

Estado da publicação: O preprint foi publicado em um periódico como um artigo  
DOI do artigo publicado: <https://doi.org/10.1590/2236-8906-98/2019>

# Extrato de *Melia azedarach* L. induz a produção de uma fitocistatina em folhas de maracujá

Alyne Bispo Rodrigues, César Luis Siqueira Junior

<https://doi.org/10.1590/2236-8906-98/2019>

Submetido em: 2021-05-24

Postado em: 2021-05-25 (versão 1)

(AAAA-MM-DD)

***Melia azedarach* L. extract induced the production of phytocystatin in passion fruit leaves**

Título resumido: Plant extract induced production of phytocystatin

Alyne Bispo Rodrigues <sup>1</sup> and César Luis Siqueira Junior <sup>1,2</sup>

NÚMEROS DOS ORCID<sup>s</sup> DE CADA AUTOR

Alyne Bispo Rodrigues: 0000-0002-4090-1888

César Luis Siqueira Junior: 0000-0001-8668-2222

Recebido: 28.10.2019; aceito: 05.11.2020

---

1. Universidade Federal do Estado do Rio de Janeiro, Departamento de Botânica, Laboratório de Bioquímica e Função de Proteínas Vegetais, Avenida Pasteur, 458, Urca, 22290-240 Rio de Janeiro, RJ, Brasil

2. Corresponding author: [cesarjunior@unirio.br](mailto:cesarjunior@unirio.br)

ABSTRACT - (*Melia azedarach* L. extract induced the production of phytocystatin in passion fruit leaves). Phytocystatins belong to the family of cysteine proteinases inhibitors. These proteinase inhibitors have crucial functions in plant defense against insects and pathogens attack. In the present study *Melia azedarach* L. extract acts as an elicitor of plant defense responses in passion fruit seedlings inducing the increase of a phytocystatin levels in of these plants. The leaf protein content of the seedlings was extracted 24 hours after treatment with plant extract and analyzed for the induction of proteinase inhibitor activity. The results demonstrated the induction of a ~ 60 kD phytocystatin (*Pfcyst*) that has in vitro inhibitory effects against papain. These findings suggest that *M. azedarach* plant extracts may act as a potential activator of induced resistance against insects and pathogens by upregulating cystatin proteins synthesis in passion fruits plants. Thus, the use of plant extracts as biodefensives could reduce the use of pesticides in economically important agricultural crops.

Keywords: cinamomo, cystatin, plant defense, plant extract

RESUMO - (Extrato de *Melia azedarach* L. induz a produção de uma fitocistatina em folhas de maracujá). As fitocistatinas pertencem à família de inibidores de proteinase cisteínica. Esses inibidores de proteinase têm função crucial na defesa de plantas contra o ataque de insetos e patógenos. No presente estudo, extrato de *Melia azedarach* L. atuam como um eliciador da resposta de defesa em plântulas de maracujá induzindo o aumento dos níveis de fitocistatinas nessas plantas. O conteúdo proteico foliar dessas plântulas foi extraído 24 horas após o tratamento com o extrato vegetal e analisado quanto a indução da atividade do inibidor de proteinase. Os resultados demonstraram a indução de uma fitocistatina (*Pfcyst*) de ~60 kDa que possui efeito inibitório *in vitro* contra a papaína. Essas descobertas sugerem que o extrato da planta *M. azedarach* pode atuar como um potencial ativador da resistência induzida contra insetos e patógenos por regular positivamente a síntese de proteínas cistatinas em plantas de maracuja. Dessa forma, o uso de extratos vegetais como biodefensivos poderia reduzir o uso de agrotóxicos em culturas agrícolas economicamente importantes.

Palavras chave: cinamomo, cistatina, defesa vegetal, extrato vegetal

## Introduction

It is estimated that in Brazil there are about 120 native species of passion fruit that constitute the family Passifloraceae. Among these, the most cultivated species is the *Passiflora flavicarpa*, popularly known as sour passion fruit, or yet, yellow passion fruit. As in every plant, diseases and pests that attack the passion fruit plant cause significant economic losses (Leão 2011).

Plants, despite being sessile organisms, can develop defensive strategies that promote the reduction or even elimination of herbivorous attack. One of the most studied strategies is the production of defensive proteins, such as protease inhibitors (PIs) that act by inhibiting digestive proteases, reducing the availability of amino acids to synthesis of proteins necessary to the growth and development of the insect (Marinho *et al.* 2008). PIs have been studied as being produced as a defensive response in a large plant variety through the octadecanoid pathway (Ryan 1990). Siqueira Junior *et al.* (2008) demonstrated the production of a cysteine PI (cystatin) in passion fruit leaves as a result of the production of allene oxide synthase in passion fruit seedlings in response to injury or plant exposure to methyl jasmonate (MeJa).

Recent studies have demonstrated that plant extracts can trigger in other plants the induction of resistance to attack (Carvalho 2010). According to (Schwan-Estrada *et al.* 2000), this reaction involves the activation of defensive pathways in response to treatment with elicitors presents in the extract. *Melia azedarach* Linn (Meliaceae), popularly known as chinaberry, is a fast-growing ornamental tree of world distribution, being native to Persia, India and China, but widely spread in Africa, South America, Australia, Japan, among others (Kingsbury 1964). According to (Hassanein *et al.* 2008) the plant is well known for its pharmacological properties, such as anticancer, antiviral, antimalarial, antibacterial, and antifeedant activities (Tan *et al.* 2010).

## Materials and methods

Sowing of seeds - Passion fruit seeds were obtained from fruit purchased in local market of Campos dos Goytacazes, a city located in the northern region of Rio de Janeiro State, Brazil. Seeds were collected, dried at room temperature and stored at 4 °C in the dark until used. Passion fruit seeds were sown in earthworm humus and grown in a growth chamber at 28 °C ± 5 °C, 60% RH with 12 h photoperiod. Six-week-old plantlets were used for the study.

Preparation of aqueous *Melia Azedarach* leaf extract - *Melia azedarach*'s leaves were obtained from plants from the São João da Barra district (21°44'59''S- 41°10'01''W). Leaves (10 g) were washed twice with sterile water and dried in aseptic conditions at 40 °C until total dehydration. Leaves were macerated in a pre-chilled pestle and mortar. The powdered leaves were mixed in 20 mL of sterilized distilled water and vigorously shaken for 2 h. The extract obtained was filtered through two folds of muslin cloth and the filtered was centrifuged at 12.000 xg at 4 °C for 20 min. The supernatant was used as aqueous leaf extract.

Treatment of passion fruit plantlets - Four group of plants having three replicates each were treated as following based on the methodology described by (Siqueira\_Junior *et al.* 2008). Each replicate had nine plants. Group 1: The Leaves from each plant was sprayed with sterile distilled water and used as control. Group 2: Leaves from each plant was sprayed with sterilized distilled aqueous leaf extract. Group 3: Leaves from each plant was not treated and used as negative control. *Group 4.* Leaves from each plant was sprayed with methyl jasmonate 1 mM (125 µl in 500 ml of 0.125% triton x-100 solution). The leaves from plants were collected at 24 h after treatment and analyzed for the cysteine proteinase inhibitors (CysPI) activity.

Preparation of crude extract, gel electrophoresis and immuno-blot analysis - The passion fruit leaves were ground to a fine powder in liquid N<sub>2</sub>, 1% (w/w) insoluble polyvinylpolypyrrolidone (PVPP) was added to it and total protein was extracted by addition of 3 mL ice-cold extraction buffer (50 mM Tris-HCl, pH 6.0) to 1 gram of powdered tissue. The homogenate was centrifuged at 10,000 xg for 30 min at 4 °C. Otherwise stated, the recovered crude supernatant was used for the following

experiments. Protein concentration was determined using the Bradford assay kit and bovine serum albumin as standard protein according to the manufacturer's instructions (BioRad). Proteins were analyzed in 10% (w/v) SDS polyacrylamide gel according to (Laemmli 1970). The transfer of proteins to nitrocellulose membranes was carried out as described by (Towbin *et al.* 1979). Membranes were probed with immune serum against tomato cystatin (gift from Dra. Tania Jacinto, Universidade Estadual do Norte Fluminense, Brazil). Blots were developed using Protein A-peroxidase (Invitrogen) and TMB 1-component membrane peroxidase substrate (Sinapse biotecnologia) following the manufacturer's instructions.

Estimation of CysPI in leaves extracts - Measurements of the inhibition activity of crude leaf extract (70 µg) were performed using N-benzoyl-1-arginine-2-naphthylamide (BANA, Sigma), following the procedure described by (Siqueira\_Junior *et al.* 2008). In all experiments, 2 µg of papain was used. Inhibitory activity was detected as a reduction of BANA hydrolyzing activity of papain.

Statistical Analysis - Data are presented as the mean (S.D.) of three independent experiments, and the differences from controls were assessed with Student's t-test; statistically significant at  $P < 0.05$ .

## Results and discussion

It is suggested from the preview results that defensive proteins could be synthesized in passion fruit leaves in response to injury and methyl jasmonate treatment (Siqueira\_Junior *et al.* 2002). Exposure to mechanical injury or treatment with methyl jasmonate vapors induces synthesis of an allene oxidase synthase (AOS) in the leaves of treated plants. In this present work, we suggest that treatment passion fruit plants with cinamomo extract induced an increase in the activity of a proteinase inhibitor which would lead to increase resistance to insect and pathogen attack, since the secondary metabolites contained in the cinamomo extract apparently act as elicitors of the natural defense response of passion fruit plants.

Treatment with plant extract increased cystatin activity by almost 3-fold compared to control (untreated) plants after 24 h. As positive control was used MeJa, a known elicitor of the plant defense response (figure 1 a). The idea of cystatin synthesis in response to treatment with cinamomo extract is reinforced by Western blot analysis (figure 1 b) which demonstrated that the ~60 kD protein band reacts with antibodies produced against tomato cystatin (Madureira *et al.* 2006) and the amount of the recognized protein was consistent with the *in vitro* inhibitory assays. Previously, Siqueira\_Junior *et al.* (2008) verified that the increase in the activity of AOS, in response to the treatment with MeJa, caused the increase of the activity of this same cystatin in the leaves of passion fruit plants. Nothing can be said from our studies regarding which compounds in the plant extract are responsible for the observed effects. Further studies will be needed to find out which plant compounds present in cinamomo extract are involved in eliciting the defense mechanism in passion fruit plants. Secondary plant compounds have been studied for their repellent action, or even for being lethal to insects. Lovatto *et al.*, (2012), in his work, reinforces the idea that the use of plant extracts in agriculture could contribute to the natural control of pests based on the agroecological concept.

Together, our data provides important information about plant defense system elicitation. This plant extract could potentially be used as an appropriate biological control agent induction under controlled laboratory conditions, but field trials are underway to evaluate the potential effectiveness of open field passion fruit crops.

### **Conflict of interests**

The authors declare no conflicts of interest.

### **Author's Contribution**

Alyne Bispo Rodrigues: Methodology, Formal analysis, Investigation, Validation. **César Luis** Siqueira-Junior: Project administration, Conceptualization, Resources, Methodology, Supervision, Writing - Original Draft, Writing - review & editing.

### Literature cited

- Carvalho, P.R.S. 2010.** Extratos vegetais: potencial elicitador de fitoalexinas e atividade antifúngica em antracnose do cajueiro. Tese de Doutorado, Universidade Estadual Paulista, Jaboticabal.
- Hassanein, N.M., Abou Zeid, M.A., Youssef, K.A. & Mahmoud, D.A. 2008.** Efficacy of leaf extracts of neem (*Azadirachta indica*) and chinaberry (*Melia azedarach*) against early blight and wilt diseases of tomato. Australian Journal of Basic and Applied Sciences 2: 763-772.
- Kingsbury, J.M. 1964.** Poisonous plants of the United States and Canada. Englewood Cliffs: Prentice-Hall.
- Laemmli, U.K. 1970.** Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227: 680-685.
- Leão, A.J.P. 2011.** Formação de mudas de maracujazeiro por enxertia em espécies silvestres e em híbridos inter e intraespecíficos. Dissertação de Mestrado, Universidade de Brasília, Brasília.
- Lovatto, P.B., Schiedeck, G., & Garcia, F.R.M. 2012.** A interação co-evolutiva entre insetos e plantas como estratégia ao manejo agroecológico em agrossistemas sustentáveis. Interciência 37: 657-663.
- Madureira, H.C., Da Cunha, M. & Jacinto, T. 2006.** Immunolocalization of a defense-related 87 kDa cystatin in leaf blade of tomato plants. Environmental and Experimental Botany 55: 201-208.
- Marinho, J.S., Oliveira, M.G.A., Guedes, R.N.C., Pallini, A. & Oliveira, C.L. 2008.** Protease inhibitors of native and exotic hosts and their action on *Thyrinteina leucoceraea* caterpillar intestines. Revista Árvore 32: 1125-1132.
- Ryan, C.A. 1990.** Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annual Review of Phytopathology 28: 425-449.
- Schwan-Estrada, K.R.F., Stangarlin, J.R. & Cruz, M.E.S. 2000.** Use of plant extracts in the control of plant pathogenic fungi. Floresta 30: 129-137.
- Siqueira\_Junior, C.L., Fernandes, K.V.S., Machado, O.L.T., Cunha, M., Gomes, V.M., Moura, D. & Jacinto, T. 2002.** 87 kDa Tomato cystatin exhibits properties of a defense protein and forms protein crystals in prosystemin overexpressing transgenic plants. Plant Physiology and Biochemistry 40: 247-254.
- Siqueira-Júnior, C.L., Jardim, B.C., Ürményi, T.P., Vicente, A.C.P., Hansen, E., Otsuki, K., Da Cunha, M., Madureira, H.C., De Carvalho, D.R. & Jacinto, T. 2008.** Wound response in passion fruit (*Passiflora f. edulis flavicarpa*) plants: gene characterization of a novel chloroplast-targeted



allene oxide synthase up-regulated by mechanical injury and methyl jasmonate. *Plant Cell Reports* 27: 387-397.

**Tan, Q.G., Li, X.N., Chen, H., Feng, T., Cai, X.H. & Luo, X.D.** 2010. Sterols and terpenoids from *Melia Azedarach*. *Journal of Natural Products* 73: 693-697.

**Towbin, H., Staehelin, T. & Gordon, J.** 1979. Eletrophoretic transfer of proteins form polyacrilamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* 76: 4350.

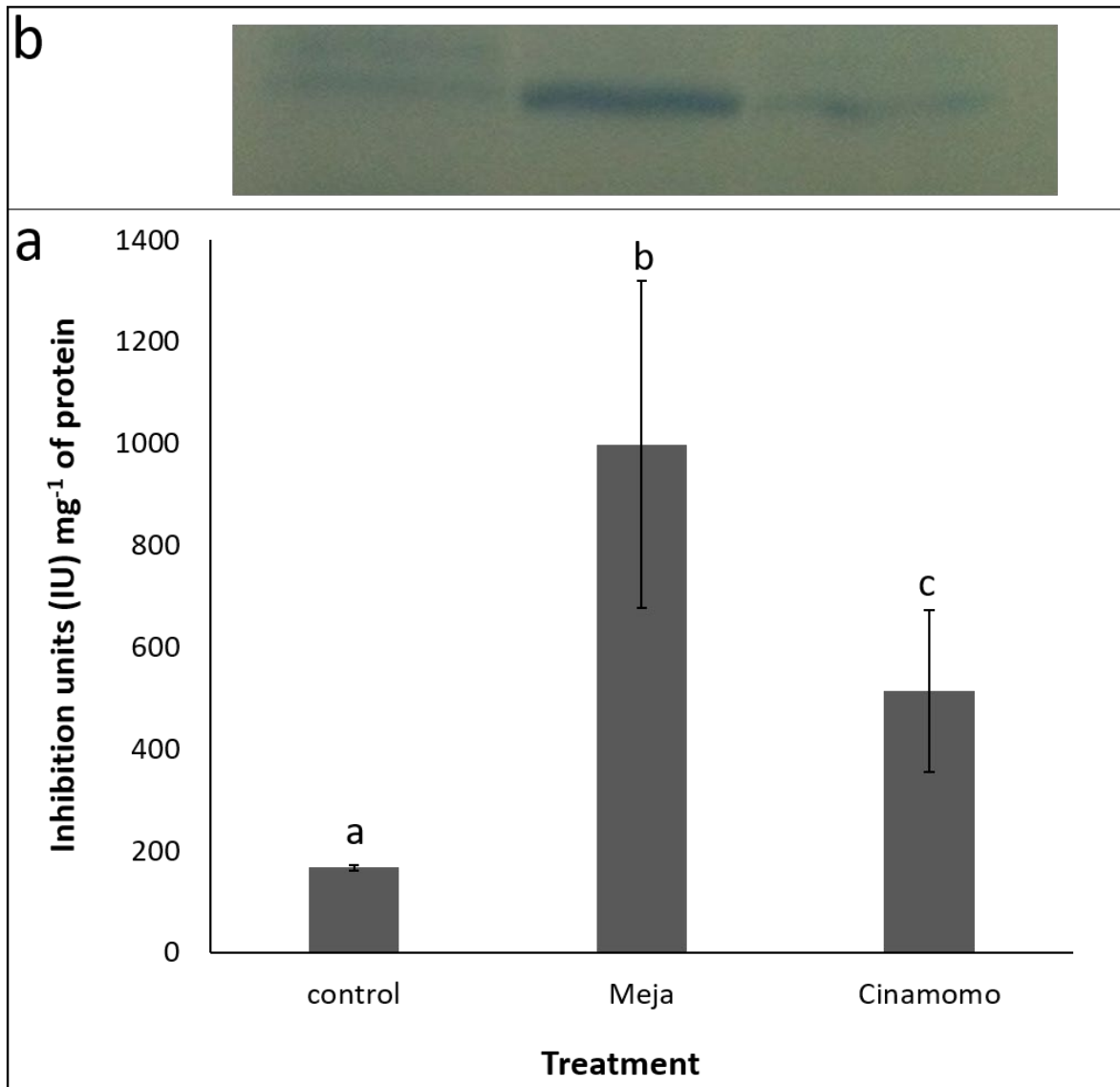


Figure 1. a. Cystatin activity in passion fruit leaves. From each sample 100 ug of crude leaf extract were combined with 5 ug papain and the proteolytic activity evaluated using BANA as substrate. Samples were analyzed 24 h after stimuli. Data are a mean of three independent experiments ratios of standard error to mean were 5% or less. b. Western blot analysis of crude leaf proteins extracts. Left to right control leaves; MeJa treated leaves; cinamomo extract treated leaves. For each sample 50 ug of total protein were loaded. Proteins were probed with immune serum against tomato cystatin 10,000 fold diluted. In all cases leaves from three plants were pooled for each protein extraction to obtain an average pattern. Letters a, b e c from Figure 1 b denote significant difference at  $P < 0.05$  (Student's t-test).

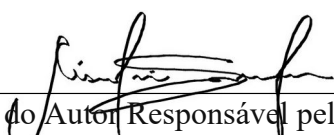
## CARTA DE AUTORIZAÇÃO DE PUBLICAÇÃO NO PORTAL DE PREPRINTS DO SCIELO

Ao Comitê Editorial de HOEHNEA

Declaro, em meu próprio nome e nos dos demais Autores que concordo com a publicação do artigo Aceito pelo Corpo Editorial de Hoehnea, intitulado “*Melia azedarach* L. extract induced the production of phytocystatin in passion fruit leaves” de autoria de Alyne Bispo Rodrigues e César Luis Siqueira Junior, no Portal de Preprints do SciELO Brasil ([Biological Sciences | SciELO Preprints](#)).

Declaro, ainda, que o referido artigo é original, sendo que o conteúdo não foi ou não está sendo considerado para publicação em outra Revista, quer seja no formato impresso e/ ou eletrônico.

São Paulo, 27 de abril de 2021.

  
Assinatura do Autor Responsável pelo Artigo César  
Luis Siqueira Junior

gov.br

Documento assinado digitalmente  
Cesar Luis Siqueira Junior  
Data: 28/04/2021 10:14:23-0300  
CPF: 053.654.837-40