrade Castro[c], Jacy Gameiro[c], Maria Cristina Caporrino[d], Ana Maria Moura-da-Silva[d], Marcelo de Oliveira Santos[a*].

[a]Laboratorio de Genética, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, Rua José Lourenço Kelmer, S/n - Martelos, Juiz de Fora - MG, 36036-330

[b]CONICET-Universidade Maimónides (CEBBAD)– Hidalgo 775, lab 603, Buenos Aires - Argentina

[c]Laboratorio de Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, Rua José Lourenço Kelmer, S/n - Martelos, Juiz de Fora - MG, 36036-330

[d]Laboratorio de Imunopatologia, Instituto Butantan, Av. Vital Brazil, 1500, Butantã, CEP 05503-900 São Paulo, SP, Brazil

* Corresponding author e-mail: marcelo.santos@ufjf.edu.br

ABSTRACT

Human accidents with venomous snakes represent an overwhelming public health problem, mainly in rural populations of underdeveloped countries. Their high incidence and the severity of the accidents result in 81,000 to 138,000 deaths per year. The treatment is based on the administration of purified antibodies, produced by hyper immunization of animals to generate immunoglobulins (Igs), and then obtained by fractionating hyper immune plasma. The use of recombinant antibodies is an alternative to conventional treatment of snakebite envenoming, particularly the Fv fragment, named the single-chain variable fragment (scFv). We have produced recombinant single chain variable fragment scFv against the venom of the pit viper *Bothrops asper* at high levels expressed transiently and stably in transgenic plants and *in vitro* cultures that is reactive to BaP1 (a metalloproteinase from *B. asper* venom). The yield from stably transformed plants was significantly ($p > 0.05$) higher than the results in from transient expression. In addition, scFvBaP1 yields from systems derived from stable transformation were: transgenic callus 62 µg/g (± 2); biomass from cell suspension cultures 83 µg/g (± 0.2); culture medium from suspensions 71.75 mg/L (± 6.18). The activity of scFvBaP1 was confirmed by binding and neutralization of the fibrin

degradation induced by BnP1 toxins from *B. neuwiedi* and by Atroxlysin Ia from *B. atrox* venoms. In the present work, we demonstrated the potential use of plant cells to produce scFvBaP1 to be used in the future as a biotechnological alternative to horse immunization protocols to produce anti-venoms to be used in human therapy against snakebites.

Key words: *Bothrops,* Molecular Farming, scFv, BaP1, i*n vitro* plant cultures, heterologous expression.

Financial Support

"This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001"

Abbreviations list:

ADAM - A Disintegrin and Metalloprotease;

BAP1 *Bothrops asper* metalloproteinase 1;

BnP1 – *Bothrops neuwiedi* metalloproteinase 1;

CTAB - Cetyltrimethylammonium bromide;

ELISA - Enzyme-Linked Immunosorbent Assay;

GFP – Green fluorescence protein;

MMA - MacConkey agar;

PCR – Polymerase chain reaction;

scFv – single chain variable fragment;

SVMP - snake venom metalloproteinase;

TCV-CP - CP - Coat protein - Turnip crinkle virus.

1 - INTRODUCTION

Accidents with snakes represent a public health issue, considering their high incidence and the severity of their effects. They are considered to be a neglected problem since they mostly affect the rural populations in underdeveloped countries from tropical areas such as Asia, Africa and Latin America (Sachett et al., 2017). According to the World Health Organization (WHO), epidemiological data on snake envenoming are estimated at between 1.8 and 2.7 million per year, resulting in 81,000 to 138,000 deaths (Laustsen et al., 2018). In South America, most of the envenoming events are caused by snakes of the genus *Bothrops*, namely, *B. asper* and *B. atrox* being included in the group of species with high medical importance (Gois et al., 2017).

At present, passive immunotherapy is the sole available treatment against ophidian accidents. The treatment is based on the administration of purified antibodies, produced by hyper immunization of animals, generally horses, with snake venoms (WHO, 2012). These anti-venoms are preparations of immunoglobulins (Igs), or Ig fragments such as $F(ab)_2$ or Fab, obtained by fractionating hyper immune plasma either by treatment with caprylic acid to obtain whole IgG preparations (Gutiérrez et al., 2005) or by enzymatic digestion followed by ammonium sulfate precipitation and chromatographic steps to obtain IgG fragments (WHO, 2010).

Anti-venoms are generally very efficient for neutralizing the most relevant systemic effects of snakebite envenoming. Therapeutic hindrances may be due to early onset of these effects, as well as to poor distribution of the Igs and their fragments to the local tissues where venom is injected (Gutiérrez et al., 1998). Furthermore, administration of anti-venom may be associated, in a variable percentage of cases, with early and late adverse reactions to the heterologous proteins (León et al., 2013).

An alternative to conventional treatment of snakebite envenoming is the use of recombinant monoclonal antibodies. Costs of production in CHO cultivation methods were compared leading economical basis to make a decision in which platform the anti-venom production should be built on (Lausten et al., 2017). The scFv antibody is another form of recombinant antibody for envenoming treatment (Lausten et al 2016a). Such molecules presents several distinctive features when compared to the whole antibody, such as higher diffusion to the affected tissues, low immunogenicity, faster elimination, and lower

immunocomplexes formation (Azzazy and Highsmith, 2002; Zhang et al., 2014; Yu et al., 2014).

Castro et al. (2014) has described the generation of a recombinant scFv against BaP1 (scFvBaP1) from the venom of the pit viper *B. asper,* a medically important species in Central America and regions of South and North America. BaP1 is an abundant P-I snake venom metalloproteinase (SVMP) in the venom of *B. asper*. This toxin plays a relevant role in the associated local tissue damage. The scFvBaP1 was produced from the mRNA isolated from the BaP1-8 monoclonal antibody producing cells (MABaP1-8), expressed in *Escherichia coli* cytoplasm, and possesses neutralizing activities similar to those of the original monoclonal antibody. In this respect, the fragment was able to recognize the Bap1 toxin present in the venom of *B. asper* and neutralize its hemorrhagic, fibrinolytic, myotoxic and displayed pro-inflammatory properties (Castro et al., 2014). However, the main disadvantage of that scFvBaP1 was its low yield, which made its large-scale pharmaceutical production unfeasible.

Plant systems stand as an alternative platform to produce pharmaceutical interest proteins. When compared to other recombinant protein production systems, plants should be highlighted due to some advantages, such as easy genetic manipulation, high biomass yields, post-translational modifications, low production cost, and possibility of production scheduling through the use of industrial bioreactors (Moussavou et al., 2015; Yao et al., 2015; Juarez et al., 2016; Besufekad; Malaiyarsa, 2017), although the glycosylation patterns still are a challenge to be overcome for therapeutic uses (Montero-Morales et al., 2017). An interesting remark is that the first plant-made therapeutic drug for human use was approved by the Food and Drug Administration (FDA) in 2012 (Fox, 2012) and that over 16 plant-manufactured proteins in phase I, II, and III clinical trials are in progress (Sack et al 2015). The venom of the snake *B. asper* was successfully used to transiently produce a cocktail of antibodies in a *N. benthamiana* based model, a more in depth preclinical trial should be done before its use as a biopharmaceutical (Julve Parrenõ et al., 2018). Although the plantibody technology would be far to be commercially viable its potential have being demonstrated.

Herein we describe high production levels of a recombinant single chain antibody fragment (scFv) deduced and modified from scFvBaP1 (Castro et al., 2014), expressed transiently and stably in transgenic plants and *in vitro* cultures (callus and suspension cells). The antibodies produced by *N. benthamiana* cells possess neutralizing activities similar to

those of the original scFvBaP1 antibody. In addition to its potential, we propose its use as a molecular tool to assess function of SVMP present in venoms of different snakes.

2 - MATERIALS AND METHODS

2.1 - Venoms and enzymes

The Herpetology Laboratory of Instituto Butantan provided *Bothrops neuwiedi* and *B. atrox* venoms. The venoms corresponded to pools obtained from many specimens and were lyophilized and stored at -20°C. The SVMPs BnP1 and Atroxlysin Ia were purified as previously described by Baldo et al. (2008) and by Freitas-de Sousa et al. (2017).

2.2 - Cloning the expression vector

We used the amino acid sequence of the fragment scFvBaP1 (Castro et al., 2014) as template to optimize the nucleotide sequence by reverse genetics for its expression in *Nicotiana benthamiana*. We used the optimization tool in Integrated DNA Technologies (IDT- https://www.idtdna.com/site) to determine the coding sequence and digestion sites. After the synthesis, the fragment was digested with the restriction enzymes *NcoI* and *XhoI* and cloned into the pENTR4® vector (Invitrogen). The ligation product was inserted by electroporation into *Escherichia coli* Top 10 *One Shot* and the transformed cells were selected with 50mg/L kanamycin. The assembly of the expression vector occurred from the LR cloning of the target vector pK7WG2D with the cloning vector, in the presence of the enzyme LR clonase (Invitrogen) selected under 50mg/L of spectinomycin in *E. coli* top 10.

2.3 - Transient Expression

For transient expression of the fragment, we used 6 week-old *N. benthamiana* plants, cultivated in plastic vessel filled with a mixture of autoclaved substrate Plantmax ® and sand (1:1) placed in growing chamber at 25 ± 2°C under a 16 h photoperiod.

The *Agrobacterium* GV3101 strain was grown in Lysogeny Broth (LB) medium (5 mL), the expression vector was inserted in *Agrobacterium tumefaciens* GV3101 and cultivated under 50 mg/L of spectinomycin, 10 mg/L rifampicin and 30 mg/L of gentamicin.

The pre inoculum was maintained at 28° C under agitation of 210 rpm for 24 h. 100 µl of bacterial suspension was transferred to 7.5 ml of LB medium containing the appropriate antibiotics plus acetosyringone 20 µM and MES 10 mM, pH 5.6. The same procedure was performed with *A. tumefaciens* EHA101 transformed with plasmid pPZP212 containing the anti-silencer TCV-CP (capsid protein from Turnip Crinkle Virus). Bacterial suspensions were centrifuged at 5,000 rpm for 15 minutes at room temperature. The pellet was then suspended in 20 mL of MMA medium (5 g/L) + MES (1.95 g/L), sucrose (20 g/L), acetosyringone (200 µM), pH = 5.6 incubated at 28^{o} C during 3 h without stirring until they reached $O.D_{600}$ (0.4-0.8). Subsequently, *N. benthamiana* leaves were infiltrated with that suspension using 1 ml syringes. The leaves were collected between 3-9 days post agroinfiltration (dpi), and the expression of the GFP reporter gene was monitored under UV illumination at 480 nm. Expression of the scFvBaP1 fragment was detected by Dot Blotting immunoassay using the rabbit primary antibody anti-HIS (1:1000).

2.4 - Stable Transformation

In order to achieve the stable transformation, *N. benthamiana* leaf explants from *in vitro* propagated plants were co-cultivated with agrobacteria suspension. Briefly, the *A. tumefaciens* GV3101 strain was grown in LB medium (5 mL), containing the expression vector as previously described. Then, the pre inoculum was maintained at 28 °C under agitation of 150 rpm for 16 h. Finally, the bacterial suspension was adjusted to $O.D_{600}$ 0.6 and was co-cultivated with *N. benthamiana* leaves during 15 minutes in MS liquid medium according to the protocol described by Hallwass et al. (2014). Afterwards, to select the cells that have received the transgene, we used a selection medium composed by 4.4 g/L of Murashige & Skoog Medium (MS) (Sigma basal medium with Gamborg vitamins) (Murashige and Skoog, 1962), 20 g/L glucose, 7 g/L agar, and 50 mg/L of Kanamycin, 1mg/L of Benzyl amino purine (BAP) at pH 5.8 during 28 days when the transgenic the shoots emerged. These shoots were transferred to rooting media (MS without growth regulators plus 50 mg/L of Kanamycin) and propagated *in vitro*.

2.5 - PCR analysis

Total DNA was extracted from the leaves of plants that survived and were rooted on selection media, based on the protocol described by CTAB modified method (Porebski et al., 1997). In order to confirm the stable transformation of *N. benthamiana* with the scFvBaP1 gene, a polymerase chain reaction was performed using the enzyme Easy Taq DNA polymerase (LGC Biotechnology), according to the manufacturer's recommendations. The primers used for fragment amplification were, respectively, F 5 'TACCCGGGGATCCTCTAGAG and R 5'CGGCCGCACTAGTGATACA flanking transfer-DNA border.

2.6 - Callus induction and cell suspensions

Leaf explants of $1cm^2$ from three transgenic plants lineages were placed in Petri plates containing 20 mL of MS medium (4.4 g/L MS basal medium, 30 g/L sucrose and 7 g/L Agar) supplemented with 0.5 mg/L 2,4- dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/L kinetin to induce *callus* development. The explants were sub-cultured to fresh media every 14 days during six weeks. The plates were kept in a growing room at $25^oC \pm 2$, under a photoperiod of 16h. After six weeks of culture, 100 mg from each friable *calli* were separately transferred to 250 mL Erlenmeyer containing 50 mL of the same culture medium used for callus induction but without agar. The suspensions were maintained in a shaker table at 120 rpm for 8 days, at the same temperature and light conditions described before. Then, each suspension in triplicate were collected and centrifuged for 10 minutes at 2,000, 4 °C to obtain the fractions (precipitate and supernatant) that were weighted and separated for further protein analysis and productivity.

2.7 - Purification of the recombinant scFvBaP1

The total protein of the extract obtained from the infiltrated leaves were concentrated using the concentration column Amicon®Ultra (Millipore), the columns were centrifuged at 4,000 rpm for 30 minutes at 4 °C. After centrifugation, the concentrate was applied in a HisTrapHP 5mL (GE Healthcare) column. Purification was performed following the instructions of manufacturers, using for elution a gradient of imidazole concentration (80 mM, 100 mM, 250 mM, and 500 mM). After standardization of the best imidazole concentration (500 mM) for elution of the scFvBaP1 fragment, the following samples were selected: 1. total

extract from leaves of transgenic plants; 2. total extract from callus induced from transgenic plants; 3. total extract from cell suspension biomass (precipitate); 4. total extract from cell suspension culture medium (supernatant). In order to remove the excess of imidazole used in the elution, the samples were dialyzed using Amicon® Ultra (Millipore) concentration columns. The columns were centrifuged in a refrigerated centrifuge (4 ° C) for 20 min / 4,000 rpm. The purified proteins were stored at 4 °C. The concentration of proteins obtained in each of the samples was determined by the Lowry et al. (1951) dosing method. The proteins were analyzed in SDS – PAGE 10% and their expression was detected by the western blotting technique using the rabbit primary antibody Anti-His (1:1000).

2.8 - Ability of scFvBaP1 to recognize SVMPs BnP1, Atroxlysin Ia and whole *B. asper* venom, by ELISA

We employed the Enzyme-Linked Immunosorbent Assay (ELISA) in order to evaluate the specificity of the scFvBaP1 fragment. Polystyrene plates were previously sensitized with 2 μg/mL of *B. asper* total venom, BnP1 toxin (*B. neuwiedi*) and Atroxlysin Ia toxin (*B. atrox*) and subsequently incubated at 4 °C overnight. Afterwards, they were washed 3 times with PBS/0.05 % Tween, blocked with 3 % BSA for 2 h at 37°C, and washed again with the same solution. Then, the scFvBaP1 fragment totalizing 100 μg was added to the wells (2 μg/well) and the plates incubated for 1h at 37°C. The antigen-antibody reaction was detected using anti-His rabbit peroxidase followed by the substrate ortho-phenylenediamine (OPD) and H_2O_2. The reaction was quenched with 50 μL of H_2SO_4 and the absorbance read at 492 nm in Varioskan system (Thermo ®).

2.9 - Neutralization of fibrinolytic activity of BnP1, Atroxlysin Ia and whole *B. asper* venom by scFvBaP1 antibodies

Fibrinolytic activity was assayed by the fibrin-plate method as used by Castro et al., (2014). Briefly, a fibrin agarose gel was prepared by mixing 1 mg/mL solution of human fibrinogen (Calbiochem) with a pre-heated solution of 2 % agarose in 50 mM of Tris-HCl, pH 7.3, buffer containing 200 mM of NaCl, 50 mM of $CaCl_2$ and 2 U/mL of thrombin. The samples (5 μg- total venom *B. asper*, BnP1 toxin, Atroxlysin Ia toxin) were incubated with the fragment scFvBaP1 (20:1) for 15 minutes at 37°C. Finally, the samples were applied to

wells pierced in the solidified gel. Plates were incubated at 37° C overnight, and then the area of fibrin hydrolysis was measured. The results were expressed in mm^2 of fibrinolytic area.

2.10 - Statistical analysis

The significance of the differences of two mean values was analyzed by the Student's t-test. When more than two experimental groups were compared, the significance of the differences was determined by ANOVA, followed by Tukey test (p values < 0.05 were considered significant).

3 - RESULTS

3.1 - Transient expression

The expression of the GFP reporter gene was monitored during nine days being its expression visible from 3 to 9 days after infiltration (DPI). The leaves infiltrated with the genomic silencing suppressor (TCV-CP) apparently showed GFP more green spots (Figure 1 B, D) but the recombinant protein yield was not different. In the Dot-Blotting immunoassay the expression of the scFvBaP1 fragment has become detectable only at the third day following the agro-infiltration (Figure 2). The expression of the scFvBaP1 fragment was observed both in the presence and in the absence of the suppressor TCV-CP.

3.2 - Stable transformation

The PCR from the regenerated explants demonstrated that the stable transformation of *N. benthamiana* was successful (Figure 3). Three transgenic plants (P2, P3 and P4) with detectable expression were obtained from three different explants of the same transformation experiment. The transformed plants showed in Western blotting assay from the total extracts detected the expression of the scFvBaP1 fragment.

The band corresponding to the scFvBap1 fragment were shown at ~ 58kDa, thus suggesting the formation of dimers during the electrophoresis process (Figure 4).

3.3 - Purification of the recombinant scFvBaP1

A trial for Imidazole purification was carried out (Figure 5). The total yielding of the scFvBap1 fragment by the agro-infiltration method was 43 µg/g (± 0.7) from agro-infiltrated fresh leaf seven days post infiltration (dpi). The statistical analysis from the yield of the two treatments (presence and absence of the gene silencing suppressor TCV-CP) did not show significant difference (Figure 6). The Western Blotting analysis demonstrated the presence of the scFvBaP1 fragment in the purified samples, with the band at approximately 58 kDa. The yield obtained from fresh leaves from transgenic plants was 270 µg/g (± 5) 15 days after sub-culture. The statistical analysis comparing the total production yield of the two production methods (transient and stable) showed a significant difference (Figure 7). The yield from stably transformed plants was significantly ($p > 0.05$) higher than the results in from transient expression. Additionally, scFvBaP1 yields from systems derived from stable transformation were: transgenic callus 62 µg/g (± 2); biomass from cell suspension cultures 83 µg/g (± 0.2); culture medium from suspensions 71.75 mg/L (± 6.18) eight days after culture (Table 1). The analysis demonstrated that the suspension culture medium presented higher scFv yields and productivity. As expected, the scFvBaP1 protein, after being produced was exported to the culture medium since the coding sequence was built with the signal sequence 2S2 from *Arabidopsis thaliana* that drives the protein to the secretory pathway.

3.4 - Ability of scFvBaP1 to recognize SVMPs BnP1, Atroxlysin Ia and whole *B. asper* venom, by ELISA

The ability of the scFvBaP1 fragment to recognize the venom toxins was demonstrated by the ELISA assay. As expected, the fragment was capable of recognizing the homologous BaP1 toxin, present in the total venom of *B. asper*, as well as the isolated BnP1 and Atroxlysin Ia toxins, two related P-I-class SVMPs isolated from venoms of other species of *Bothrops* snakes: *B. neuwiedi* and *B. atrox*, respectively. Greater recognition was observed for the BnP1 toxin, as shown in Figure 8.

3.5 - Neutralization of enzymatic activity of BnP1, Atroxlysin Ia and whole *B. asper* venom by scFvBaP1 antibodies

After the incubation, the scFvBap1 fragment was able to neutralize the fibrin degradation induced by total venon of *B. asper*. In addition, the scFvBap1 neutralized the

fibrinolysis caused by the homologous BaP1 toxin, BnP1 toxins of *B. neuwiedi* and by Atroxlysin Ia (ATX) from *B. atrox* venoms. By scoring the halos, we demonstrated that the scFvBaP1 fragment was stable for 24 hours, not allowing the expansion of the halos measured. Moreover, when scFvBaP1 was applied separately, the fragment did not cause any type of damage to the fibrin, also showing no significant differences in relation to the control (Figure 9).

4 - DISCUSSION

BaP1 is an enzyme that comprises approximately 10% of *B. asper* venom from the Pacific region of Costa Rica (Alape-Giron et al., 2009). BaP1 has low systemic toxicity (Escalante et al., 2004), but exerts significant local tissue damage associated with hemorrhage, myonecrosis (Rucavado et al., 1995), dermonecrosis and blistering, pain (Fernandeset al., 2007), and inflammation (Fernandes et al., 2006). Anti-venoms are relatively inefficient for controlling the extent of local tissue damage, including local hemorrhage and myonecrosis (Gutiérrez et al., 1998; Otero-Patino, 2009). This is likely to depend on several factors, such as rapid development of local pathology after the bite and poor access of anti-venom antibodies to the affected tissues. Thus, neutralization of venom-induced local tissue damage remains one of the most difficult challenges to be improved in the management of viper snakebite envenoming. To this end, the use of recombinant scFv fragments offers a promising alternative to improve treatment, mainly owing to the pharmacokinetic properties of such small molecular mass antibody fragments, which enable them to reach extravascular spaces in the affected tissues much more readily than whole IgG or its fragments. Some recent studies point to the scFv fragments as promising molecules in the recognition and neutralization of toxins present in viper snake venoms. Recombinant antibodies are promising for envenoming treatment since local lesions starts damages at hospital admission, thus smaller antibodies like scFv, FAB and VHH are able to rapidly reach target tissues such as neuromuscular junctions so as to neutralize toxins in circulation. Their larger volumes of distribution allow these smaller fragments to be highly effective (Lausten et al. 2018). Their potential has been demonstrated in the treatment of lesions caused by snakes (Laustsen et al., 2016; Pessenda et al. 2016). Lee et al. (2016) expressed scFv fragments capable of recognizing and neutralizing the effects of venoms of *Crotalus durissus*, *Bungarus multicinctus* and some snakes of the *Bothrops* genus. According to Laustsen et al. (2016), the

use of recombinant molecules in the treatment of ophidian accidents may help to solve this neglected public health problem. According to the author, such molecules tend to have a lower production cost compared to conventional antibodies and can be expressed in easily controllable systems having an effective action in a small amount.

The *E. coli*-expressed scFvBaP1 fragment, proposed by Castro et al. (2014), was shown to be effective against BaP1 and BnP1 toxins, isolated from *B. asper* and *B. neuwiedi* venoms, respectively. However, in this expression system scFvBaP1 yields were 280 μg/L of culture medium, which makes it impossible its use on an industrial scale. Therefore, in this work, we proposed the plant system as an alternative platform for producing the scFvBaP1 in which the production reached 8.96 mg/L/day in suspension cell culture.

Currently, within the panorama of plant systems, the most efficient platforms for the production of recombinant antibodies are tobacco leaves, either by transient or stable expression. The clinical trial using plant made antibodies may be starting a new era in protein production for pharmaceutical industries (Ma et al, 2015). When produced in leaves *N. benthamiana* protein yielding shows reduced alkaloid contamination and this plant species have been accepted by the U. S. Food and Drug Administration (FDA) as a safety system for antibody production (Stephan et al., 2017). In order to avoid degradation of recombinant proteins, some strategies have been adopted, such as the use of signal peptides targeting proteins to some cellular compartments, such as the apoplast or the endoplasmic reticulum, as reported by Goulet et al. (2010); Jutras et al. (2015); Robert et al. (2013). In this work, the recombinant antibodies were directed to the periplasmic space of the cells, in order to reduce their degradation. The targeting was performed with the addition of a signal peptide to the expression cassette. The transient expression, monitored for nine days, was detected after 3 dpi, as shown in the study by Pêra and collaborators (2015). The average yield of the scFvBaP1 fragment by transient expression was 43 μg/g (± 0.7). Some authors, such as Lacombe and collaborators (2018), reported a lower production of recombinant proteins by a plant system, which was associated to gene silencing and protein degradation produced by proteases by host plant (Grosse-Holtz et al., 2018). Another factor to be taken into account is the mechanism of gene silencing that tends to happen as a form of plant's response to infection, and drastically reduces the levels of recombinant protein expression (Rasool et al., 2016).

In an attempt to mitigate gene silencing on scFvBaP1, the TCV-CP viral suppressor was used; however, we observed that the suppressor did not increase the yield of the

scFvBaP1 fragment. The statistical analysis showed that there were no significant differences between both treatments. These data are contrary to those reported by Qu et al. (2003) working with the same viral suppressor. In that study, significant differences were found when co-infection was performed using viral suppressors.

Transgenic plants (stable-transformed) have shown to be promising and efficient in the production of proteins of therapeutic interest, including monoclonal antibodies and scFv fragments (Moussavou et al., 2015; Xu et al., 2016). The transgenic lines that we have obtained, besides integrating the transgene, were able to express the scFvBaP1 fragment, demonstrating its potential use as a biological reactor for its production. Many studies report the use of *N. benthamiana* species for the production of biopharmaceutical proteins (Marusic et al., 2016; Niemer et al., 2015; Alkanaimsh et al., 2016).

We obtained a total yield of 270 μg/g of fresh weight leaves (± 5) of scFvBaP1 in transgenic plants (stable-transformed). That yield was higher than in the transient production system. Since in the stable transformation the proteins were directed to the periplasmic space of the cells, they were protected from proteases, culminating in a higher concentration of proteins. These results demonstrate a greater efficiency of the stable transformation in relation to the transient expression and suggest a better efficiency of such a system of production, as it is reported in the review paper by Merlin et al. (2014). Furthermore, another advantage of the system is the possibility of obtaining cell suspensions (Yoshikawa, 2016) which not only are capable large-scale production, but they also guarantee a homogeneous, clean, and less expensive production, free from the action of interfering secondary metabolites (e.g. alkaloids) and overcoming the step of extraction from biomass by directing the secretion of proteins to the culture medium (Muthamilselvan et al., 2016). In this work, we obtained 83 μg/g (± 0.2) of scFvBaP1 in the biomass and 8.96 mg/L/day (± 0.77) in the culture medium. As the protein concentration in the culture medium was significantly higher ($p > 0.05$) than the concentration of proteins found in the biomass, it is clear that the produced antibodies are being released into the culture medium.

Furthermore, the production yields of this system can be improved by adjusting the culture conditions, such as culture medium composition, luminosity, CO_2 concentration, among others. These parameters can be adjusted with the use of semi-industrial bioreactors, which allow controlling the culture conditions over a period of time, optimizing the protein production both quantitatively and also qualitatively (Magy et al., 2014).

In summary, we have initially transiently expressed the scFvBaP1 fragment in leaves of *N. benthamina*, with a final yield of 43 μg/g (± 0.7) of infiltrated leaf. Additionally, three transgenic plant lines capable of expressing the scFvBap1 fragment were obtained, totaling a production yield of 270 μg/g (± 5).

Moreover, its biological activity against BaP1, BnP1 and Atroxlysin Ia toxins was verified by the ELISA assay. The scFvBaP1 fragment was able to recognize the BaP1 toxin present in the total venom of *B. asper* and also the isolated toxins BnP1 and Atroxlysin Ia. There was a greater affinity between the scFvBaP1 fragment and the BnP1 toxin, which may be due to the fact that we employed the total venom of *B. asper* and not the isolated BaP1 toxin, such metalloproteinase corresponds to 10% of the total venom dry weight (Watanabe, 2003).

The ability of the scFvBaP1 fragment to neutralize the fibrinolytic activity of BaP1, BnP1 and Atroxlysin Ia toxins was also evaluated. Castro et al. (2014) demonstrated the neutralization of the fibrinolytic activity of the BaP1 toxin by the scFvBaP1 fragment produced in *E. coli*. The scFvBaP1 fragment, expressed in plants, not only recognized and inhibited the activity of the BaP1 toxin but also was able to recognize and inhibit the action of BnP1 and Atroxlysin Ia toxins. This recognition of toxins from different species can be attributed to the high phylogenetic conservation that the metalloproteinases present, as reported by Tanjoni et al. (2003). According to the authors, the metalloproteinases are extremely conserved among snake venoms and derive from common ancestor ADAM proteins, thus justifying the presence of common epitopes shared amongst different species of snakes.

Taking our results into account, we may suggest that the scFvBaP1 fragment may be used not only to inhibit the effects of the snakebite envenoming caused by *B. asper*, but also by other snakes. Possibly, such a fragment exhibits polyvalent recognition, thus demonstrating itself as a promising molecule to be used in therapy against ophidian accidents.

In conclusion, the plant systems showed a greater efficiency for producing the neutralizing scFvBaP1 antibody when compared to the prokaryotes production system. Additionally, the scFvBaP1 fragment was able to recognize and neutralize the fibrinolytic activity of BaP1, BnP1 and Atroxlysin Ia toxins. That observation demonstrates its potential uses to produce the antibody for serum therapy in future research and development for pre-clinical trials in cases of envenoming events caused by snakes *B. asper*, B. *neuwiedi* and *B. atrox*. Furthermore, this plant system produced-scFv antibody may become a useful tool for

exploring novel therapeutic alternatives for the neutralization of the local tissue damage induced by SVMPs abundant in venoms of snakes as *B. asper*, B. *neuwiedi* and *B. atrox*. Herein, we successfully describe the production of high levels of recombinant single chain antibody fragment – scFv by transgenic plants. Future studies are necessary to test the therapeutic potential of this scFv antibody in preclinical experimental models.

5 - REFERENCES

Alape-Girón A, Flores-Díaz M, Sanz L, Madrigal M, Escolano J, Sasa M, Calvete JJ. Studies on the venom proteome of Bothrops asper: Perspectives and applications. Toxicon, 2009;54: 938–948.

Alkanaimsh S, Karuppanan K, Guerrero A, Tu AM, Hashimoto B, Hwang MS, Phu ML, Arzola L, Lebrilla CB, Dandekar AM, Falk BW, Nandi S, Rodriguez RL, McDonald KA.Transient Expression of Tetrameric Recombinant Human Butyrylcholinesterase in Nicotiana benthamiana. Front Plant Sci, 2016;7:743.

Azzazy H ME.; Highsmith WE. Phage display technology : clinical applications and recent innovations. Clin Biochem, 2002;35: 425–445.

Baldo C, Tanjoni I, León IR, Batista IF, Della-Casa MS, Clissa PB, Weinlich R, Lopes-Ferreira M, Lebrun I, Amarante-Mendes GP, Rodrigues VM, Perales J, Valente RH, Moura-da-Silva AM. BnP1, a novel P-I metalloproteinase from Bothrops neuwiedi venom: Biological effects benchmarking relatively to jararhagin, a P-III SVMP. Toxicon, 2008;51:54–65.

Besufekad Y, Malaiyarsa P. Production of Monoclonal Antibodies in Transgenic Plants. J Adv Biol Biotechno, 2017;12:1-8.

Castro JM, Oliveira TS, Silveira CR, Caporrino MC, Rodriguez D, Moura-da-Silva AM, Ramos OH, Rucavado A, Gutiérrez JM, Magalhães GS, Faquim-Mauro EL, Fernandes I. A neutralizing recombinant single chain antibody, scFv, against BaP1, A P-I hemorrhagic metalloproteinase from Bothrops asper snake venom. Toxicon, 2014;87: 81–91.

Escalante T, Rucavado A, Kamiguti AS, Theakston RD, Gutiérrez JM. *Bothrops asper* metalloproteinase BaP1 is inhibited by a2- macroglobulin and mouse serum and does not

induce systemic hemorrhage or coagulopathy. Toxicon, 2004;43: 213-217.

Fernandes CM, Pereira Teixeira Cde F, Leite AC, Gutiérrez JM, Rocha FA. The snake venom metalloproteinase BaP1 induces joint hypernociception through TNF-a and PGE2-dependent mechanisms. Br. J. Pharmacol., 2007;151:1254-1261.

Fernandes CM, Zamuner SR, Zuliani JP, Rucavado A, Gutiérrez JM, Teixeira Cde F. Inflammatory effects of BaP1 a metalloproteinase isolated from *Bothrops asper* snake venom: leukocyte recruitment and release of cytokines. Toxicon, 2006;47: 549- 559.

Freitas-de-Sousa LA, Colombini M, Lopes-Ferreira M, Serrano SMT, Moura-da-Silva AM. Insights into the Mechanisms Involved in Strong Hemorrhage and Dermonecrosis Induced by Atroxlysin-Ia, a PI-Class Snake Venom Metalloproteinase. Toxins, 2017;9:pii: E239.

Fox JL. First plant-made biologic approved. 2012; Nat Biotech 30:472-472.

Gois PHF, Martines MS, Ferreira D, Volpini R, Canale D, Malaque C, Crajoinas R, GirardACC, Massola Shimizu MH, Seguro AC. Allopurinol attenuates acute kidney injury following Bothrops jararaca envenomation. PLoS Neglect Trop D, 2017 ;11:e0006024.

Goulet C, Benchabane M, Anguenot R, Brunelle F, Khalf M, Michaud D. A companion protease inhibitor for the protection of cytosol-targeted recombinant proteins in plants. Plant Biotechnol J, 2010;8:142–154.

Grosse-Holz F, Kelly S, Blaskowski S, Kaschani F, Kaiser M, van der Hoorn RAL. The transcriptome, extracellular proteome and active secretome of agroinfiltrated *N. benthamiana* uncover a large, diverse protease repertoire. Plant Biotechnol J. 2018;16(5):1068-1084.

Gutiérrez JM, León G, Rojas G, Lomonte B, Rucavado A, Chaves F. Neutralization of local tissue damage induced by Bothrops asper (terciopelo) snake venom. Toxicon, 1998 ;36:1529-1538.

Gutierrez, JM., Rojas, E., Quesada, L., Leon, G., Núnez, J., Laing, GD., Sasa, M., Renjifo, JM., Nasidi, A., Warrell, DA., Theakston, RD., Rojas, G. Pan-African polyspecific antivenom produced by caprylic acid purification of horse IgG: an alternative to the antivenom crisis in Africa. Trans. R. Soc. Trop. Med. Hyg. 2005;99, 468e475.

Hallwass M, de Oliveira AS, de Campos Dianese E, Lohuis D, Boiteux LS, Inoue-Nagata AK, Resende RO, Kormelink R. The Tomato spotted wilt virus cell-to-cell movement

protein (NSM) triggers a hypersensitive response in Sw-5-containing resistant tomato lines and in Nicotiana benthamiana transformed with the functional Sw-5b resistance gene copy. Mol Plant Pathol. 2014;15:871-880.

Juarez P, Virdi V, Depicker A, Orzaez D. Biomanufacturing of protective antibodies and other therapeutics in edible plant tissues for oral applications. Plant Biotechnol J, 2016;14:1791-1799.

Julve Parreño JM, Huet E, Fernández-Del-Carmen A, Segura A, Venturi M, Gandía A, Pan WS, Albaladejo I, Forment J, Pla D, Wigdorovitz A, Calvete JJ, Gutiérrez C, Gutiérrez JM, Granell A, Orzáez D. A synthetic biology approach for consistent production of plant-made recombinant polyclonal antibodies against snake venom toxins.Plant Biotechnol J, 2018;16:727-736.

Jutras PV, D'Aoust MA, Couture MM, Vézina LP, Goulet MC, Michaud D, Sainsbury F. Modulating secretory pathway pH by proton channel co-expression can increase recombinant protein stability in plants. Biotechnol. J. 2015;10:1478–1486.

Lacombe S, Bangratz M, Brizard JP, Petitdidier E, Pagniez J, Sérémé D, Lemesre JL, Brugidou C. Optimized transitory ectopic expression of promastigote surface antigen protein in *Nicotiana benthamiana*, a potential anti-leishmaniasis vaccine candidate. J Biosci Bioeng, 2018;125:116-123.

Laustsen AH, María Gutiérrez J, Knudsen C, Johansen KH, Bermúdez-Méndez E, Cerni FA, Jürgensen JA, Ledsgaard L, Martos-Esteban A, Øhlenschlæger M, Pus U, Andersen MR, Lomonte B, Engmark M, Pucca MB. Pros and cons of different therapeutic antibody formats for recombinant antivenom development. Toxicon, 2018; 146:151-175.

Laustsen AH, Johansen KH, Engmark M, Andersen MR. Recombinant snakebite antivenoms: A cost-competitive solution to a neglected tropical disease? PLoS Negl Trop Dis, 2017; 11(2): e0005361.

Laustsen AH, Engmark M, Milbo C, Johannesen J, Lomonte B, Gutiérrez JM, Lohse B. From Fangs to Pharmacology: The Future of Snakebite Envenoming Therapy, 2016a; Curr Pharm Des. 2016;22(34):5270-5293.

Laustsen AH, Solà M, Jappe EC, Oscoz S, Lauridsen LP, Engmark M. Biotechnological Trends in Spider and Scorpion Antivenom Development. Toxins, 2016;8: pii: E226.

Lee CH, Lee YC, Leu SJ, Lin LT, Chiang JR, Hsu WJ, Yang YY. Production and Characterization of Neutralizing Antibodies against Bungarus multicinctus Snake Venom. Appl Environ Microbiol, 2016;pii: AEM.01876-1.

León G, Herrera M, Segura Á, Villalta M, Vargas M, Gutiérrez JM. Pathogenic mechanisms underlying adverse reactions induced by intravenous administration of snake antivenoms.Toxicon, 2013;76:63-76.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. The folin by oliverProtein measurement with the Folin phenol reagent. J Biol Chem, 1951;217:220–230.

Ma, JKC, Drake PMW; Christou P. The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet, 2003;4:794–805.

Magy B, Tollet J, Laterre R, Boutry M, Navarre C. Accumulation of secreted antibodies in plant cell cultures varies according to the isotype , host species and culture conditions. Plant Biotechnol J, 2014;12:457–467.

Marusic C, Novelli F, Salzano AM, Scaloni A, Benvenuto E, Pioli C, Donini M. Production of an active anti-CD20-hIL-2 immunocytokine in *Nicotiana benthamiana*. Plant Biotechnol J, 2016;14:240-251.

Merlin M, Gecchele E, Capaldi S, Pezzotti M, Avesani L. Comparative Evaluation of Recombinant Protein Production in Different Biofactories: The Green Perspective. Biomed Res Int, 2014:136419.

Montero-Morales L, Maresch D, Castilho A, Turupcu A, Ilieva KM, Crescioli S, Karagiannis SN, Lupinek C, Oostenbrink C, Altmann F, Steinkellner H. Recombinant plant-derived human IgE glycoproteomics. J Proteomics, 2017;161:81-87.

Moussavou G, Ko K, Lee JH, Choo YK. Production of monoclonal antibodies in plants for cancer immunotherapy. Biomed Res Int, 2015:306164.

Murashige T, Skoog F. A revised medium for rapid growth and bloassaym with tobacco tissue cultures. Physiol Plantarum 1962;15:473-497

Muthamilselvan T, Lee CW, Cho YH, Wu FC, Hu CC, Liang YC, Lin NS, Hsu YH. A transgenic plant cell-suspension system for expression of epitopes on chimeric Bamboo mosaic virus particles. Plant Biotechnol J, 2016;14:231-239.

Niemer M, Mehofer U, Verdianz M, Porodko A, Schähs P, Kracher D, Lenarcic B, Novinec M, Mach L. *Nicotiana benthamiana* cathepsin B displays distintic enzymatic features which differ from its human relative and aleurain-like proteasis. Biochimie, 2015 ;122: 119-125.

Otero-Patino R. Epidemiological, clinical and therapeutic aspects of Bothrops asper bites. Toxicon, 2009;54:998-1011.

Pêra FF, Mutepfa DL, Khan AM, Els JH, Mbewana S, van Dijk AA, Rybicki EP, Hitzeroth II. Engineering and expression of a human rotavirus candidate vaccine in Nicotiana benthamiana. Virol J, 2015;12: 205.

Pessenda G, Silva LC, Campos LB, Pacello EM, Pucca MB, Martinez EZ, Barbosa JE. Human scFv antibodies (Afribumabs) against Africanized bee venom: Advances in melittin recognition. Toxicon, 2016;112:59-67.

Porebski S, Bailey LG, Baum BR. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol Biol Rep, 1997;15: 8–15.

Qu F; Ren T, Morris TJ. The Coat Protein of Turnip Crinkle Virus Suppresses Posttranscriptional Gene Silencing at an Early Initiation Step. J Virol, 2003;77, 1:511–522.

Rasool G, Yousaf S, Amin A, Mansoor S and Saeed M. Transient expression of synthetic coat protein gene of cotton leaf curl burewala virus in tobacco (*Nicotiana benthamiana*). J Agric Res, 2016;54:21-34.

Robert S, Khalf M, Goulet MC, D'Aoust MA, Sainsbury F, Michaud D. Protection of recombinant mammalian antibodies from development-dependent proteolysis in leaves of *Nicotiana benthamiana*. PloS One, 2013;8:e70203.

Rucavado A, Lomonte B, Ovadia M, Gutiérrez JM. Local tissue damage induced by BaP1, a metalloproteinase isolated from *Bothrops asper* (Terciopelo) snake venom. Exp Mol Pathol, 1995;63:186-199.

Sachett JAG, da Silva IM, Alves EC, Oliveira SS, Sampaio VS, do Vale FF, Romero GAS, Dos Santos MC, Marques HO, Colombini M, da Silva AMM, Wen FH, Lacerda MVG, Monteiro WM, Ferreira LCL Poor efficacy of preemptive amoxicillin clavulanate for

preventing secondary infection from *Bothrops* snakebites in the Brazilian Amazon : A randomized controlled clinical trial. PLoS Neglect Trop D, 2017;11:e0005745.

Sack M, Hofbauer A, Fischer R, Stoger E. The increasing value of plant-made proteins. 2015; Current Opinion in Biotechnology 32:163-170.

Stephan A, Hahn-Löbmann S, Rosche F, Buchholz M, Giritch A, Gleba Y. Simple Purification of Nicotiana benthamiana-Produced Recombinant Colicins: High-Yield Recovery of Purified Proteins with Minimum Alkaloid Content Supports the Suitability of the Host for Manufacturing Food Additives. 2017, Int J Mol Sci;29:19(1). pii: E95.

Tanjoni I, Butera D, Spencer PJ, Takehara HA, Fernandes I, Moura-da-Silva AM. Phylogenetic conservation of a snake venom metalloproteinase epitope recognized by a monoclonal antibody that neutralizes hemorrhagic activity. Toxicon, 2003;42:809-816.

Watanabe L, Shannon JD, Valente RH, Rucavado A, Alape-Girón A, Kamiguti AS, Theakston RD, Fox JW, Gutiérrez JM, Arni RK. Amino acid sequence and crystal structure of BaP1 , a metalloproteinase from Bothrops asper snake venom that exerts multiple tissue-damaging activities. Protein Sci, 2013;12:2273–2281.

WHO. Animal Bites.WorldWealth Organization, Geneva, Switzerland, 2012.

WHO. Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins. World Wealth Organization, Geneva, Switzerland, 2010.

Xu J., Towler M., Weathers P.J. (2016) Platforms for Plant-Based Protein Production. 1-40. In: Pavlov A., Bley T. (eds) Bioprocessing of Plant In Vitro Systems. Reference Series in Phytochemistry. Springer, Cham

Yao J, Weng Y, Dickey A, Wang KY. Plants as Factories for Human Pharmaceuticals: Applications and Challenges. Int J Mol Sci, 20015;16:28549-28565.

Yoshikawa M. Pre□microRNA processing activity in nuclear extracts from Arabidopsis suspension cells. J Plant Res, 2016;130:75–82.

Yu F, Wang Y, Xiao Y, He Y, Luo C, Duan D, Li C, Xu S, Xiang T. RP215 single chain fragment variable and single domain recombinant antibodies induce cell cycle arrest at G0/G1 phase in breast cancer. Mol Immun, 2014;59:100-109.

Zhang L, Crawford F, Yu L, Michels A, Nakayama M, Davidson HW, Kappler JW, Eisenbarth GS. Monoclonal antibody blocking the recognition of an insulin peptide-MHC complex modulates type 1 diabetes. P Natl Acad Sci Usa, 2014;111:2656–2661.

Table 1 – Average of scFvBaP1 yield and productivity by different methods in *N.benthamiana*. Yield is expressed in recombinant purified protein weight/fresh tissue weight from different sources, except in the cell suspension culture medium that contain only the MS medium with secreted antibody. Productivity is expressed in recombinant purified protein weight/fresh tissue weight/day of culture.

Sample (days after induction)	Yield	Productivity (days)
Infiltrated leaves	43 µg/g (± 0.7)	6.1 µg/g/d
Transgenic plants	270 µg/g (± 5)	18 µg/g/d
Transgenic callus	62 µg/g (± 2)	4.1 µg/g/d
Cell suspension biomass	83 µg/g (± 0,2)	10.37 µg/g/d
Cell suspension culture medium	71.75 mg/L (± 6.18)	8.96 mg/L/d

Highlights:

1 – Successful expression of a fibrinolytic inhibitor venom serum in *N. benthamiana*.

2 – scFvBAP1 was active agains the metalloproteinase and total venom from 3 snakes

3 – Higher expression in stable transgenic plants compared to transient expression.

4 – Liquid culture medium displayed higher scFvBAP1 yield.