

## ***Euphorbia trigona* latex nematicidal activity on the root-knot nematode *Meloidogyne incognita***

Atividade nematicida do látex de *Euphorbia trigona* sobre o nematoide das galhas *Meloidogyne incognita*

Actividad nematicida del látex de *Euphorbia trigona* en el nematodo agallador *Meloidogyne incognita*

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### **Abstract**

The aim of this study was to test nematicidal activity of the *Euphorbia trigona* latex on *Meloidogyne incognita* juveniles and to partially purify and characterize three proteases present in this latex. Three distinct proteases were partially purified from *E. trigona* latex. They were named here trigonin 1, 2 and 3. Their molecular weights were estimated at: 36, 31 and 29 kDa, for trigonin 1, 2 and 3, respectively. The pH and temperature that provided highest protease activity were pH values of 4.0, 6.0 and 9.0, and 70 °C. The crude extract containing the three proteases present in *E. trigona* latex reduced significantly ( $p < 0.01$ ) the number of live *M. incognita* juveniles in 96% after 24 hours treatment. The present study is the first report of *E. trigona* latex with nematicidal activity, therefore more studies regarding latex proteases action on nematodes are needed.

**Keywords:** Nematicidal; Protease; *Meloidogyne incognita*; Trigonin.

### **Resumo**

O objetivo deste estudo foi testar a atividade nematicida do látex de *Euphorbia trigona* sobre juvenis de *Meloidogyne incognita* e purificar parcialmente e caracterizar três proteases presentes neste látex. Três proteases distintas foram parcialmente purificadas do látex de *E. trigona*. Eles foram nomeadas como trigonina 1, 2 e 3. Seus pesos moleculares foram estimados em: 36, 31 e 29 kDa, para trigonina 1, 2 e 3, respectivamente. O pH e a temperatura que proporcionaram maior atividade de protease foram os valores de pH 4,0, 6,0 e 9,0 e 70 °C. O extrato bruto contendo as três proteases presentes no látex de *E. trigona* reduziu significativamente ( $p < 0,01$ ) o número de juvenis vivos de

*M. incognita* em 96% após 24 horas de tratamento. O presente estudo é o primeiro relato do látex de *E. trigona* com atividade nematocida, portanto, mais estudos sobre a ação das proteases do látex sobre os nematoides são necessários.  
**Palavras-chave:** Nematicidal; Protease; *Meloidogyne incognita*; Trigonin.

### Resumen

El objetivo de este estudio fue evaluar la actividad nematocida del látex de *Euphorbia trigona* sobre las larvas de *Meloidogyne incognita* y purificar y caracterizar parcialmente tres proteasas presentes en este látex. Tres proteasas distintas fueron purificadas parcialmente del látex de *E. trigona*. Ellas fueron nombradas aquí trigonina 1, 2 y 3. Sus pesos moleculares se estimaron en: 36, 31 y 29 kDa, para trigonina 1, 2 y 3, respectivamente. El pH y la temperatura que proporcionaron la actividad de proteasa más alta fueron valores de pH de 4.0, 6.0 y 9.0, y 70 ° C. El extracto crudo que contiene las tres proteasas presentes en el látex de *E. trigona* redujo significativamente ( $p < 0.01$ ) el número de juveniles vivos de *M. incognita* en 96% después de 24 horas de tratamiento. El presente estudio es el primer informe de látex de *E. trigona* con actividad nematocida, por lo que se necesitan más estudios sobre la acción de las proteasas del látex sobre los nematodos.

**Palabras clave:** Nematicida; Proteasa; *Meloidogyne incognita*; Trigonina.

## 1. Introduction

*Euphorbia* is a cosmopolitan genus which shares in common the characteristics of having specialized, highly reduced, flowerlike inflorescences, and the presence of milky white latex (Fonseca et al., 2010; Gunawardana et al., 2015). *Euphorbia trigona* is a succulent plant from Africa, known as African milk tree because of its high latex production, cultivated in various countries for ornamental purposes (Villanueva et al., 2015). Many studies suggest that the latex of plants from *Euphorbia* genus is a rich source of proteases (Yadav et al., 2012; Badgular & Mahajan, 2013; Mahajan & Adsul, 2015; Rezanejad et al., 2015; Flemmig et al., 2017). Recently, our research group reported for the first time proteases from *E. milii* latex with nematocidal activity on *Panagrellus* sp. larvae (Sufiate et al., 2017). However, there are no studies of the *E. trigona* latex regarding nematocidal action.

*Meloidogyne spp.* (root knot nematodes) are responsible for reducing the production of several plants with economic importance, causing many losses to agriculture. Although chemical nematicides are efficient in nematodes control, they are extremely toxic and non-specific (Adegbite, 2011). Thus, there is the need to develop new eco-friendly strategies to combat these nematodes.

Therefore, the aim of this study was to test nematocidal activity of the *E. trigona* latex on *Meloidogyne incognita* juveniles and to partially purify and characterize three proteases present in this latex.

## 2. Methodology

### 2.1 Latex obtainment

*Euphorbia trigona* latex was collected by means of superficial cuts on plants from Viçosa, Minas Gerais, Brazil. The latex was collected in microtubes and immediately stored at -20 °C. After five hours, the latex was thawed at room temperature, and the clear supernatant was collected, which was denominated as crude extract.

### 2.2 Obtaining of *Meloidogyne incognita* juveniles

Pure population of *Meloidogyne incognita* collected in Lavras, Minas Gerais, identified by analysis of esterase phenotypes (Carneiro and Almeida, 2001), were maintained in soybean plant during 60 days. After this period, the root system of the plants was submitted to Baermann funnel for hatching eggs and obtaining second stage juveniles (J<sub>2</sub>), which were quantified in Peters' chamber. The nematode suspensions were calibrated to 50 J<sub>2</sub>/mL.

### 2.3 Protease and protein assay

Protease activity was measured (Soares et al., 2013). One protease unit was defined as the amount of enzyme required to release 1.0 µg of tyrosine per minute under the assay conditions. Protein content was determined according to Bradford (1976).

### 2.4 Purification

The crude extract was applied in a gel filtration column Sephacryl® S-300 previously equilibrated with citrate-phosphate buffer 25 mM (pH 6.0), at 4 °C. The flow was adjusted to 0.5 mL/min. Protease elution was monitored by protease activity and by protein content. Fractions with high protease activity were pooled, constituting the proteases partially purified (PPP).

SDS-PAGE 10% was used to monitor the purification (Laemmli, 1970), and the gel was stained with coomassie blue. A zymogram was prepared from a PAGE (10%) containing casein 0.1% co-polymerized. The gel was incubated in citrate phosphate buffer 100 mM (pH 6.0) at 70 °C during 30 minutes. Then, it was stained with coomassie blue, and the protease activity was observed due to degradation halos.

### 2.5 PPP characterization

PPP activity was determined in different pH values, ranging from 2.2 to 10.0, using citrate-phosphate buffer 100 mM (pH 2.2 to 8.0), and glycine-sodium hydroxide buffer 100 mM (pH 8.0 to 10.0). For temperature effect characterization in the protease activity, different temperature values ranging from 40 to 80 °C were utilized.

PPP activity was measured in presence of the following inhibitors at 10 mM concentration: iodoacetamide, phenylmethylsulfonyl fluoride (PMSF) and ethylenediamine tetraacetic acid (EDTA). This assay was conducted using citrate-phosphate buffer 100 mM (pH 3.0 and 6.0), and glycine-sodium hydroxide buffer 100 mM (pH 9.0), at 70 °C. All the protease activity assays were performed in triplicate.

### 2.6 Nematicidal assay

The effect of *E. trigona* latex on *M. incognita* juveniles was tested. Two groups were formed in microtubes, a treated group containing crude extract and approximately 50 *M. incognita* J<sub>2</sub>, and a control containing the same number of *M. incognita* J<sub>2</sub>, without latex crude extract. This assay had seven replicates for each group. The microtubes were incubated at 28 °C, in a dark room, during 24 hours. After this period, the number of live *M. incognita* J<sub>2</sub> was counted in each tube of both groups. For data analysis, analysis of variance was used at significance levels of 1 and 5%. The destruction efficiency of *M. incognita* juveniles in relation to control was evaluated by Tukey test at 1% significance level. Subsequently, the percent reduction of juveniles' number was calculated according to the following equation:

$$\text{Reduction} = \frac{(\bar{X} \text{ juveniles from control} - \bar{X} \text{ juveniles from treatment})}{\bar{X} \text{ juveniles from control}} \times 100 \%$$

## 3. Results and Discussion

The purification methodology allowed partially purification (Table 1) of three distinct proteases, which were named here trigonin 1, 2 and 3 (Figure 1a). In the SDS-PAGE, there are three protein bands present in the pool formed after the gel filtration chromatography (Figure 1a). In the zymogram, there are also three degradation halos (Figure 1b), indicating that the three proteins present in the pool are three proteases. However, the methodology used in this study did not allow that each of

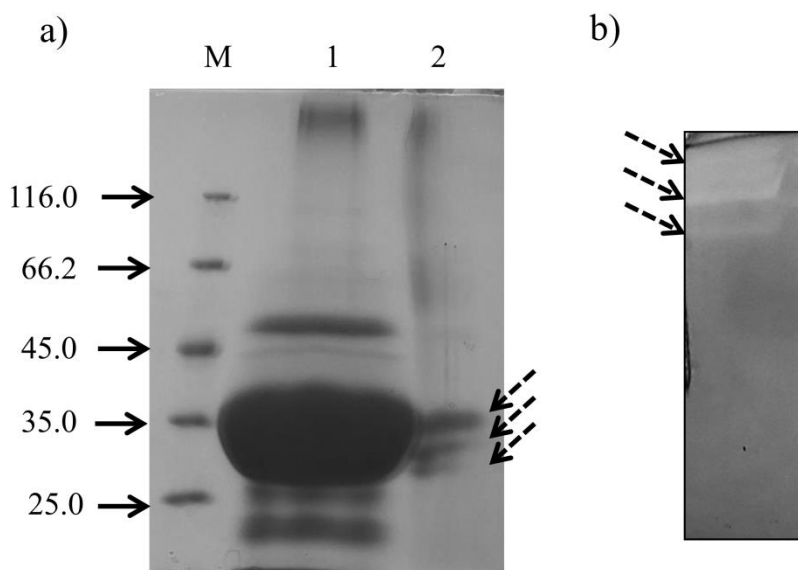
the enzymes were purified separately. The molecular weight of proteases was estimated at approximately: 36, 31 and 29 kDa for trigonin 1, 2 and 3, respectively (Figure 1a). These molecular weights are similar to those related for hirtin from *E. hirta* (34 kDa) (Patel et al., 2012), eumiliin from *E. milii* (30 kDa) (Fonseca et al., 2010), and neriifolin from *E. neriifolia* (35 kDa) (Yadav et al. 2012).

**Table 1.** Purification steps of proteases from *Euphorbia trigona* latex.

Step	Total enzyme activity (U) <sup>(1)</sup>	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	309.44	3.53	87.58	100	1.00
Gel filtration	170.53	0.52	327.17	55	3.74

<sup>(1)</sup> One protease unit (U) was defined as the amount of enzyme required to release 1.0 µg of tyrosine per minute under the assay conditions. Source: Authors.

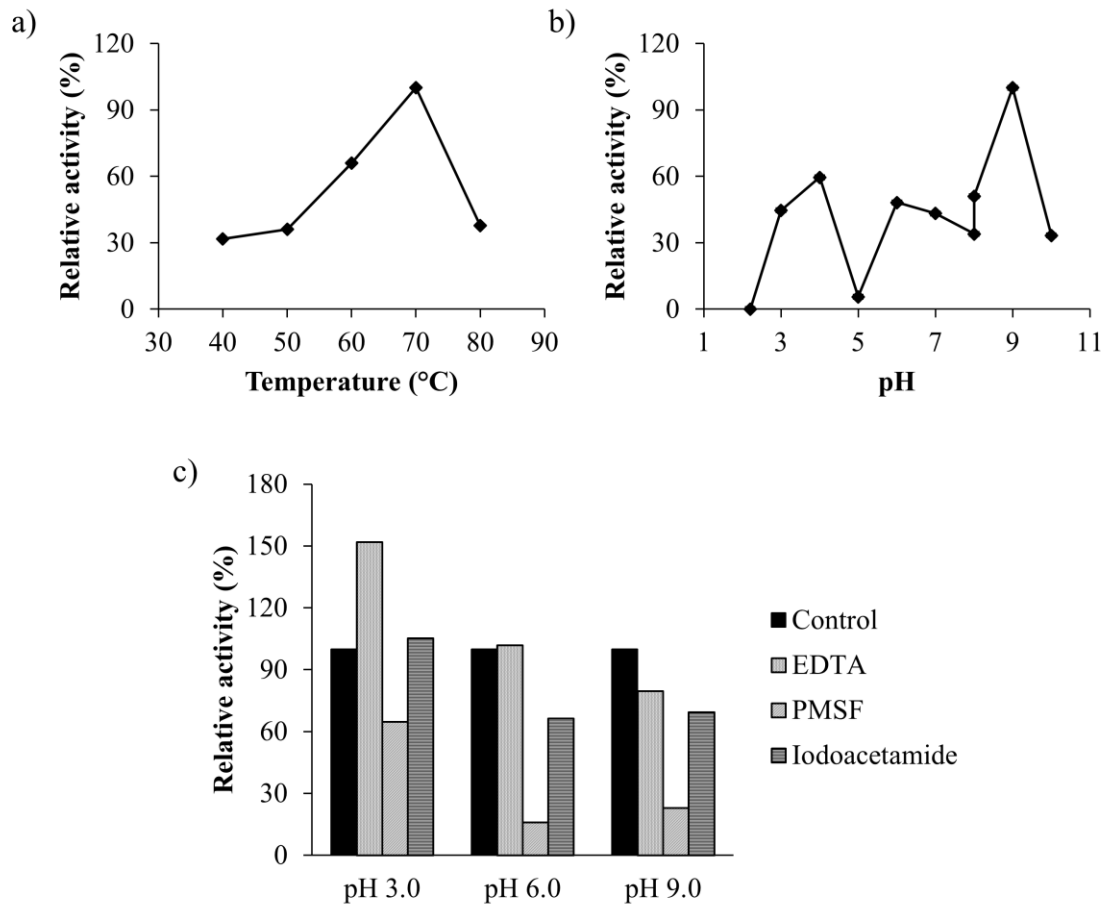
**Figure 1.** a) Purification analysis of proteases from *Euphorbia trigona* latex by SDS-PAGE 10%. Lane M: Protein molecular weight markers; Lane 1: crude extract; Lane 2: proteases partially purified. Dashed arrows indicate the three proteases from *E. trigona* latex. b) Zymogram of the proteases partially purified from *E. trigona*. Dashed arrows indicate the clear bands formed by proteases activity.



Source: Authors.

PPP showed highest activity at 70 °C. After this temperature, there was an abrupt decline in protease activity (Figure 2a). In relation to the pH effect on protease activity, the pH values of 4.0, 6.0 and 9.0 resulted in highest activity, also suggesting the presence of three enzymes (Figure 2b).

**Figure 2.** Characterization of the proteases partially purified from *Euphorbia trigona*. a) Temperature effect on the proteases partially purified activity from *Euphorbia trigona*. b) pH effect on the proteases partially purified activity from *E. trigona* latex. c) Effect of inhibitors on enzymatic activity of proteases partially purified from *E. trigona* latex.



Source: Authors.

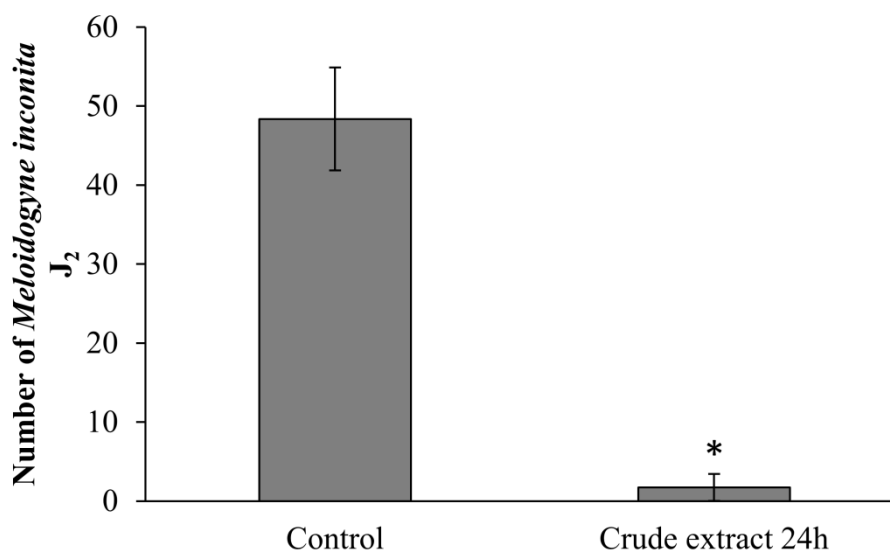
These results are similar from those observed by Fibriana and Upaichit (2015), which, evaluating the crude extract from *E. trigona*, determined that the pH 6.0 and temperature of 50 °C resulted in highest protease activity. Proteases from the present study showed higher optimum temperature in comparison with different proteases from *Euphorbia* genus (Badgular and Mahajan, 2012; Patel et al., 2012; Yadav et al., 2012; Moro et al., 2013; Rezanejad et al., 2015). The pH optimum values observed in this study, 4.0, 6.0 and 9.0, were similar to described for microsciadin from *E. microsciadia* (pH 4.5) (Rezanejad et al., 2015), nivulian from *E. nivulia* (pH 6.6) (Badgular & Mahajan, 2012), and miliin from *E. milii* (pH 9.0) (Moro et al., 2013).

PMSF inhibited PPP activity in 35, 84 and 77% at pH values 3, 6 and 9, respectively. Iodoacetamide reduced PPP activity in 34 and 31% at pH 6 and 9, respectively, and it had no effect on PPP activity at pH 3. EDTA presence was responsible for increased activity in 52% at pH 3 (Figure 2c). These results indicate presence of serine and cysteine proteases in *E. trigona* latex. Serine proteases were described in *E. hirta* and *E. neriifolia* (Patel et al., 2012; Yadav et al., 2012), and cysteine proteases were observed in *E. microsciadia* and *E. nivulia* (Badgular & Mahajan, 2012; Rezanejad et al., 2015).

The crude extract containing the three proteases present in *E. trigona* latex reduced significantly ( $p < 0.01$ ) the number of live *M. incognita* J<sub>2</sub>. The efficiency of *E. trigona* crude extract in nematodes reduction is showed by the high reduction percentage observed, with 96% reduction in 24 hours of treatment, when compared to control (Figure 3). In a similar

study, the proteases from *Synadenium grantii* reduced the number of *M. incognita* juveniles in 100% after 24 hours of treatment (Gomes et al., 2019). The present study is the first report of *E. trigona* latex with nematicidal activity, therefore more studies regarding latex proteases action on nematodes are needed.

**Figure 3.** Average number of *Meloidogyne incognita* J<sub>2</sub> after 24 hours treatment with the extract obtained from *Euphorbia trigona* latex. The control had no enzymes. Asterisk indicates significant difference ( $p < 0.01$ ) between the treated group and the control by Tukey test at 1% significance level.



Source: Authors.

#### 4. Conclusion

*E. trigona* latex contains three distinct proteases, which were named here trigonin 1, 2 and 3, with an estimated molecular weight of 36, 31 and 29 kDa, respectively. The results indicate that, among these three proteases, there are serine and cysteine proteases. The pH and temperature that provide highest protease activity are pH values of 4.0, 6.0 and 9.0, and temperature of 70 °C. The crude extract containing the three proteases from *E. trigona* latex has nematicidal activity on *M. incognita* J<sub>2</sub> larvae.

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