Research Article   **Open Access**

Interaction of Extracellular Proteinases of *Phytophthora cinnamomi* with Proteinase Inhibitors Produced by Avocado Root

Jonatan Encino-López, Elda Castro-Mercado, Gerardo Rangel-Sánchez and Ernesto García-Pineda*

Universidad Michoacana de San Nicolás de Hidalgo, Instituto de Investigaciones Químico Biológicas, Edif. B1, Ciudad Universitaria, 58040, Morelia, Michoacán, México

Abstract

The interaction avocado plant/*Phytophtora cinnamomi* has not been explored at the level of defense responses induced by this pathogen. Here, we show that the infection of avocado plants by *Phytophthora cinnamomi* induced proteinase inhibitor, which had inhibitor activity against extracellular proteases of *P. cinnamomi*. Extracellular proteinase activity was not increased with the addition of the avocado root cell wall to liquid culture but decreased in the presence of root of healthy avocado plants. The pretreatment of avocado roots with the avocado inhibitor before the infection by *P. cinnamomi* inhibited root colonization by the pathogen. Avocado inhibitor had no effect on *in vitro* growth of the oomycete, suggesting that the inhibition of root colonization could be due to its interaction with pathogen proteinases.

Keywords: *Phytophthora cinnamomi*; Avocado; Proteinases; Avocado inhibitor

Introduction

Most plants are resistant to most microbes and only specialist organisms have evolved the capacity to overcome plant defenses. Mechanisms of resistance in plants can be subdivided into two categories, passive (constitutive) and active (induced). Passive mechanisms involve both structural elements, such as the cuticle and pre-formed antimicrobial chemical compounds within the plant termed phytoanticipins. These form the initial layers of protection against microbial attack. Inducible defense mechanisms include the hypersensitive response (local plant cell death) and induction of specific gene expression within the plant, including genes for the biosynthesis of additional antimicrobial compounds, and localized induction of genes encoding hydrolytic enzymes and other defense-related proteins [1].

Oomycetes form a diverse group of fungus-like eukaryotic microorganisms that includes both saprophytes and pathogens of plants. They cause devastating diseases in numerous crop, ornamental, and native plants. They include more than 60 species of the genus Phytophthora that are arguably the most devastating pathogens of dicotyledonous plants [2]. Virtually every dicot plant is affected by one or more species of Phytophthora. Oomycetes are known to secrete a range of degradative enzymes [3], but there is only limited direct demonstration of the role of cell wall-degrading enzymes in plant penetration by oomycete pathogens.

Phytophthora cinnamomi Rands is an oomycete pathogen with a broad host range, and the diseases it causes are of economic importance throughout the temperate and tropical zones [4].

This pathogen causes the root rot disease in avocado plants (*Persea americana* Mill), one of the most destructive and important diseases worldwide of this plant. The pathogen attacks trees of all ages, including those in nurseries, and destroys the small, absorbing roots of the avocado which become black and brittle and eventually die [5], leaving the affected tree with little uptake ability. The aboveground symptoms of the disease, resemble droughting, and are not obvious until the roots are heavily infected [4].

Some phytopathogenic microorganisms produce active extracellular proteinases that, along with other enzymes, play important roles in pathogenesis. These proteins include proteolytic enzymes of different catalytic types [6]. The important role of proteinases is indicated by direct dependence between the activity of extracellular proteinases in microorganisms and intensity of plant disease [7]. The participation of extracellular phytopathogenic proteinases in pathogenesis can be of different nature: from degradation of cell wall proteins and other defense proteins in plants [8] to processing of inherent extracellular proteins of microorganism, which are significant for progression of a disease [9].

Proteinases are classified based on their catalytic mechanisms into Ser, Cys, aspartic, and metallo proteinases [10]. Papain-like cysteine proteinases (PLCPs) play crucial roles in plant-pathogen interactions. PLCPs are usually 23-30 kDa in size, and use a catalytic cysteine residue to cleave peptide bonds in protein substrates. This catalytic cysteine is part of a catalytic triad situated in the middle of a cleft that binds the substrate through specific interactions [11]. Many PLCPs of plants and their invaders are produced with a signal peptide, indicating that they are secreted or localized in the endomembrane system [12].

On another hand, it has been reported that plant inhibitors of proteinases not only suppress the activity of enzymes secreted by the phytopathogenic microorganisms, but also inhibit their growth and development [13]. The interactions of proteinases with their substrates and inhibitors can be seen as a molecular battle-field because during parasitic interactions the selection of counteracting inhibitors and non-cleavable substrates could be used to evade proteolysis.

Recently, some defense responses were studied in avocado roots after infection with *P. cinnamomi*, which included the monitoring reactive oxygen species, total phenols, epicatechin and nitric oxide

[14]. A burst of reactive oxygen species (ROS) was observed. Total phenols and epicatechin content showed an important decrease, but lignin, and procyanidins did not exhibit changes after inoculation. Furthermore, an increased nitric oxide (NO) production was observed 72 h after treatment. The role of proteinases in the *P. cinnamomi*/ avocado plant interaction has not been elucidated. The aim of the present study was to analyze the interaction of the avocado proteinase inhibitors with exoproteinases secreted by *P. cinnamomi*.

Materials and Methods

Plant material and soil

These studies were performed using seedlings of a commercial avocado rootstock, *Persea americana* Mill var. *Drymifolia* which is susceptible to avocado root rot. Four-month old seedlings were planted into soil that had been inoculated with *P. cinnamomi* as follows: ten Petri dishes containing mycelia of *P. cinnamomi* (provided by Dr. Rafael Salgado-Garciglia from the Universidad Michoacana de San Nicolás de Hidalgo, and isolated from avocado plant roots showing characteristic symptoms of the disease) growing for 7 days in V8 juice agar media, were homogenized by blending for 1 min with Hamilton Beach blender (Hamilton Beach/Proctor-Silex, Inc.) and mixed into the sterilized soil Miracle-Gro (2 kg), and irrigated as needed [15]. At least five plants were used per treatment. Replicate experiments were performed and always included control plants that were mock-inoculated appropriately with sterile distilled water.

All biochemical analysis were performed with feeder avocado roots harvested from inoculated plants at different times of incubation, washed in water, and used immediately for experiments which were conducted at room temperature (23-25°C), with four replicates per treatment. *P. cinnamomi* was also cultured in liquid medium using V8 juice media without agar, as described [16].

Inhibitor extraction and papain activity

Inhibitor activity was measured by observing its effect on the endopeptidase activity of papain as described [17]. This method follows the breakdown of casein by papain spectrophotometrically. Protease inhibitor was partially purified from plant tissue by grinding weighed tissue (0.5 g) in 50 mM Tris buffer, pH 8, containing 0.15 M NaCl and 2 mM sodium EDTA and centrifuged for 30 min at 350,000g. The supernatant was used in papain activity assays. Papain (0.25 μM) was incubated with 100 μL of plant extract in the presence of 5 mM Cys for 10 min at 37°C in a total volume of 200 μL using the same buffer. After incubation the assay medium was cooled to 4°C before addition of 100 μL of 1% ice-cold casein. The reaction medium was then incubated at 37°C for 10 min and rapidly cooled to 4°C while 700 μL of 8% trichloroacetic acid (TCA) was added to stop the proteolytic action of papain. The tubes were centrifuged at 15,000 rpm in an Eppendorf 5415C for 5 min and the absorbance of the supernatant was measured at 280 nm. One unit will hydrolyze casein to produce peptides equivalent to 1.0 μmole of tyrosine per minute at pH 8 at 37°C. Controls with and without papain were performed to ensure that the plant extract had no direct effect on casein.

Extracellular proteinases analysis of *P. cinnamomi*

Extracellular proteinases were isolated from the liquid culture [18]. V8 juice liquid medium (125 ml) was inoculated with 5 plugs (0.5 cm diameter) of *P. cinammomi* growing for 7 days in V8 juice agar media and incubated at 23°C for 3 days. The culture was filtered and proteins were precipitated with (NH4)2SO4 at 80% saturation. The precipitate was left at 4°C overnight and then collected by centrifugation at 10,000g

for 30 min. The precipitate was dissolved in appropriate buffers for protease assays.

Quantification of infection by root plating

For experiments to analyze the effect of avocado inhibitor on root colonization by *P. cinnamomi*, four (1-cm-long) root tips were excised and incubated for 1 h with avocado inhibitor (2.5 μg of protein), 0.25 μM papain, 100 μg *Phytophthora* exoproteinase, or water (control) and then infected *in vitro* by dipping into a mycelial aqueous solution (10 mL), incubated for 24 h and planted on a medium selective for *Phytophthora* spp. containing (per litre of corn meal agar): 10 mg piramicin, 250 mg ampicillin, 10 mg rifampicin, 100 mg pentachloronitrobenzene, and 75 mg hymexazol. After 3 days, the root tips were scored for infection based on the growth of *P. cinnamomi*. To prepare the inoculum, *P. cinnamomi* mycelia from one Petri dish grown for 7 days in V8 juice agar medium was collected with 5 mL sterilized distilled water. The optical density (OD600) of the inoculum was adjusted to 1.5–2 to obtain a uniform mycelium concentration [16].

In vitro sensitivity assay

Avocado root inhibitor or H_2O_2 were added at different concentrations to plates containing autoclaved V8 juice agar (15 g/L). Plates were inoculated with plugs of agar containing 7-day-old mycelium of *P. cinnamomi* with the mycelium facing up. Mycelial growth on these plates was measured every 24 h as colony radius [19].

Isolation of avocado cell wall

Cell walls were prepared from the avocado root [20]. Briefly, 0.5 g of root were ground with liquid nitrogen to a fine powder using a pestle and mortar and suspended in boiling ethanol (1% w/v) for 15 min to deactivate any enzymes present. The powder was recovered by filtration and subjected to a series of extractions to remove lipids, polyphenols, and other low-Mr metabolites as follow: the residues were extracted for 12 h at room temperature twice with methanol-chloroform (1:1; v/v), twice with methanol-acetone (1:1; v/v), and finally with acetone-water (4:1; v/v). The residue was air dried at 80°C, and stored at -70°C until analysis.

Gel electrophoresis

Agar discs of *P. cinammomi* growing for 7 days in V8 juice agar media (5 x 0.5 cm diameter) were cultured in 125 ml of liquid medium using V8 juice media [16] and after three days of growing health avocado root (0.5g), previously washed with sterile water was added to liquid culture, incubated for 24 h and then extracellular proteinase activity assayed immediately. Proteolytic activity was visualized by SDS-PAGE (12%, w/v). To visualize proteolitic activity, after electrophoresis gels were washed with Triton X-100 in 25 mM Tris-HCI, pH 8.0 and then immersed in 50 ml of 2% (w/v) casein in 50 mM Tris-HCI, pH 8.0, for 30 min at 20°C to allow diffusion of the substrate into the gel. Then the temperature was raised to 37°C and the gel was incubated for 90 min for protein digestion. The gel was fixed and stained with a solution of 40% (v/v) methanol, 10% (v/v) acetic acid and 0.25 (w/v) Coomassie Brilliant Blue R-250. Bands of caseinase activity appeared as clear areas against the blue back-ground.

Data analysis

All experiments were performed at least two times with four replicates. Values are expressed as means with standard deviation. Separation of means was determined by the *t* test.

Results

Proteinase inhibitor activity in avocado after infection with *P. cinnamomi*

In a previous work, we characterized the symptoms in avocado plants infected with *P. cinnamomi* [14] and based on these symptoms, plant responses were assayed up to 4 days after infection. An increase in inhibitory activity was observed in root tissue after 4 days of infection (35%) (Figure 1).

In the course of plant diseases, plant inhibitors can suppress the activity of pathogen enzymes. This prompted us to analyze the interaction between *P. cinnamomi* exoproteinases with avocado inhibitor extracted from non infected root tissue (Figure 2). A strong decrease of extracellular proteolytic activity of the pathogen was detected with avocado root extract relative to control. Proteinase activity dropped to very low levels concomitant with the increase of inhibitor concentration analyzed.
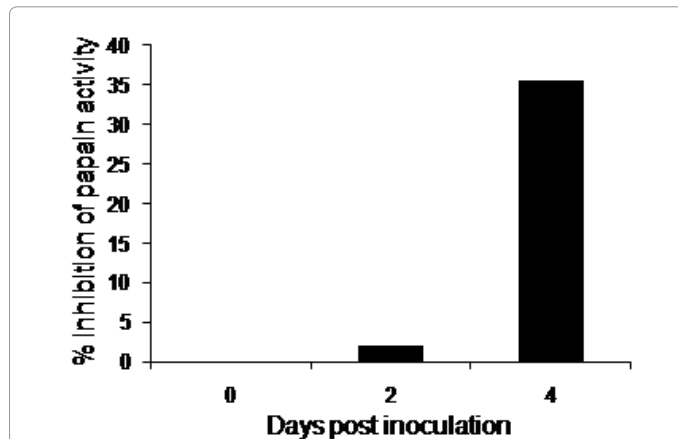
Table 1. Root infection by *P. cinnamomi* of avocado roots treated with avocado inhibitor*

Treatment	Root infection (%)
Control	100 a†
Papain	75 a
PP	95 a
PP+AI	25 b

*Values represents the mean of 8 replications from two experiments.
† Means followed by the same letter are not significantly different (*P*=0.05).
PP=Phytophthora exoproteinase
AI= Avocado inhibitor

Figure 1: Effect of infection with *P. cinnamomi* on avocado root proteinase inhibitor. Root extracts were obtained at different times after infection with *P. cinnamomi* and papain inhibitor activity was assayed. Each value represents the mean of four replications.
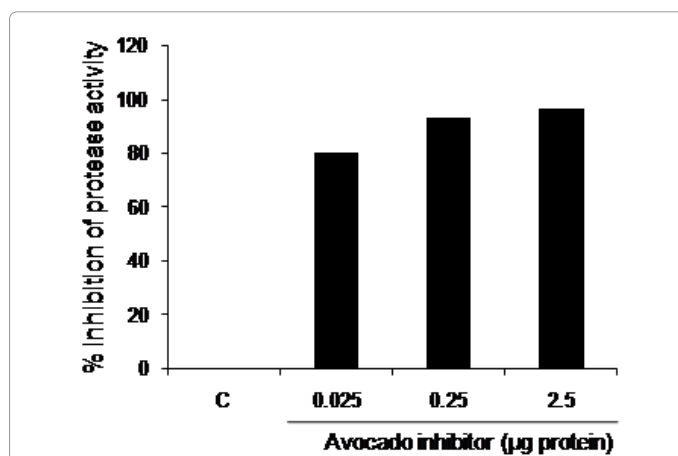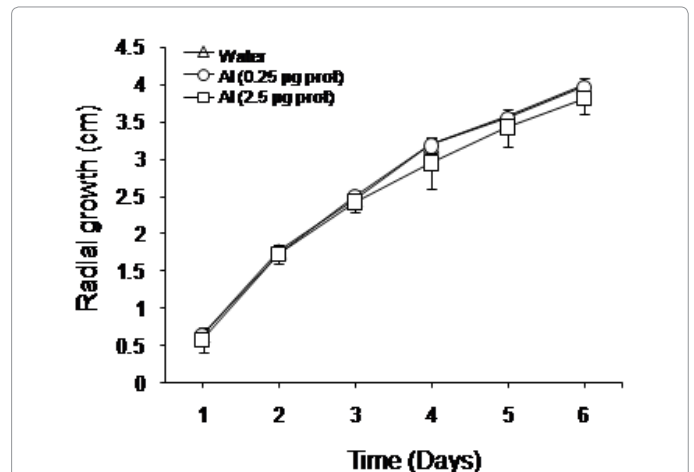

Figure 3: Effect of avocado inhbitor on *P. cinnamomi* mycelial growth. Each value represents the mean of four replications ± standard deviation.

Figure 2: Effect of avocado root inhibitor on extracellular proteinases of *P. cinnamomi. P. cinnamomi* was grown by three days in V8 liquid culture and then extracellular proteins were precipitated with $(NH4)_2SO_4$ at 80% saturation and proteinase activity assayed in the presence of different avocado inhibitor concentrations. Each value represents the mean of four replications.

Effect of avocado inhibitor on root infection

The treatment of avocado roots with avocado inhibitor significantly affected the root colonization by *P. cinnamomi* (Table 1). Infection of avocado roots by *P. cinnamomi* was 25% over control when exoproteinase plus avocado inhibitors were added together to avocado roots. The exoproteinase of *P. cinnamomi* or papain, which was used to compare with exoproteinase of *P. cinnamomi*, did not affect root infection.

Sensitivity of *P. cinnamomi* to avocado inhibitor

To analyze the sensitivity of *P. cinnamomi* to avocado inhibitor, mycelium was grown in Petri dishes in the presence of two externally added inhibitor concentrations: 0.25 and 2.5 µg of protein. Radial growth was measured daily (Figure 3). Our results show that these two inhibitor concentrations had no influence on *P. cinnamomi* radial growth *in vitro*.

Extracellular proteinases production by *P. cinnamomi*

To analyze if the presence of avocado tissue could stimulate the extracellular proteinase production in *P. cinnamomi*, we added different

concentrations of avocado root cell wall to *P. cinnamomi* growing in liquid culture and analyzed the extracellular proteolytic activity three days after growing (Figure 4). No changes in proteinase activity were observed with any cell wall concentration used in this experiment.

A casein-gel showed at least three extracellular proteinases of *P. cinnamomi*, which were not detected when avocado root was incubated for 24 h with the pathogen in liquid culture (Figure 5).

Discussion

Because only a few works of biochemical responses have been done on avocado roots infected with *P. cinnamomi*, to know how this plant regulates the biochemical battery of defense responses in the presence of the pathogen, we explore the role of proteinases during this interaction. After 4 days of infection with *P. cinnamomi*, proteinase inhibitor activity was observed to increase in avocado roots. Extracts of avocado root showed proteinase-inhibiting activity on extracellular proteinases of *P. cinnamomi*. Phytopathogenic microorganisms produce active extracellular proteinases that along with other enzymes play an important role in pathogenesis, e.g., polygalacturonase, pectolyases, and xylanases. When extracellular proteinases are actively involved in pathogenesis, their functions can be widely diversified including participation in microorganism intrusion into the plant,

irreversible inactivation of the protective proteins, and participation in transformations of the pathogen´s own proteins [21]. Proteinases found in pathogens can also play an active role in the degradation of other proteins involved in plant protection, for instance, such enzymes as chitinases and glucanases [22]. Proteinase inhibitors in plants are able to suppress the enzymatic activity of phytopathogenic microorganisms [23], affecting pathogenesis process.

In the presence of avocado root tissue, the extracellular proteinase activity of *P. cinnamomi* decreased in liquid culture, perhaps due to the presence of constitutive proteinase inhibitors present in the health avocado root. The significance of the decrease in exoproteinase activity during the interaction of avocado with the oomycete is unknown until now, and additional work is needed to this respect, however, because the infection strategies used by oomycetes to attack and colonize plants involve the production of germ tubes to enter directly into the intercellular spaces through wound openings on leaves, or form swellings that allow penetration between epidermal cells on root surfaces [24], high levels of extracellular proteinases, specifically during the avocado infection, could be not necessary.

Also, although cell wall-degrading enzymes and other extracellular proteins are thought to be delivered to and secreted during plant infection by oomycetes like *P. cinnamomi*, there is still limited direct demonstration of the role of cell wall-degrading enzymes in plant penetration even other proteins including proteinases.

To better understand the mechanism of interactions between avocado and *P. cinnamomi*, is necessary to intensify the search for targets of inhibitors in the pathogen. Progress in this field, along with the available data, will allow designing more efficient strategies to generate plants with a higher resistance to this pathogen on the basis of mechanisms such as proteinase inhibitors.

References

1. Dickinson M (2003) Resistance mechanisms in plants. Molecular Plant Pathology, London: Taylor & Francis Group, 145-156.

2. Erwin DC, Ribeiro OK (1996) Phytophthora diseases worldwide. St. Paul, Minn, APS Press.

3. Yan HZ, Liou RF (2005) Cloning and analysis of pppg1, an inducible endopolygalacturonase gene from the oomycete plant pathogen *Phytophthora parasítica*. Fung Genet Biol 42: 339-350.

4. Zentemeyer GA (1980) *Phytophthora cinnamomi* and the diseases it causes. Monograph No. 10. St Paul, MN: The American Phytopathological Society.

5. Pegg KG, Coates LM, Korsten L, Harding RM (2002) Foliar and soilborne diseases. In: Whiley AW, Schaffer B, Wolstenholme BN, editors. The avocado: Botany, Production and Uses: CAB International, 299-338.

6. Valueva TA, Mosolov VV (2004) Role of inhibitors of proteolitic enzymes in plant defense against phytopathogenic microorganisms. Biochem (Mosc) 69: 1305-1309.

7. Olivieri FP, Maldonado S, Tonon CV, Casalongue CA (2004) Hydrolytic activities of *Fusarium solani* and *Fusarium solani* f. sp. *eumartii* associated with the infection process of potato tubers. J Phytopathol (Berlin) 152: 337-344.

8. Olivieri F, Zanetti ME, Oliva CR, Covarrubias A, Casalongué C (2002) Characterization of a novel extracellular serine protease of *Fusarium eumartii* and its action on pathogenesis related proteins. Eur J Plant Pathol 108: 63-72.

9. Shevchik VE, Boccara M, Vedel R, Hugouvieux-Cotte-Pattat N (1998) Processing of the pectate lyase PelI by extracellular proteases of *Erwinia chrysanthemi* 3937. Mol Microbiol 29: 1459-1469.

10. Powers JC, Asgian JL, Ekici OD, James KE (2002) Irreversible inhibitors of serine, cysteine, and threonine proteases. Chem Rev 102: 4639-4750.
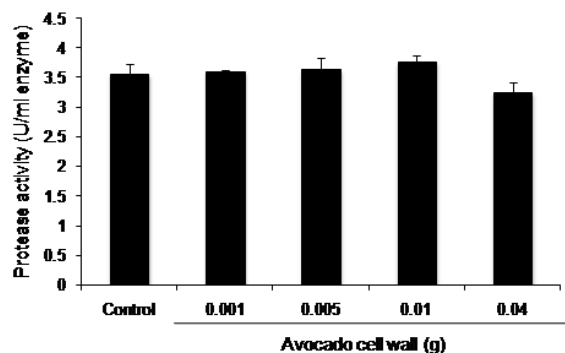
Figure 4: Effect of the avocado cell wall on extracellular proteinase activity of *P. cinnamomi*. Avocado root cell wall was extracted from 3-5 months health plants and added to liquid culture of *P. cinnamomi*, incubated for 24 h and then proteinase activity assayed. Each value represents the mean of four replications ± standard deviation.
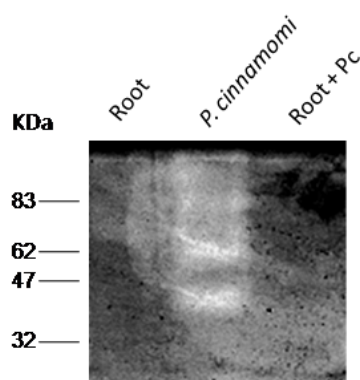

Figure 5: Analysis of proteolytic activity on SDS-PAGE. Culture supernatant was electrophoresed in a polyacrylamide gel and incubated with casein. Light areas indicate protease capable of degrading the casein substrate. Pc=*P. cinnamomi*.

11. Drenth J, Jansonius JN, Koekoek R, Swen HM, Wolthers BG (1968) Structure of papain. Nature 5145: 929-932.

12. Shindo T, Van Der Hoorn RA (2008) Papain-like cysteine proteases: key players at molecular battlefields employed by both plants and their invaders. Mol Plant Pathol 9: 119-125.

13. Chen ZY, Brown RL, Lax AR, Cleveland TE, Russin JS (1999) Inhibition of plant pathogenic fungi by a corn trypsin inhibitor overexpressed in *Escherichia coli.* Appl Environ Microbiol 65: 1320-1324.

14. García-Pineda E, Benezer-Benezer M, Rangel-Sánchez G, Gutiérrez-Segundo A, Arreola-Cortés A, et al. (2010) Regulation of defence responses in avocado roots infected with *Phytophthora cinnamomi* (Rands). Plant Soil 331: 45-56.

15. Messenger BJ, Menge JA, Pond E (2000) Effects of gypsum soil amendments on avocado growth, soil drainage, and resistance to *Phytophthora cinnamomi*. Plant Dis 84: 612-616.

16. Chen Y, Wang D (2005) Two convenient methods to evaluate soybean for resistance to *Sclerotinia sclerotiorum*. Plant Dis 89: 1268-1272.

17. Bolter CJ (1993) Methyl jasmonate induces papain inhibitor(s) in tomato leaves. Plant Physiol 103: 1347-1353.

18. Gvozdeva EL, Volotskaya AV, Sof´in AV, Kudryavtseva NN, Revina TA, et al. (2006) Interaction of proteinase secreted by the fungal plant pathogen *Rhizoctonia solani* with natural proteinase inhibitors produced by plants. Appl Biochem Microbiol 42: 572-579.

19. Schouten A, Tenberge KB, Vermeer J, Stewart J, Wagemakers L, et al. (2002) Functional analysis of an extracellular catalase of *Botrytis cinerea.* Mol Plant Pathol 3: 227-238.

20. Moore JP, Nguema-Ona E, Chevalier L, Lindsey GG, Brandt WF, et al. (2006) Response of the leaf cell wall to desiccation in the resurrection plant *Myrothamnus flabellifolius*. Plant Physiol 141: 651-662.

21. Showalter AM (1993) Structure and function of plant cell wall proteins. Plant Cell 5: 9-23.

22. Alexander D, Lawton K, Uknes S, Ward E, Ryals J (1994) Defense-related gene induction in plants. In: Setlow JK, editor. Genetic Engineering. New York: Plenum Press, 195-212.

23. Mosolov VV, Loginova MD, Fedurkina NV, Benken II. (1976) The biological significance of proteinase inhibitors in plants. Plant Sci Lett **7**: 77-80.

24. Attard A, Gourgues M, Galiana E, Panabieres F, Ponchet M, et al. (2008) Strategies of attack and defense in plant-oomycete interactions, accentuated for *Phytophthora parasitica* Dastur (syn. *P. Nicotianae* Breda de Haan). J Plant Physiol 165: 83-94.