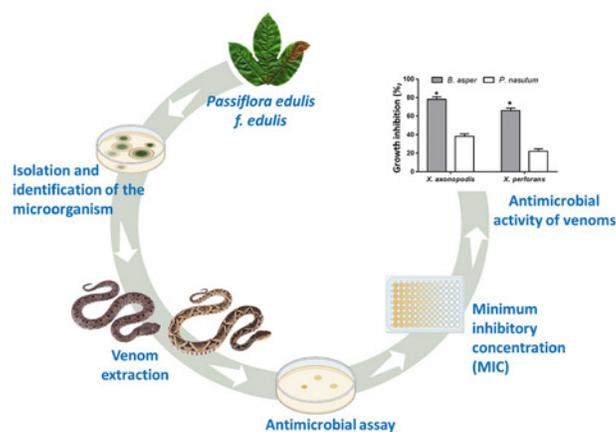


# Antimicrobial activity of *Bothrops asper* and *Porthidium nasutum* venom on purple passion fruit (*Passiflora edulis* f. *edulis*) phytopathogens

## Actividad antimicrobiana del veneno de *Bothrops asper* y *Porthidium nasutum* sobre fitopatógenos de gulupa (*Passiflora edulis* f. *edulis*)



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**Snake venom on purple passion fruit.**

Source: P. Rey-Suárez

### ABSTRACT

The most important pathogens causing high damage to fruit and vegetable crops are bacteria and fungi; among them *Xanthomonas* and *Fusarium* respectively are in the phytopathogens responsible of diseases in those crops. In Colombia, the crop of *Passiflora edulis* f. *edulis* occupies a position preferential within international market. However different pathologies affect their production, one of them is the infectious diseases and are caused by microorganisms as *Xanthomonas* and *Fusarium*. The control includes application of pesticides or antibiotics, but to some of them the microorganisms have develop resistance is widespread, and others are restricted associated to the impact on the environment and effects in animal and human healthy. Then find other alternatives is necessary, in this work the snake venoms of the vipers *Bothrops asper* and *Porthidium nasutum* were evaluated in ability to inhibit growth of these microorganism isolated of purple passion fruit crops with typical symptoms of diseases associated with infections by microorganisms. For these antimicrobial assays were performed in agar culture and broth microdilution. The results showed growth inhibition of 80% to *B. asper* against *Xanthomonas axonopodis* and 70% *Xanthomonas perforans*. The minimum inhibitory

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concentration was  $31,2 \mu\text{g mL}^{-1}$  to *B. asper* and  $500 \mu\text{g mL}^{-1}$  to *P. nasutum* to both bacteria. Nevertheless, the activity against *F. oxysporum* was low. The isolation of compounds responsible of this activity will contribute to the development of new molecules for antimicrobial agents against phytopathogens affecting purple passion fruit crops.

**Key words additional:** snake venoms; plant infectious diseases; *Xanthomonas*; *Fusarium*; bioprospecting.

## RESUMEN

Los patógenos más importantes que causan grandes daños a los cultivos de frutas y hortalizas son las bacterias y hongos; entre ellos se encuentran *Xanthomonas* y *Fusarium*, respectivamente. Estos fitopatógenos son responsables de varias enfermedades en los cultivos. En Colombia, el cultivo de *Passiflora edulis* f. *edulis* ocupa una posición preferencial dentro del mercado internacional. Sin embargo, diferentes patologías afectan su producción, una de ellas son las enfermedades infecciosas y son causadas precisamente por microorganismos como *Xanthomonas* y *Fusarium*. Su control incluye la aplicación de pesticidas o antibióticos, pero los microorganismos han desarrollado resistencia a algunos de ellos y otros están restringidos debido al impacto en el medio ambiente y por sus efectos en la salud animal y humana. Por ello, es necesario encontrar otras alternativas. Así, en este trabajo se evaluó la capacidad de los venenos de las víboras *Bothrops asper* y *Porthidium nasutum* para inhibir el crecimiento de microorganismos aislados de gulupa. Para ello, se realizaron ensayos antimicrobianos en cultivos en agar y microdilución en caldo. Los resultados mostraron una inhibición del crecimiento del 80% de *B. asper* frente a *Xanthomonas axonopodis* y del 70% de *Xanthomonas perforans*. La concentración mínima de inhibición fue de  $31,2 \mu\text{g mL}^{-1}$  para *B. asper* y de  $500 \mu\text{g mL}^{-1}$  para *P. nasutum* para ambas bacterias. Sin embargo, la actividad contra *F. oxysporum* fue baja. El aislamiento del compuesto responsable de esta actividad contribuirá al desarrollo de nuevas moléculas de agentes antimicrobianos contra fitopatógenos que afectan los cultivos de gulupa.

**Palabras clave adicionales:** venenos de serpientes; enfermedades infecciosas de las plantas; *Xanthomonas*; *Fusarium*; bioprospección.

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## INTRODUCTION

*Passiflora edulis* f. *edulis*, commonly known as purple passion fruit, is cultivated in many subtropical and tropical regions 1,500 m a.s.l. (Joy and Sherin, 2016), and especially in Colombia in altitudes between 1,600 and 2,300 m (Fischer *et al.*, 2022). Is one of the most sought-after exotic fruits in the global market, primarily due to its organoleptic properties, nutritional characteristics (macro and micronutrients), and the accumulation of secondary compounds with medicinal potential (Corrêa *et al.*, 2016; He *et al.*, 2020; Lozano-Montaña *et al.*, 2021; Fonseca *et al.*, 2022). Besides its culinary uses, there is emerging pharmaceutical interest, as recent studies have demonstrated its antioxidant, anti-inflammatory, antidiabetic, anxiolytic, anticancer, and antihypertensive properties (Carmona-Hernández *et al.*, 2019, Alves *et al.*, 2021,

Hettiarachchi *et al.*, 2022). In Colombia, the national production is increasing and is related to a higher percentage of exports every year. In the last years the production was of 24,798 t (Rodríguez-Polanco *et al.*, 2022). However, in Colombia the diseases are serious problem for passion fruit plants production, causing losses in quantity and quality of the fruit, which can be very high when there are environmental conditions that favor the development of pathogens, such as higher altitudes and rainfall, allow the increase of the bacterium inoculum, which can spread from the leaves to the stems and fruits (Benítez and Hoyos, 2009; Rodríguez-Polanco *et al.*, 2022).

Many factors contributing to the reduction in longevity and productivity in purple passion fruit plants,

especially due to diseases caused by viral (Sepúlveda *et al.*, 2022), bacterial or fungal etiologies (Joy and Sherin, 2016). Among these microorganisms *Xanthomonas* bacteria and *Fusarium* fungus are frequently involved (Fernández Barbosa and Suarez Meza, 2009; Benítez *et al.* 2011). Additionally, prolonged cultivation, the use of antibiotics, and poor agricultural practices have led to the development of resistance in some of these phytopathogens (Farfán *et al.*, 2014).

The *Xanthomonas* genus, is a gram-negative bacteria and near 27 species are pathogenic to several plants including cane, beans, cabbage, banana, citrus, tomatoes, pepper, and rice between others and the genus is responsible for many crop diseases, causing economic losses worldwide (Nakayinga *et al.*, 2021). Distinct genomic groups have been defined with *X. axonopodis* with many pathovars causing diseases on different host plants of agronomic significance. The symptoms include angular leaf lesions, blight, and wilt, stem exudates and stem canker. Other species is *X. perforans* pathogen that colonizes different growth stages of several plants in greenhouse or fields and causal agent of bacterial spot of tomato (Abrahamian *et al.*, 2021).

Currently, host resistance is the most effective way to control this disease (Mansfield *et al.*, 2012). Other methods include uprooting, burying and burning of infected plant tissues, sterilization of garden tools, and application of pesticides or antibiotics such streptomycin and copper-based compounds; however, resistance is widespread (Abrahamian *et al.*, 2021).

In the case of the fungus, *Fusarium oxysporum* is one of the most destructive pathogens in agriculture, causing vascular disease in economically important crops (Abrahamian *et al.*, 2021). The application of synthetic pesticides is the primary strategy to control disease. Nevertheless, fungicides have benefited crop production for decades, currently the use of such chemicals is restricted associated to overapplication has raised impact on the environment, contamination of drinking water, imbalance in the ecosystem, cause neurological effects in humans, severe lung injuries and the development of resistance is other of the principal problems (Seepe *et al.*, 2021).

The foregoing motivates the search for new molecules as alternatives for the control of phytopathogens. The snake venoms have demonstrated antimicrobial activity over different microorganism

including bacteria and fungus. These compounds include peptides and proteins with enzymatic function and the studies suggest that the components show diverse antimicrobial efficacy depending on the component and classes of microorganisms (Charvat *et al.*, 2018). For instance, a PLA<sub>2</sub> phospholipase obtained from the venom of *Crotalus adamanteus* demonstrated antimicrobial activity *in vitro* against *S. aureus* and *Enterobacter aerogenes* (Samy *et al.*, 2014). Also, a lectin obtained from *Bothrops leucurus* was effective against *Staphylococcus aureus* and *Enterococcus faecalis* (Nunes *et al.*, 2011). Likewise, a peptide derived from crotoamine from *Crotalus durissus terrificus* (South American rattlesnake) induced marked antimicrobial activity (Falcao and Radis-Baptista, 2020).

Synthetic cationic peptides derived from a PLA<sub>2</sub>, from *Bothrops asper*, such as pEM-2 have shown activity against bacteria and fungus (Santamaría *et al.*, 2005; Murillo *et al.*, 2007). Part of the sequence of that same peptide allowed the construction of a new one called V3, which increased its activity and improved its therapeutic index (Memariani *et al.*, 2017). A PLA<sub>2</sub> with activity against *S. aureus* was identified from the venom of *Porthidium nasutum* from Antioquia-Colombia (Vargas *et al.*, 2012). Likewise, from elapids of the genus *Micrurus*, a component was isolated that showed antibacterial activity on ATCC strain (Rey-Suárez *et al.*, 2018). In the same way, venom Cathelicidins have demonstrated *in vitro* and *in vivo* antimicrobial activity (de Barros *et al.*, 2019).

The aim of this work was to evaluate the antimicrobial properties of *Bothrops asper* and *Porthidium nasutum* venoms against phytopathogenic fungi and bacteria affecting *Passiflora edulis* f. *edulis*, given the need for alternatives to control of diseases in crops caused by these microorganisms.

## MATERIALS AND METHODS

### Venoms

Venom was obtained by manual extraction of *Bothrops asper* and *Porthidium nasutum* specimens collected in Antioquia and Choco, Colombia; and maintained in captivity at the serpentarium of Universidad de Antioquia (Medellin, Colombia). Venoms were centrifuged at 800 g for 15 min, and supernatants were lyophilized and stored at -20°C until use.

### Isolation and identification of the microorganism

Samples of leaves from purple passion fruit (*Passiflora edulis* f. *edulis*) crops exhibiting disease symptoms caused by *Xanthomonas* or *Fusarium* were collected for the isolation of these microorganisms (Fig. 1).

For isolation, the diseased tissue was washed, and dried and several segments were cut in small pieces (0,5 cm) and placed on the surface of a potato dextrose agar PDA (Becton-Dickinson, USA) plate for *Fusarium* or nutrient agar (Becton-Dickinson, USA) plate for *Xanthomonas*. To obtain pure cultures, tips from development colonies were transferred to fresh PDA media or chocolate agar PolyViteX (BioMérieux, Brazil) for *Xanthomonas*. When a pure culture was observed, the identification of the microorganism was

performed in the laboratory of Collection of Microorganism, Microbiology School, Universidad de Antioquia (CM-EM-UDEA)-Unique National Registry of Biological collections (RNC) No 250. For them, different strategies were used: direct with blue of lactophenol, gram stain; oxidase and catalase test. Finally, MALDI-TOF (MALDI-Biotyper) was employed to identify each species, as recommended by manufacturer.

### Antimicrobial assay

Antimicrobial susceptibility testing was performed as described by Vargas *et al.* (2012), with some modifications. For the agar diffusion assay, *Fusarium* or *Xanthomonas* grown on PDA or Chocolate agar were suspended in sterile saline solution, turbidity was



**Figure 1.** Typical alterations caused by phytopathogens in purple passion fruit. A) damage caused by *Fusarium*, showing plant chlorosis, growth retardation, wilting, fruit darkening, and dry stem. B) Bacteriosis caused by *Xanthomonas*, depicting leaves and fruits with necrotic spots and oily exudate. Photos by Rey-Torres A., from a purple passion fruit crop in Colombia.

measured at 600 nm and adjusted to McFarland patron (absorbance of 0.5), then were seeded on agar on Mueller-Hinton agar plates (MERCK Darmstadt, Germany). After 10  $\mu\text{L}$  of each *P. nasutum* or *B. asper* venom was added to bacterial agar media and incubated for 24 h to *Xanthomonas* or 48 h to *Fusarium* at room temperature (RT). A blank with sterile saline solution instead of venom served as negative control and antibiotic-antimycotic solution (SIGMA) served as a positive control or reference. Diameters of the bacterial growth inhibition zones were measured using a ruler. Each assay was performed in duplicate.

### Minimum inhibitory concentration (MIC) by broth microdilution susceptibility test

The assay was performed as described by Vargas *et al.* (2012). Serial dilutions of each venom (500-0.97  $\mu\text{g mL}^{-1}$ ) were prepared in 96-well microliter plates at a final volume of 50  $\mu\text{L}$  saline solution; then, 100  $\mu\text{L}$ /well of Mueller-Hinton broth, then 50  $\mu\text{L}$  of the bacterial inoculum adjusted to McFarland patron (absorbance of 0.5) were added to each well and incubated at RT for 24 h. Inhibition of bacterial growth was determined by absorbance reading at 600 nm (microplate reader Awareness Technology). The MIC was defined as the lowest concentration of venom required to inhibit microbial growth. Each dilution series included control wells, which consisted of 50  $\mu\text{L}$  of saline solution or antibiotic (chloramphenicol 6  $\mu\text{g mL}^{-1}$ ). All assays were run by duplicates and three assays were performed.

### Statistical analysis

Results were presented as mean  $\pm$  SD (standard deviation). Normality was assessed using the Shapiro-Wilk test, and analysis was conducted using the T-student test or two-way ANOVA with Bonferroni post hoc test. Statistical significance was established at  $P < 0.05$ .

## RESULTS

### Isolation, identification, and growth inhibition from *Fusarium*

The isolates of microorganisms showed a violet cotton colony. The microscopy analysis showed septate

hyphae, macroconidia and microconidia compatible with *Fusarium spp.* The MALDI-TOF analysis indicated *Fusarium oxysporum*.

The inhibitory activity of venoms in agar plates against this microorganism was reduced and only doses of 1 mg/10  $\mu\text{L}$  to *B. asper* showed inhibition halos of 8 mm (data not shown). *P. nasutum* did not shown inhibitory activity.

### Isolation, identification, and growth inhibition from *Xanthomonas*

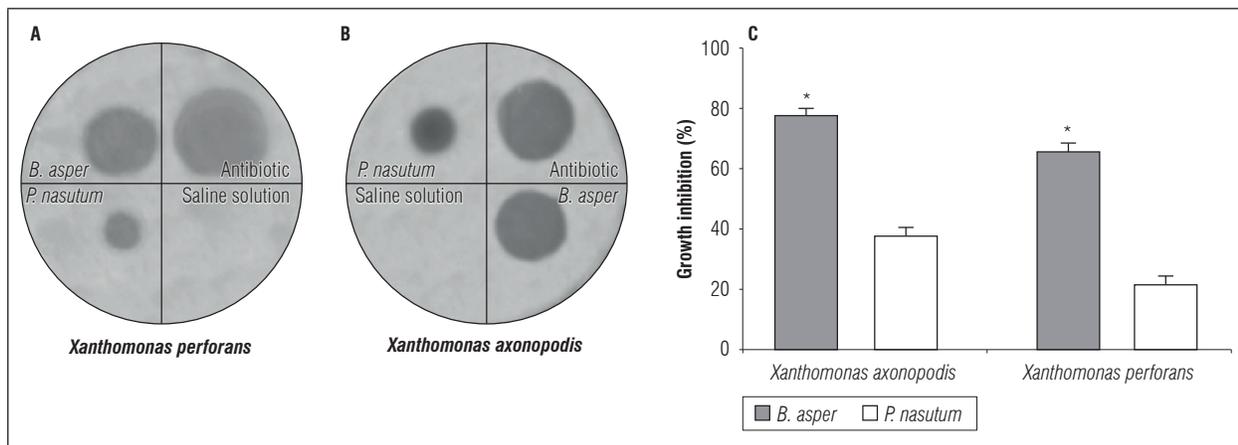
Two isolates of bacteria showed yellow mucus colonies, and Gram staining indicated gram negative rod shaped bacterium. Test of oxidase and catalase were negative and the MALDI-TOF analysis indicated one isolated for *Xanthomonas axonopodis* and another for *Xanthomonas perforans* (Tab. 1).

**Table 1. Isolation and identification of *Xanthomonas* from *Passiflora edulis f. edulis* samples.**

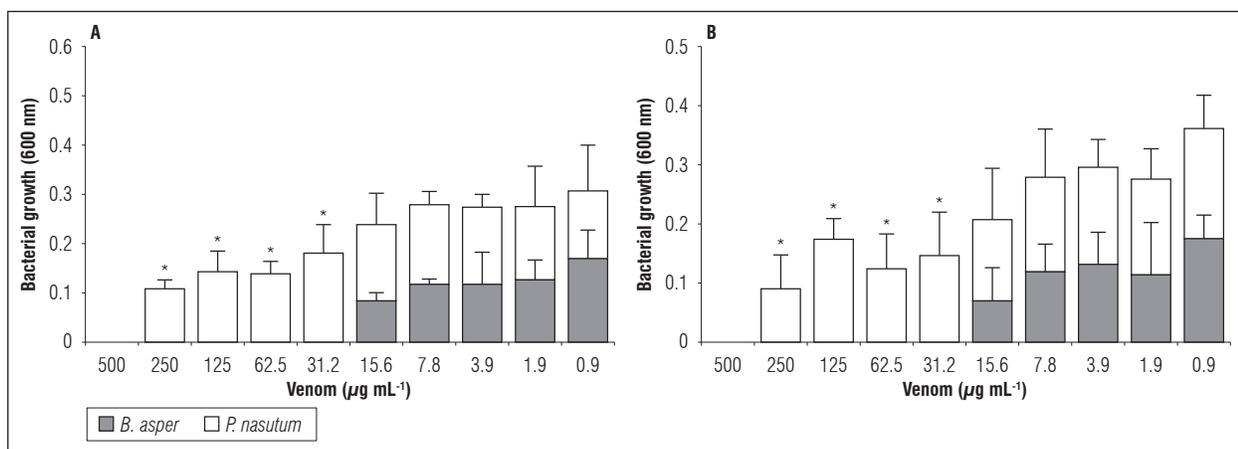
Test	Isolate 1	Isolate 2
Strain morphological characteristics	Mucous yellow colony	Mucous yellow colony
Gram stain	Gram negative bacillus	Gram negative bacillus
Oxidase	Negative	Negative
Catalase	Negative	Negative
MALDI-TOF	<i>Xanthomonas axonopodis</i>	<i>Xanthomonas perforans</i>

The inhibitory activity of venoms in agar plates against this microorganism was dependent on the species and doses used. The *B. asper* venom showed the highest activity against both bacterial species. Doses of 250  $\mu\text{g}/10 \mu\text{L}$  produced inhibition halos of 20 mm for *X. axonopodis* and 17 mm for *X. perforans*, representing 80 and 70% inhibition, respectively, compared to the antibiotic used as control. In contrast, *P. nasutum* only showed 40 and 20% inhibition respectively at the same doses (Fig. 2).

In the broth microdilution assay both venoms showed growth inhibition in all doses used (Fig. 3). However, the MIC to *B. asper* venom was 31.2  $\mu\text{g mL}^{-1}$  while *P. nasutum* exhibited a MIC of 500  $\mu\text{g mL}^{-1}$  (Tab. 2) against both bacteria. Furthermore, the venoms showed statistical differences ( $P < 0.001$ ) in the absorbance obtained in concentration below the MIC



**Figure 2.** Antimicrobial activity in agar diffusion assay of *B. asper* and *P. nasutum* venom against *Xanthomonas* isolated from *Passiflora edulis* samples. A) and B) inhibition halos. C) inhibition percentage in relation to the inhibition halo showed by antibiotic used. Bars represent mean  $\pm$  SD of duplicate \* Represents statistically significant differences ( $P < 0.05$ ).



**Figure 3.** Antimicrobial activity in broth microdilution assay of *B. asper* and *P. nasutum* venom against *Xanthomonas* isolated from *P. edulis f. edulis* samples. A) *X. perforans*; B) *X. axonopodis*. Bars represent mean  $\pm$  SD of triplicate, asterisk indicate statistically significant differences in antimicrobial activity of both venoms ( $P < 0.05$ ).

**Table 2.** Minimal inhibitory concentration (MIC) (in  $\mu\text{g mL}^{-1}$ ) of *B. asper* and *P. nasutum* venom against *X. axonopodis* and *X. perforans*. The MIC was lowest doses of venom required to inhibit totally microbial growth.

Venom	<i>Xanthomonas axonopodis</i>	<i>Xanthomonas perforans</i>
<i>B. asper</i>	31.2	31.2
<i>P. nasutum</i>	500	500

compared to the growth control. The positive control (chloramphenicol) completely inhibited growth at the concentration used.

## DISCUSSION

Snake venoms are complex mixtures of proteins and peptides and exhibit a wide range of pharmacological activities, and could be used to develop new

treatments in different areas for example in agricultural sector. Plant diseases represent a growing problem concern for global food security, and their control is crucial to produce food, and others as flowers, fibers and biomaterials (Rahman *et al.*, 2018). The annual losses of crops cause high economic impact on the food market. The most important pathogens that cause high damage to fruit and vegetable crops are bacteria and fungi (Rahman *et al.*, 2018; Shah *et al.*, 2022).

Every day many efforts are done to reduce bacterial and fungal infections that affect crop yield. *Xanthomonas* is a gram negative bacterial genus contains species. Occurrences of *Xanthomonas* diseases have been reported from multiple hosts worldwide and had caused major losses in different crops. *X. axonopodis* pv. *passiflorae* caused bacterial spot in passion fruit, it attacks the purple and yellow passion fruit as well as sweet passion fruit (Munhoz *et al.*, 2011).

In this work, *B. asper* and *P. nasutum* venom demonstrated inhibitory activity against two *Xanthomonas* species. This bactericidal activity is consistent with previous reports of these venoms exhibiting efficacy against both gram-positive and gram-negative bacteria (Yu *et al.*, 2010; Santamaría *et al.*, 2005; Vargas *et al.*, 2012). The MIC of *B. asper* venom was found to be lower compared to *P. nasutum* venom, which could be attributed to differences in the protein composition of their venoms (Alape-Girón *et al.*, 2009; Lomonte *et al.*, 2012).

Thus, the possibility of finding molecules that in the future allow the development applications in agricultural field for the control of this bacterium that causes losses in crops, such as purple passion fruit. Although they preliminary results it is not unthinkable, since there is other example of development of molecules from snake venom in other areas. Majority of the clinical and to date several snakes venom based treatments have been approved for cardiovascular diseases and other news uses currently being investigated in clinical trials as example, chronic pain, cancer and infections (Pérez-Peinado *et al.*, 2020). In relation to infections disease several venoms had been assay and demonstrated activity against different microorganisms related with clinical pathologic, examples include enzymes and proteins as L-aminoacid oxidase (LAO), metalloproteinases, desintegrins, lectins and antimicrobial peptides (cathelicidin). The mechanism of action included direct toxic action, free radical generation, induction of apoptosis, protease inhibitors,

among others (Shamsi *et al.*, 2016; de Barros *et al.*, 2019; Pérez-Peinado *et al.*, 2020).

The evaluation of snake venoms against microorganisms involved in plant pathologies is scarce. Toyama *et al.* (2006) evaluated a LAO from *Crotalus durissus cascavella* (CascaLAO) against *X. axonopodis* pv. *passiflorae* and observed an inhibition of 50% growth using 35  $\mu\text{g mL}^{-1}$ . This activity was demonstrated through transmission electron microscopy, which revealed the rupture of the plasma membrane of microorganisms, leading to the leakage of cytoplasmic content and cell death. However, a PLA<sub>2</sub> isolated from this venom only inhibited 9.8% their growth (Oliveira *et al.*, 2008). The lectins CTC and CVX-like isolated from *Crotalus durissus terrificus* species inhibited 87.8% growth of this bacteria (Rádis-Baptista *et al.*, 2006). Additionally, the venom and PLA<sub>2</sub>s from *Bothrops jararacussu* showed antibacterial effects on this bacterium, with the whole venom demonstrating better activity (Barbosa *et al.*, 2005).

In the same way Gomes *et al.* (2005) obtained a peptide from *Bothrops jararaca* venom with antifungal activity over *F. oxysporum* and *Colletotrichum lindemuthianum* phytopathogenic fungi. However, the activity was minor in relation with other fungi evaluated. Similarly, viper venoms evaluated in this work demonstrated low activity over *F. oxysporum* isolated from purple passion fruit.

The discovery of new potent antimicrobial agents with different mechanisms of action remains of high priority. The expectation to find such agents in snake venoms increases every day. In this study, the venoms of *B. asper* and *P. nasutum* demonstrated inhibitory activity against *X. perforans* and *X. axonopodis*, two microorganisms that cause significant damage in *Passiflora edulis* f. *edulis* crops. Further studies to isolate the compound responsible for this action are desirable.

## CONCLUSION

In this study, we evaluated the antimicrobial activity of venoms from *B. asper* and *P. nasutum* against isolates of *Xanthomonas* and *Fusarium* from *Passiflora edulis* f. *edulis*. The results showed MIC values of 31.2 and 500  $\mu\text{g mL}^{-1}$  for *B. asper* and *P. nasutum*, respectively, against *X. axonopodis* and *X. perforans* species. The activity of both venoms on *Fusarium* isolates was low, with only *B. asper* showing activity at high

doses. The significant antibacterial activity against *Xanthomonas* indicates the potential of these venoms as new sources for alternative treatments against this phytopathogens that cause considerable losses in agriculture, especially in passion fruit crops.

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**Authors' contributions:** VN was responsible for the conceptualization, data curation, formal analysis, investigation, methodology design, supervision, writing of the original draft, review, and editing of the manuscript. ART identify diseases plants and collect plant material. PRS investigation, data curation, statistical analysis, review, editing of the manuscript. AS, JGR and EM investigation. All authors read and approved the final manuscript.

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