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Analysis of protease activity in *Aspergillus flavus* and *A. parasiticus* on peanut seed infection and aflatoxin contamination

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Abstract *Aspergillus flavus* and *A. parasiticus* are aflatoxin-producing fungi that can infect peanut seeds in field crops. An association between *A. parasiticus* proteolytic enzyme activities and peanut fungal infection was examined. For this study, a model of inductive and non-inductive culture media to produce *A. parasiticus* extracellular protease before infection was used. These *A. parasiticus* cultures were used to infect peanut seeds of cultivars resistant and susceptible to aflatoxin contamination. Peanut seeds of both cultivars exposed to fungi grown on casein medium (inductive medium) showed higher internal and external infection and a higher fungal protease content than those observed on potato dextrose agar (PDA) and sucrose medium (non-inductive media). A further study showed higher fungal colonisation and aflatoxin contamination in seeds of the resistant cultivar pre-incubated with *Aspergillus* extracellular proteases than in those incubated without proteases. Moreover, protease activities affected the viability of non-infected resistant cultivar seeds, inhibiting germination and radicle elongation and enhancing seed tissue injury. The results strongly suggest that protease production by *A. parasiticus* is involved in peanut seed infection and aflatoxin contamination resulting in seed tissue damage, affecting seed viability and facilitating the access of fungi through the testa. The analysis of fungal extracellular proteases formed on peanut seed during infection showed that *A. flavus* and *A. parasiticus* produced metallo and serine proteases; however, there were differences in the molecular masses of the enzymes between both species. The greatest activity in both species was by serine protease, that could be classified as subtilase.

Keywords *Arachis hypogaea* · Extracellular proteases · Seed infection

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Introduction

Aspergillus flavus and *A. parasiticus* are fungi that infect cereal and oil seeds and produce aflatoxins as secondary metabolites which are highly poisonous, teratogenic and carcinogenic (Williams et al. 2004; Abnet 2007). These fungi are found in the soil as saprophytes. However, they can invade peanut seeds, seedlings, and pods during the pre–and post–maturity stages (Middleton et al. 1994). The highest levels of *A. flavus* and *A. parasiticus* infection and aflatoxin contamination are associated with seed and pod damage and when plants are drought-stressed (Dowell et al. 1990; Holbrook et al. 2000; Horn 2006). However, *Aspergillus* can also colonise undamaged

peanut pods and seeds as observed in histological studies (Xu et al. 2000).

Plant pathogens are able to cross the seed cuticle by secreting hydrolytic enzymes that degrade the component of plant cell walls (Sieber et al. 2000; Roncero et al. 2003). It has been reported that pectinases and cutinase secreted by *A. flavus* during cotton boll and corn infection are related to *Aspergillus* virulence (Brown et al. 1992; Guo et al. 1996, Mellon et al. 2007). Proteases are other enzymes identified in a number of plant pathogenic fungi and may play a critical role in successful host colonisation (Di Pietro et al. 2001; Pekkarinen and Jones 2002; Bindschedler et al. 2003). Physiological studies have suggested that proteases could have a direct effect on the proteins of a plant's plasma membrane or cell wall (Movahedi and Heale 1990; Carlile et al. 2000). *Aspergillus* spp. proteases have been shown to be involved in invasive aspergillosis in mammals (Reichard et al. 1990; Monod et al. 2002; Reed and Kita 2004; Mellon et al. 2007). However, there is limited evidence of the role of *A. flavus* and *A. parasiticus* proteases in plant infection. In a comparative study of virulent and avirulent *A. flavus* in cotton boll infection, the virulent strain showed higher proteases and α-amylases activities, suggesting some correlation between enzyme activity and *A. flavus* virulence (Brown et al. 2001). In a preliminary study, *A. parasiticus* and *A. flavus* were induced to produce different extracellular enzymes (pectinases, amylases, cutinases and proteases) by growing the fungus in specific substrate culture media and exposing peanut seeds to infection (unpublished work). Only the fungus with protease and cutinase induction showed a significant higher fungal infection in peanut seed.

In the present work, a laboratory study was performed to evaluate the role of extracellular fungal proteases during *A. parasiticus* infection on peanut seed as an approach to understanding their role during natural infection. This paper details (*i*) an association between *A. parasiticus* extracellular protease induction and the ability of this species to infect peanut seed and (*ii*) the effects of exogenously-applied extracellular *A. parasiticus* proteases on fungal infection, aflatoxin contamination and viability of peanut seeds. Furthermore, the extracellular proteases synthesised by *A. flavus* and *A. parasiticus* during peanut seed colonisation were characterised.

Materials and methods

Peanut cultivars

Two peanut cultivars were provided by the Instituto Nacional de Tecnologia Agropecuaria (INTA), Manfredi Experimental Station, Cordoba, Argentina. The PI337394 cultivar was characterised as resistant and the Florman INTA cultivar was characterised as susceptible to *Aspergillus* infection (Asis et al. 2005).

Aspergillus source

Isolates 6 (*A. flavus*) and 18 (*A. parasiticus*) were obtained from peanut seed and pods of peanut plants grown in Cordoba, Argentina and were identified and classified as virulent *Aspergillus* spp. (Asis et al. 2005). A reference strain of *A. parasiticus* (NRRL 2999) characterised as an aflatoxigenic strain was also used. The fungi were grown on potato-dextrose agar (PDA) (Merck) at 30°C for 7 days and the spore suspensions obtained by washing the surface of the cultures with Tween 80 (0.5 ml l^{-1}.)

Aspergillus extracellular protease production on inductive and non-inductive culture media

Casein medium (CM) containing casein as the only carbon source was used to induce the synthesis of *A. parasiticus* extracellular proteases (inductive medium). In contrast, sucrose medium (SM) containing sucrose as the only carbon source and PDA containing mainly starch as the carbon source and a low protein content were defined as non-inductive.

CM contained (l^{-1} of phosphate buffer 50 mM, pH 8) the following: 10 g casein from bovine milk (Fluka), 0.5 g NH_4SO_4, 0.05 g $MgSO_47H_2O$, 1 ml of trace elements (700 mg $Na_2B_4O_7$, 680 mg $Na_2MoO_4.2H_2O$, 10 g $Fe_2(SO)_2.6\ H_2O$, 300 mg $CuSO_4.4\ H_2O$, 1.76 g $ZnSO_4.7\ H_2O$, 110 mg $MnSO_4$), and 15 g agar. SM contained 10 g sucrose (Merck) instead of casein. PDA (Merck) was prepared following the manufacturer's instructions. One ml of *A. parasiticus* isolate 18 spore suspension ($1x10^7$ spores ml^{-1}) were seeded and spread on a Petri plate containing 15 ml culture medium and incubated for 10 days at 30°C to allow fungal colonisation and protease production. The assay was conducted in ten

replicates. After 10 days, the proteolytic activity was measured in each culture medium and thereafter, the Petri plates with fungus were used for the seed infection assay.

Proteases were extracted from two circular plugs (1.3 cm diam) that were cut from each culture medium. The plugs were placed in a container with 5 ml phosphate buffer saline (PBS) pH 7.2 and shaken for 1 h at room temperature. The extraction solution was filtered and used to measure the enzymatic activity. Proteolytic activity in culture media was measured against fungal biomass estimated by ergosterol content. Ergosterol was extracted from culture media plugs (0.9 cm diam) with 500 µl methanol by sonicating for 10 min, and determined by HPLC (Asis et al. 2005).

Proteolytic activity measurement

Proteolytic activity of enzymes was estimated with azocasein (Sarath et al. 1989). One hundred microlitres of enzyme extract was incubated with 50 µl of enzyme buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl and 1.8 mM $CaCl_2$) and 25 µl 10 g l^{-1} azocasein (Sigma-Aldrich) solution (in 0.1 M NaOH) for 1h at 25°C. Reaction was stopped with 25 µl of 100% trichloroacetic acid. The samples were centrifuged at 11,000 g for 5 min and the absorbance recorded at 415 nm. One proteolytic activity unit (PAU) was defined as the amount of enzyme required to produce an absorbance change of one unit under the assay conditions.

Study of the fungal infection and protease content in peanut seeds exposed to *A. parasiticus* grown on inductive and non-inductive media.

Seeds of resistant and susceptible cultivars were examined closely for visible injury, and undamaged peanut seeds were selected for this assay. The seeds were disinfected by soaking in sodium hypochlorite (0.5% available chlorine) for 3 min, washed several times with sterile distilled water (SDW) and re-hydrated to 20% moisture by soaking in SDW (Asis et al. 2005). The disinfected peanut seeds of both cultivars (seven seeds of each) were added to each plate of CM, SM or PDA containing the fungi grown for 10 days and then incubated for a further 3 days at 30°C. Each treatment was replicated three times. The assay was conducted four times. After 3 days, internal and external seed infection as well as fungal extracellular protease activity on the seeds were measured in each culture medium. Natural infection was measured by exposing disinfected seeds of both cultivars to inductive and non-inductive media free of fungus under the same conditions as the inoculated culture media.

The external seed infection was measured by visual inspection using an ordinal scale from 0 to 5 (0=0% 1= < 20%, 2=21–40%, 3=41–60%, 4=61–80%, 5=81–100% of the seed surface covered by conidiophores bearing conidia) (Tubajika and Damann 2001). The fungal infection rating was determined for individual kernels of each cultivar on each culture medium and the percentage of external infection severity was calculated using the following equation:

$$\sum \left(N^0 \text{ of seeds classified in each scale value} \times \text{scale value} / \text{total } N^0 \text{ assayed seeds} \times 5 \right) \times 100$$

Fungal extracellular proteases produced on the exposed seeds were extracted as follows: seeds from each replicate were soaked with 10 ml PBS and shaken for 20 min. The extracted solution of each replicate was filtered and used to measure the enzymatic activity as described above. Thereafter, these seeds were used for internal infection analysis.

Internal infection was assayed by direct plating. Seeds exposed to fungi were surface-disinfected with sodium hypochlorite (1% available chlorine), washed several times with SDW and placed in Petri plates containing PDA medium. The seeds were incubated at 30°C for 48 h and the number of colonised seeds was measured. Internal infection incidence was expressed as the percentage of infected seeds over the total number of assayed seeds.

Isolation of proteases from inductive medium

Aspergillus parasiticus isolate 18 was incubated in CM liquid medium at 30°C for 10 days. After

incubation, the culture medium was filtered using paper filter and the extracellular proteases were precipitated from the filtered culture medium by the addition of -20°C chilled acetone (80% of total volume). The solution was shaken for 1 h at 4°C and centrifuged at 7,500 *g*. The precipitate was dissolved in phosphate buffer (50 mM, pH 7) and sterilised by filtration with a 0.22 µm filter (Millipore Corporation). The proteolytic activity of the filtered culture medium and the precipitated proteins was measured by azocasein assay and the protease molecular weights were evaluated by zymography and compared with the proteases produced on peanut seed.

The effect of *Aspergillus* protease activity on fungal infection, aflatoxin contamination and viability of peanut seed

To study the effect of protease activity on fungal infection and aflatoxin contamination, peanut seeds of the resistant cultivar were exposed to the isolated proteases from CM and then infected with the fungus. To perform this study, intact peanut seeds of the resistant cultivar were disinfected with sodium hypochlorite (0.5% available chlorine) as described above. Unwounded seeds (40 seeds per replicate) were immersed in a protease solution (0.8 PAU ml^{-1}) and incubated for 24 h at 30°C. Control seeds were left intact or the endosperm was wounded to a depth of 1 mm using a 20-gauge needle and immersed in phosphate buffer (50 mM, pH 7) alone. Unwounded and wounded controls (40 seeds per replicate) were incubated under the same conditions as seeds treated with proteases. After incubation, the unwounded and wounded controls and protease-treated seeds were washed three times with 100 ml SDW, placed on sterile Petri plates and infected with 1 ml of *A. parasiticus* isolate 18 spore suspension (1×10^{8} spores ml^{-1}). The inoculated seeds were shaken to homogenise the inoculum on the seed surface and incubated for 5 days at 30°C in a wet chamber. The assay was conducted in triplicate. Aflatoxins were extracted from peanut seeds with 80% v/v methanol, cleaned-up with Aflatest P immunoaffinity column (Vicam) and measured by HPLC (Asis et al. 2005). The fungal colonisation was determined by measuring ergosterol levels. Ergosterol was extracted from samples with methanol, saponified with 2 M sodium hydroxide, extracted with 3×3 ml of pentane and measured by HPLC (Asis et al. 2005).

A second assay was performed with unwounded seeds incubated with proteases and protease inhibitors. Two protease inhibitor preparations were added to protease solution to obtain the following inhibitor concentrations (a) 5 mM PMSF (Sigma-Aldrich) and (b) 5 mM PMSF, 2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 30 µm pepstatinA, 28 µm E-64, 80 µm bestatin, 40 µm leupeptin, and 1.6 µm aprotinin (Sigma-Aldrich protease mix P8340). Each preparation was incubated at 37°C for 30 min. After incubation, the unbounded protease inhibitors were removed by sephadex G25 NAP10 column (GE Healthcare) to avoid undesirable effects on seed viability. Simultaneously, a protease solution free of protease inhibitors was processed under the same conditions and then diluted with phosphate buffer (50 mM, pH 7) to obtain a protease activity of 0.5 PAU ml^{-1}. The protease-inhibitor mixtures were also diluted as per the protease solution with buffer plus 0.2 mM EDTA. Disinfected seeds (40 seeds per replicate) were incubated with the protease solution, protease plus inhibitors or buffer plus EDTA at 30°C for 4 h. The seeds were washed, infected and incubated as previously described and aflatoxin and ergosterol content measured.

To study the effect of fungal proteases on seed viability, seed germination and radicle elongation were measured in non-infected seeds of the resistant cultivar exposed to proteases. Undamaged seeds (40 seeds per replicate) were disinfected with sodium hypochlorite (0.5% available chlorine) and immersed in protease solutions at three concentrations (0.830, 0.416, 0.278 PAU ml^{-1}) prepared from proteases isolated from CM. Control seeds (40 seeds per replicate) were disinfected and immersed in phosphate buffer (50 mM, pH 7). As an inactivated protease control, a seed lot (40 seeds per replicate) was disinfected and immersed in a protease solution (0.830 PAU ml^{-1}) previously boiled for 10 min. Under these conditions, controls and protease-treated seeds were incubated at 30°C for 12 h. After incubation, seeds were washed three times with 100 ml SDW and incubated in a wet chamber at 30°C for 72 h. The assay was conducted in triplicate. The number of germinated seeds was measured and seed germination expressed as the percentage of germinated seeds over total number of evaluated seeds. The seed

radicle length elongation was determined and expressed as the average radicle length (cm) for each treatment. Tissue damage in the seeds immersed in 0.830 PAU ml^{-1} protease solution was evaluated by microscopy and compared with the control seeds. The analysis was carried out in the seed embryonic axis without the testa dyed overnight with 10 g l^{-1} tetrazolium salt solution at room temperature. The images were recorded using stereomicroscopy (Leica) with a 20X magnification.

Analysis of *A. flavus* and *A. parasiticus* extracellular proteases produced on peanut seeds

Fifty grams of autoclaved peanut seeds were inoculated with 1 ml of spore suspension (1×10^9 spores ml^{-1}) of *A. flavus* isolate 6 and *A. parasiticus* isolate 18, and strain NRRL 2999. The inoculated seeds were incubated for 48 h at 30°C. The extracellular proteases were extracted by washing the seeds with 50 ml PBS for 1 h. To test the possible peanut protease origin, 50 g of peanut seeds treated as described above were inoculated with 1 ml of Tween 80 (0.5 ml l^{-1}).

The activity of fungal proteases produced on peanut seed was analysed by the azocasein and zymographic tests. The protease production in CM inductive medium was also analysed by the zymography test and compared with the protease production of *A. parasiticus* isolate 18 on peanut seed. In the zymography test the enzymes were separated by electrophoresis and detected by their ability to hydrolyse casein in the migration region. The electrophoresis was carried out using 10% acrylamide co-polymerised with 1 g l^{-1} sodium casein (Sigma-Aldrich). A vertical electrophoresis system Miniprotean (Bio-Rad) for $8\times10\times0.07$ gels was used. The enzyme extracts were diluted (1:4) in sample buffer containing SDS without reducing agents. The diluted samples were not boiled before loading onto the gels. Electrophoresis was performed at a constant voltage of 100 V in an ice bath.

After electrophoresis was completed, the gel was removed and shaken at room temperature for 1 h in 25 g l^{-1} Triton X-100 to remove SDS. The gel was washed with SDW several times and incubated for 48 h at 37°C in an enzyme buffer (50 mM Tris-HCl (pH: 7.5), 200 mM NaCl, and 1.8 mM $CaCl_2$) to hydrolyse the casein. The gels were also incubated in the same buffer, but at 4.5 and 10 pH. After incubation, the gels were stained with 5 g l^{-1} coomassie brilliant blue G250 in 30% (v/v) methanol and 10% (v/v) acetic acid. The unstained regions showed the protease migration in the gels. Protein standards (Bio-Rad) with several molecular masses were myosin (200 kDa), α-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa) and carbonic anhidrase (31 kDa).

Proteases were partially characterised by an inhibition test using specific protease inhibitors. For this study, the inhibitors were added to enzyme buffers of the azocasein and zymographic tests and the inhibition was visualised by the absence or decrease of the protease activity relative to controls in the absence of inhibitors. The protease activity in the gels was estimated by densitometric analysis using Image J software. To inhibit the four classical protease groups: metallo, serine, cisteine and aspartic protease, 10 mM EDTA, 5 mM PMSF, 100 μM iodoacetamide and 1 mM pepstatin were used, respectively. The serine protease activity was also evaluated with 7.3 nM aprotinin, 100 μM antipain and 150 μM tosyl-L-lysine chloromethyl ketone (TLCK).

Statistical analysis

All data were subjected to Analysis of Variance (ANOVA). When significant differences were found by ANOVA, the Tukey test was used to compare all the treatments, and the Dunnett test was used to compare the treatments to reference controls in the study of the *Aspergillus* extracellular proteolytic activity effects on peanut fungal colonisation, aflatoxin production and seed viabilty.

Results

Aspergillus extracellular protease production on inductive and non-inductive culture media and peanut seeds

Significant differences on protease production were found among culture media ($P<0.05$) (Table 1). Casein medium (CM) was an inductive medium of *A. parasiticus* extracellular proteases, since extracellular protease activity was 45 and 11 times higher

Table 1 Protease production on culture media and peanut seeds by isolate 18 (*A. parasiticus*) after 10 days and 48 h of colonisation, respectively

	Casein medium	Sucrose medium	PDA
Culture media[a]	54.4±20 [c]	4.9±1.1	1.2±0.4
Peanut seed[b]			
Florman	7.41±4.50[cd]	0.48±0.18	0.35±0.12
PI 337394	0.89±0.13[c]	0.38±0.17	0.16±0.19

[a] Protease activity expressed as protease units μg^{-1} ergosterol

[b] Protease activity expressed as protease units per seed

[c] significant differences among culture media $P < 0.05$

[d] significant differences between cultivars $P < 0.05$

than that obtained in PDA and SM media respectively (Table 1). Similarly, a significantly higher proteolytic activity ($P < 0.05$) was found on peanut seeds of both cultivars after exposure to *A. parasiticus* isolate 18 for 3 days on CM inductive medium (Table 1). The Florman susceptible cultivar seeds also showed a significantly higher proteolytic activity ($P < 0.05$) than the resistant cultivar seeds in the inductive medium.

Fungal infection in peanut seeds exposed to *A. parasiticus* grown on inductive and non-inductive media

The extent of external and internal seed infection of the resistant and susceptible cultivars by *A. parasiticus* isolate 18 72 h post-inoculation is shown in Fig. 1. The severity of external infection on the resistant cultivar seeds was significantly lower ($P < 0.05$) than that detected on the susceptible cultivar seeds on all culture media (Fig. 1a, c and d). A significantly higher ($P < 0.05$) external infection was detected on the resistant cultivar seeds incubated on CM (65% severity) than that detected on PDA and SM (0% severity) (Fig. 1a). Similar to the resistant cultivar, the external fungal infection on the susceptible cultivar was significantly higher on CM medium (98% severity) than that detected on PDA and SM (54% and 60% severity, respectively) (Fig. 1a, c and d). A significantly higher internal infection ($P < 0.05$) was observed on the resistant cultivar seeds incubated on CM media (69% incidence) than that detected on PDA and SM media (16% and 35% incidence, respectively) (Fig. 1b). The suceptible cultivar seeds showed a significantly higher internal infection ($P < 0.05$) than the resistant cultivar over all culture media tested, except on CM medium where no differences were found (Fig. 1b). No significant differences were found in the susceptible cultivar over all culture media. Natural infection was not detected on peanut seeds of the resistant cultivar whereas 7% infection was detected in the susceptible cultivar.

The effect of *A. parasiticus* extracellular proteolytic activity on fungal infection, aflatoxin contamination and viability of peanut seeds

Fungal infection and aflatoxin contamination of unwounded or wounded seeds of the resistant cultivar pre-incubated with *A. parasiticus* proteases or phosphate buffer, and then infected with *A. parasiticus*, are shown in Fig. 2a and b. Unwounded infected seeds pre-incubated with fungal proteases (protease treatment) as well as wounded infected seeds resulted in a significant increase in fungal colonisation and aflatoxin production ($P < 0.05$) compared to unwounded infected seed without protease treatment. Neither colonisation nor aflatoxin production was observed in the unwounded infected seeds without protease treatment (Fig. 2a and b). The effects of proteases on fungal infection and aflatoxin contamination in unwounded seeds with the presence or absence of proteases inhibitors are shown in Fig. 2c and d. Only the seeds incubated with a protease solution free of protease inhibitors showed a significant increase in fungal colonisation and aflatoxin contamination ($P < 0.05$) compared to seeds incubated with buffer alone (seeds without protease treatment). Seeds incubated with proteases plus protease inhibitors did not show any differences in fungal colonisation and aflatoxin contamination compared to seeds with no protease treatment (Fig. 2c and d).

The seed germination and root elongation from uninfected and undamaged seeds of the resistant cultivar were studied at different protease concentrations (Fig. 3). Significant differences ($P < 0.05$) were found in both seed germination (Fig. 3a) and root elongation (Fig. 3b) at the three protease concentrations with lower values than the protease-free control. A negative association between protease activity and seed germination or radicle elongation was observed. The seed germination and root elongation inhibition reverted with heat inactivation (inactivated protease control) (Fig. 3a and b).

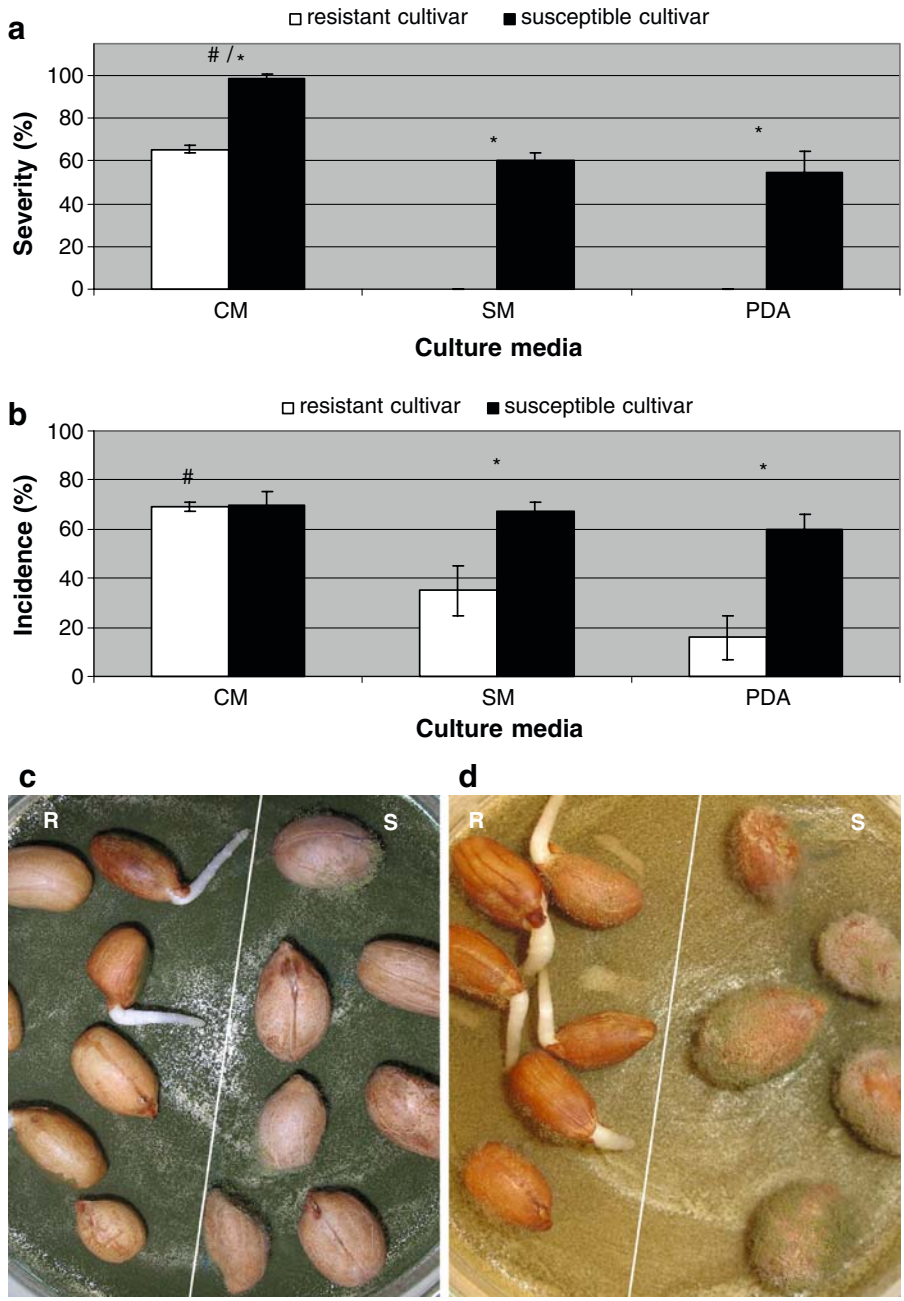

Fig. 1 Study of external and internal infection of resistant and susceptible cultivar peanut seeds after 72 h of exposure to *Aspergillus parasiticus* grown on potato dextrose agar (*PDA*), sucrose medium (SM) and casein medium (CM). **a** External seed infection was measured by visual inspection using an ordinal scale from 0 to 5 and expressed as % severity: $\sum (N^0 \textit{ of seeds classified in each scale value} \times \textit{scale value} / \textit{total } N^0 \textit{ assayed seeds} \times 5 \times 100$. **b** Internal seed infection was expressed as the percentage of infected seeds over the total number of assayed seeds (incidence). **c** Photograph of infected peanut seed on SM medium of resistant (*R*) and susceptible (*S*) cultivar after 72 h. **d** Photograph of infected peanut seed on CM medium of resistant (*R*) and susceptible (*S*) cultivar after 72 h. (*) significant differences ($P < 0.05$) between both cultivars in each culture media. (#) significant differences ($P < 0.05$) among culture media for each cultivar

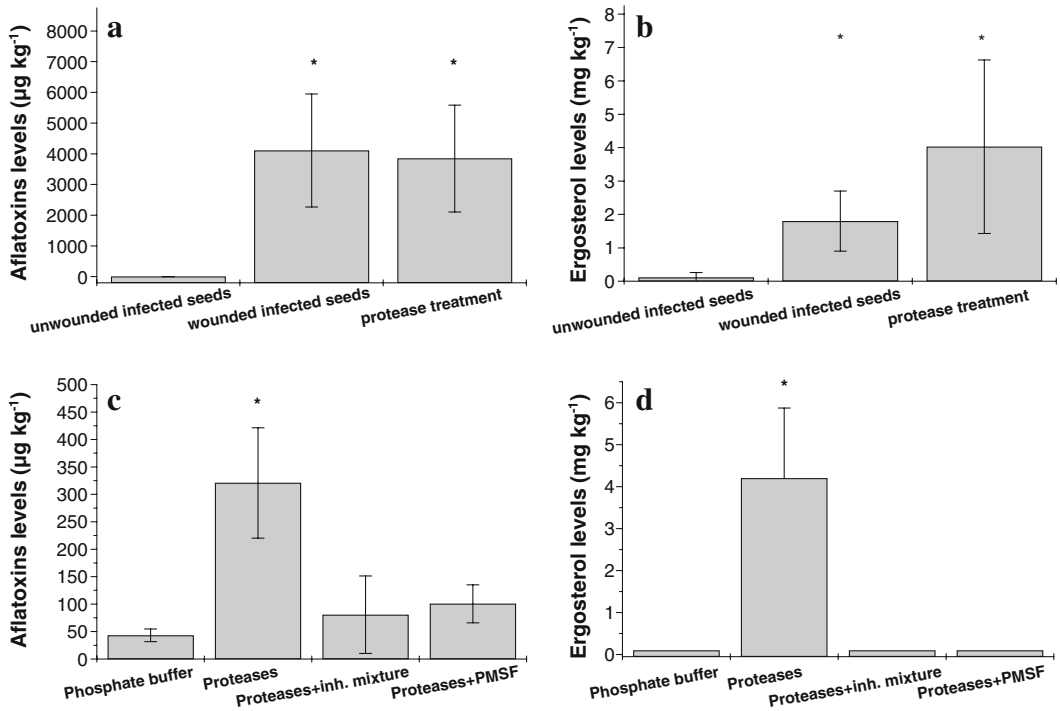

Fig. 2 Fungal biomass and aflatoxin concentration of resistant cultivar seeds infected 5 days post-inoculation with *A. parasiticus* isolate 18. **a** Aflatoxin and **b** ergosterol concentrations of: -unwounded peanut seeds pre-incubated for 12 h with proteases (protease treatment), or phosphate buffer alone (unwounded infected seeds) before infection and seeds artificially wounded before infection. **c** Aflatoxin and **d** ergosterol levels of unwounded peanut seeds pre-incubated for 4 h before infection with: fungal proteases alone (proteases) or together with PMSF/EDTA mix (proteases +PMSF) or PMSF/EDTA/commercial protease inhibitor mix (proteases+inh. mixture) or phosphate buffer alone (phosphate buffer). (*) significant differences relative to protease-free control ($P < 0.05$)

The microscopic analysis was carried out in the embryonic axis from non-infected seeds exposed to the protease solution with the highest activity (0.830 PAU ml^{-1}). The study showed that the protease treatment caused injury of the different seed tissues, such as the external and internal cotyledons and radicle (Fig. 4). The untreated seeds with proteases showed smooth and light red-dyed tissues (Fig. 4b, d and f). The damage in seeds treated with proteases appeared as eroded areas dyed from red to dark-red by tetrazolium salts (Fig. 4a, c and e).

Analysis of *A. flavus and A. parasiticus* extracellular proteases

The extracellular protease activity produced by the *A. parasiticus* NRRL 2999 strain, isolate 18 and *A. flavus* isolate 6 on peanut seeds was 0.5, 22.7, 11.1 PAU g^{-1} respectively after 48 h post-inoculation. Protease activity was not found on peanut seeds inoculated with Tween (0.5 ml l^{-1}). The *A. flavus* and *A. parasiticus* extracellular proteases extracted from infected peanut seeds were analysed by zymography. The zymogram of proteases synthesised by *A. parasiticus* showed two groups of extracellular enzymes: (*i*) a band showing the highest proteolytic activity with a molecular mass of 91 kDa and (*ii*) a band with lower proteolytic activity and a molecular mass of 43.5 kDa. *Aspergillus flavus* also synthesised two groups of extracellular enzymes: (*i*) a band with the greatest proteolytic activity with an estimated molecular mass of 186 kDa and (*ii*) a band with lower proteolytic activity with an estimated molecular mass of 43.5 kDa. In both species, the same protease activity profile was observed in gels incubated at 4.5, 7.5, and 10 pH.

The inhibition test in the zymography test showed a partial inhibition in the 91-kDa band (25% inhibition) for the *A. parasiticus* gel incubated with PMSF and in the 186-kDa band (29% inhibition) for the *A. flavus* gel, indicating the presence of serine-protease. The gel

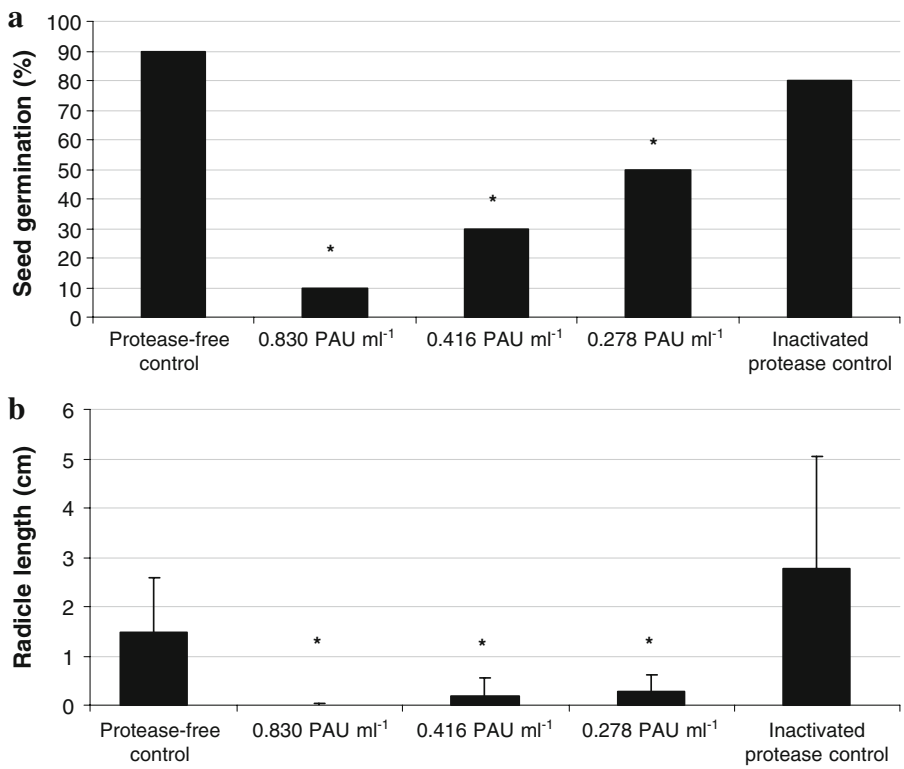

Fig. 3 Effects of *A. parasiticus* extracellular protease activity on germination and radicle elongation of resistant cultivar non-infected seeds. The seeds were immersed in protease solutions with different protease activity (PAU ml^{-1}) for 12 h at 30°C before germination. **a** Percentage of germinated seeds over the total number of assayed seeds after 72 h of incubation. **b** average radicle length (cm) of germinated seeds. Protease-free control: seeds immersed in phosphate buffer before germination. Inactivated protease control: 0.830 PAU ml^{-1} protease solution was heated in a water bath for 10 min and then incubated with the seeds before germination. (*) significant differences relative to protease-free control ($P < 0.05$)

incubated with EDTA showed a strong inhibition (100%) of *A. parasiticus* proteases and *A. flavus* proteases in the 43.5 kDa band, indicating the presence of metallo-protease. The inhibition test in the azocasein assay showed that proteolytic activity of *A. parasiticus* proteases was only inhibited with 5 mM PMSF and 10 mM EDTA by 71.4% and 9.4% respectively, whereas *A. flavus* proteolytic activity was inhibited with 5 mM PMSF and 10 mM EDTA by 62.9% and 11.9% respectively. Neither *A. flavus* nor *A. parasiticus* proteases were inhibited with iodoacetamide or pepstatin in the azocasein and zymographic assays.

The serine protease activity of *A. parasiticus* (isolate 18) was investigated with serine protease inhibitors and was partially inhibited with antipain (49.5%) apart from PMSF. Inhibition of proteolytic activity was not detected with aprotinin and TLCK. The extracellular protease activity produced on CM and peanut seeds by *A. parasiticus* isolate 18 was also analysed and compared by zymography. Two bands with protease activity corresponding to proteins with molecular masses of 91 kDa and 43.5 kDa were detected in the zymograms of protease from CM and from infected peanut seeds.

Discussion

Peanut pods are in direct contact with soil populations of aflatoxigenic fungi. Two modes of infection are proposed in peanuts. Firstly, seeds from pods without visible damage may be invaded by aflatoxigenic fungi when plants are drought-stressed. Secondly, pod and seed damage allows for direct fungal invasion from soil (Horn 2006). The success of fungal colonisation in the host depends on its ability to cross the external

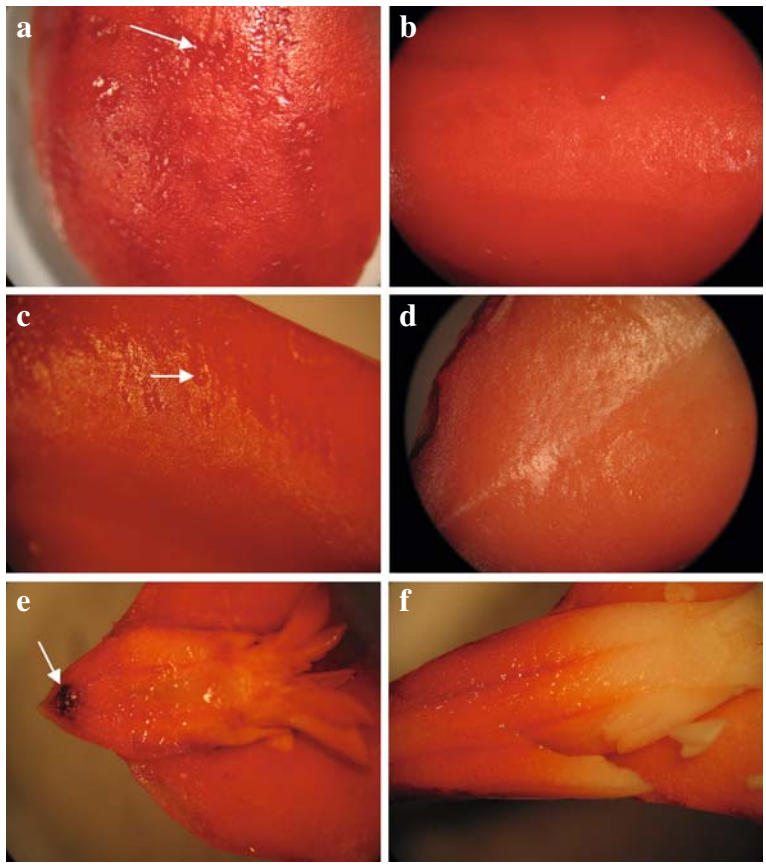

Fig. 4 Microscopic images (×20) of embryonic axis from non-infected germinated seeds of resistant cultivar incubated in 0.830 PAU ml^{-1} fungal protease solution (protease treatment) or phosphate buffer (control) at 30°C for 12 h previous to germination. Embryonic axes were dyed overnight with 10 g l^{-1} tetrazolium salt solution. **a** External cotyledon of protease treatment, **b** External cotyledon of control seeds, **c** Internal cotyledon of protease treatment, **d** Internal cotyledon of control seeds, **e** Radicle dissection of protease treatment, **f** Radicle dissection of control seeds. Arrows show the injuries in the tissues, pink to light-red area indicates normal redox activity in the tissue, red to dark-red area indicates abnormal necrotic activity in the tissue

barrier and avoid the defence mechanism to reach the substrate. Use of extracellular enzymes to degrade host barriers seems to be a common strategy used by pathogenic fungi (Sieber et al. 2000; Roncero et al. 2003). Proteases of *A. flavus* and *A. parasiticus* have been shown to be important components in the fungal infection of mammals and insects (Reichard et al. 1990; Monod et al. 2002; Reed and Kita 2004). However, there is not evidence of these enzymes being involved in *Aspergillus* plant infection. Studies of the role of fungal extracellular proteases in plant pathogenicity have been found to be controversial. In studies where protease genes were selectively disrupted, no changes in pathogenicity were reported; however, compensation for the loss of one enzyme activity by up-regulation of others was observed (Ramesh et al. 1995; Bindschedler et al. 2003; Plummer et al. 2004). Other studies have shown protease activity correlates with pathogenicity (Sreedhar et al. 1999; Brown et al. 2001). Moreover, functional studies showed that proteases could degrade proteins of plant cell walls (Movahedi and Heale 1990; Carlile et al. 2000) and pathogenesis-related (PR) proteins (Olivieri et al. 2002).

This study identified that *A. parasiticus* extracellular proteases facilitated peanut seed colonisation. To our knowledge, this is the first report of an association between *Aspergillus* extracellular protease production and non-damaged peanut seed infection. For this study, a model of inductive and non-inductive culture media to produce *A. parasiticus* extracellular protease before infection was used. Peanut seeds of resistant and

susceptible cultivars were exposed to *A. parasiticus* grown under these conditions and seed fungal infection compared. The study showed that fungi grown on an inductive medium not only produced a significantly higher protease amount ($P < 0.05$) in the culture medium but also produced significantly higher ($P < 0.05$) external and internal seed infection and protease amounts on the seeds of both cultivars after infection (Table 1 and Fig. 1). The difference in resistance between cultivars agrees with a previous report (Asis et al. 2005). The susceptible cultivar was heavily infected both externally and internally in the inductive and non-inductive media; however the highest infection was obtained on the inductive medium (Fig. 1a and b). The resistant cultivar showed resistance to infection in non-inductive media, but this resistance disappeared in the inductive medium where the highest infection levels occurred (Fig. 1a and b).

These results suggest that the up-regulation of the synthesis and activity of extracellular proteases might be the cause of the resistance breakdown on the resistant cultivar seed and the higher fungal infection in both cultivar seeds. To test this hypothesis, the effects of the proteolytic activity of *A. parasiticus* extracellular proteases on fungal infection and aflatoxin contamination on the resistant cultivar peanut seeds was investigated. The resistance in this cultivar has been associated with the testa (Asis et al. 2005). Several cultivars have shown that the testa functions as a physical or chemical barrier against fungal infection and aflatoxin contamination (Liang et al. 2006). In the present study, we found that fungal proteolytic activity raised not only seed colonisation (Fig. 2a) but also aflatoxin contamination (Fig. 2b) in the unwounded seeds to the same levels as in the wounded seeds where the testa barrier was broken. Seed damage was consistently associated with aflatoxin contamination (Dowell et al. 1990; Horn 2006). Xu et al. (2000) reported that *A. parasiticus* could colonise testa cells, but could not produce aflatoxin until it reached the cotyledons. Therefore, the higher fungal colonisation and aflatoxin contamination in seeds treated with protease suggest that protease treatment broke down the testa resistance. The presence of protease inhibitors (PMSF/EDTA mixture or PMSF/EDTA/commercial protease inhibitor mix) reduced the observed effects of proteases on fungal infection and aflatoxin contamination to the same levels as occurred on seed without protease treatment (Fig. 2c and d). These results support the theory that the effect on fungal colonisation and aflatoxin contamination are only produced by protease activity.

The effect of *A. parasiticus* extracellular proteases on seed viability was also studied. The exogenously-applied fungal proteases to non-infected seeds of the resistant cultivar caused an inhibitory effect on seed germination (Fig. 3a) and on radicle length (Fig. 3b). We found that as the protease concentration increased, the inhibitory effect also increased. When proteases were inactivated by heating, no inhibitory effects on germination and radicle elongation were observed, indicating that the inhibitory effects were produced by protease activity (Fig. 3a and b). The microscopy study of seeds treated with proteases revealed severe injury on several seed tissues (Fig. 4) that could explain the inhibition of germination and radicle elongation.

The present results show that fungal protease activity produced seed tissue damage, affecting seed viability and facilitating the access of *Aspergillus* through the testa. As a consequence, there was an increase in aflatoxin contamination. Thus, protease inhibition during seed infection is shown as an opportunity to reduce aflatoxin contamination. Past studies have shown that seed corn resistance to aflatoxin contamination is associated with seed protease inhibitors (Tubajika and Damann 2001; Chen et al. 2007). We have previously reported a partial resistance to aflatoxin contamination in cotyledons of the resistant cultivars used in the present study (Asis et al. 2005). Current work in our laboratory has shown that a seed protease inhibitor from cotyledons might be responsible for this resistance (unpublished work). Moreover, a recent study has reported the expression of four putative protease inhibitor genes from 25 up-regulated genes in peanut seeds in response to *A. parasiticus* infection (Luo et al. 2005). The relevance of the cotyledon protease inhibitor in the seed defence mechanism against *A. parasiticus* and *A. flavus* infection is a promising tool for aflatoxin contamination control.

The extracellular protease pattern of *A. flavus* described by different authors (Rhodes et al. 1990; Ramesh et al. 1994, 1995) consisted of a 36 kDa serine protease and 23 kDa metallo protease. These proteases are involved in invasive lung infections (Monod et al. 2002; Reed and Kita 2004) and they have been isolated from culture media that contained

ellastin or collagen as the sole nitrogen source (Reichard et al. 1990; Rhodes et al. 1990). In this present work, we report extracellular proteolytic activity during peanut seed infection by *A. flavus* and *A. parasiticus.* A major 186 kDa serine-protease and 43.5 kDa metallo-protease was produced by *A. flavus.* A major 91 kDa serine-protease and 43.5 kDa metallo-protease was produced by *A. parasiticus*. The same molecular weight pattern of *A. parasiticus* extracellular proteases found in seeds was also produced on casein medium.

In relation to the serine protease activity of *Aspergillus* extracellular proteases, the inhibition of azocasein hydrolysis by PMSF and antipain and the lack of inhibition by trypsin-like protein inhibitors (aprotinin, TLCK) suggest that serine protease activity could originate from a subtilisin-like protein. This class of serine proteases was identified by Ramesh (Ramesh et al. 1994) in *A. flavus* and by Kolattukudy et al. (1993) in *A. fumigatus* using sequence homology studies. Although subtilases have been related to the pathogenicity of an entomopathogen, and the trypsins to plant pathogenic fungi (St Leger et al. 1997), there are reports of subtilisin from plant pathogenic fungi being involved in plant infection (Pekkarinen and Jones 2002; Bindschedler et al. 2003). Moreover, serine proteases of the subtilisin family from *Fusarium solani* have shown the ability to degradate PR proteins and cell wall protein in potato tubers (Olivieri et al. 2002).

In summary, the present results strongly suggest that proteases produced by *A. flavus* and *A. parasiticus* are involved in peanut seed infection and aflatoxin contamination resulting in seed tissue damage affecting seed viability, and facilitating the access of *Aspergillus* through the testa. The protease analysis indicated that both *Aspergillus* species produced serine and metallo extracellular proteases. However, there were differences in the molecular masses of the enzymes between these species. The greatest activity originated from serine proteases that could be classified as subtilase.

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References

Abnet, C. C. (2007). Carcinogenic food contaminants. *Cancer Investigation*, *25*, 189–196. doi:10.1080/07357900701208733.

Asis, R., Barrionuevo, D. L., Giorda, L. M., Nores, M. L., & Aldao, M. A. (2005). Aflatoxin Production in six peanut (*Arachis hypogaea L.*) genotypes infected with *Aspergillus flavus* and *A. parasiticus*, isolated from peanut production areas of Cordoba, Argentina. *Journal of Agricultural and Food Chemistry*, *53*, 9274−9280. doi:10.1021/jf051259+.

Bindschedler, L. V., Sanchez, P., Dunn, S., Mikan, J., Thangavelu, M., Clarkson, J. M., et al. (2003). Deletion of the SNP1 trypsin protease from *Stagonospora nodorum* reveals another major protease expressed during infection. *Fungal Genetics and Biology*, *38*, 43−53. doi:10.1016/S1087-1845(02)00517-0.

Brown, R. L., Chen, Z. Y., Cleveland, T. E., Cotty, P. J., & Cary, J. W. (2001). Variation in vitro α-amylase and protease activity is related to the virulence of *Aspergillus flavus* isolates. *Journal of Food Protection*, *64*, 401–404.

Brown, R. L., Cleveland, T. E., Cotty, P. J., & Mellon, J. E. (1992). Spread of *Aspergillus flavus* in cotton bolls, decay of intercarpellary membranes, and production of fungal pectinases. *Phytopathology*, *82*, 462–467. doi:10.1094/Phyto-82-462.

Carlile, A. J., Bindschedler, L. V., Bailey, A. M., Bowyer, P., Clarkson, J. M., & Cooper, R. M. (2000). Characterization of SNP1, a cell wall-degrading trypsin, produced during infection by *Stagonospora nodorum*. *Molecular Plant-Microbe Interactions*, *13*, 538–550. doi:10.1094/MPMI.2000.13.5.538.

Chen, Z.-Y., Brown, R. L., Damann, K. E., & Cleveland, T. E. (2007). Identification of maize kernel endosperm proteins associated with resistance to aflatoxin contamination by *Aspergillus flavus*. *Phytopathology*, *97*, 1094–1103. doi:10.1094/PHYTO-97-9-1094.

Di Pietro, A., Huertas Gonzales, M. D., Gutierrez-Corona, J. F., Martinez-Cadena, G., Meglecz, E., & Roncero, M. I. G. (2001). Molecular characterization of a subtilase from the vascular wilt fungus *Fusarium oxysporum*. *Molecular Plant-Microbe Interactions*, *14*, 653–662. doi:10.1094/MPMI.2001.14.5.653.

Dowell, F. E., Dorner, J. W., Cole, R. J., & Davidson Jr., J. I. (1990). Aflatoxin reduction by screening farmers stock peanuts. *Peanut Science*, *17*, 6–8.

Guo, B. Z., Russin, J. S., Cleveland, T. E., Brown, R. L., & Damman, K. E. (1996). Evidence for cutinase production by *Aspergillus flavus* and its possible role in infection of corn kernels. *Phytopathology*, *86*, 824–829. doi:10.1094/Phyto-86-824.

Holbrook, C. K., Rucker, D. M., Wilson, J. E., Hook, J. E., & Matheron, M. E. (2000). Preharvest aflatoxin contamination in drought-tolerant and drought-intolerant peanut genotypes. *Peanut Science*, *27*, 45–48.

Horn, B. W. (2006). Relationship between soil densities of Aspergillus species and colonization of wounded peanut

seeds. *Canadian Journal of Microbiology*, *52*, 951–960. doi:10.1139/W06-050.

Kolattukudy, P. E., Lee, J. D., Rogers, L. M., Zimmerman, P., Ceselski, S., Fox, B., Stein, B., & Copelan, E. A. (1993). Evidence for possible involvement of an elastolytic serine protease in Aspergillosis. *Infection and Immunity*, *61*, 2357–2368.

Liang, X. Q., Luo, M., & Guo, B. Z. (2006). Resistance mechanisms to *Aspergillus flavus* infection and aflatoxin contamination in Peanut (*Arachis hypogaea*). *The Plant Pathology Journal*, *5*, 115–124.

Luo, M., Liang, X. Q., Dang, P., Holbrook, C. C., Bausher, M. G., Lee, R. D., & Guo, B. Z. (2005). Microarray-based screening of differentially expressed genes in peanut in response to *Aspergillus parasiticus* infection and drought stress. *Plant Science*, *169*, 695–703. doi:10.1016/j.plantsci.2005.05.020.

Mellon, J. E., Cotty, P. J., & Dowd, M. K. (2007). *Aspergillus flavus* hydrolases: their roles in pathogenesis and substrate utilization. *Applied Microbiology and Biotechnology*, *77*, 497–504. doi:10.1007/s00253-007-1201-8.

Middleton, K. J., Pande, S., Sharma, S. B., & Smith, D. H. (1994). Groundnut Crop: A scientific basis for improvement. In (Ed.), *Diseases* (pp. 336–78). London: Chapman & Hall.

Monod, M., Capoccia, S., Léchenne, B., Zaugg, C., Holdom, M., & Jousson, O. (2002). Secreted proteases from pathogenic fungi. *International Journal of Medical Microbiology*, *292* (5–6), 405–419. doi:10.1078/1438-4221-00223.

Movahedi, S., & Heale, J. B. (1990). The roles of aspartic proteinase and endo-pectin lyase enzymes in the primary stages of infection and pathogenesis of the various host tissues by different isolates of *Botrytis cinerea*. *Physiological and Molecular Plant Pathology*, *36*, 303–324. doi:10.1016/0885-5765(90)90061-2.

Olivieri, F., Zanetti, M. E., Oliva, C. R., Covarrubias, A. A., & Casalongue, C. A. (2002). Characterization of an extracellular serine protease of *Fusarium eumartii* and its action on pathogenesis related proteins. *European Journal of Plant Pathology*, *108*, 63–72. doi:10.1023/A:1013920929965.

Pekkarinen, A. I., & Jones, B. (2002). Trypsin-like proteinase produced by *Fusarium culmorum* grown on grain proteins. *Journal of Agricultural and Food Chemistry*, *50*, 3849–3855. doi:10.1021/jf020027x.

Plummer, K. M., Clark, S. J., Ellis, L. M., Loganathan, A., Al-Samarrai, T. H., Rikkerink, E. H. A., et al. (2004). Analysis of a secreted aspartic peptidase disruption mutant of *Glomerella cingulata*. *European Journal of Plant Pathology*, *110*, 265–274. doi:10.1023/B:EJPP.0000019796.78598.8c.

Ramesh, M. V., Sirakova, T., & Kolattukudy, P. E. (1994). Isolation, characterization, and cloning of the cDNA and the gene for an elastinolytic serine proteinase from *Aspergillus flavus*. *Infection and Immunity*, *62*, 79–85.

Ramesh, M. V., Sirakova, T., & Kolattukudy, P. E. (1995). Cloning and characterization of the cDNAs and genes (mep20) encoding homologous metalloproteinases from *Aspergillus flavus* and *A. fumigatus*. *Gene*, *165*, 121–125. doi:10.1016/0378-1119(95)00434-8.

Reed, C. E., & Kita, H. (2004). The role of protease activation of inflammation in allergic respiratory diseases. *The Journal of Allergy and Clinical Immunology*, *114*, 997–1008. doi:10.1016/j.jaci.2004.07.060.

Reichard, U., Buttner, S., Eiffert, H., Staib, F., & Ruchel, R. (1990). Purification and characterisation of an extracellular serine proteinase from *Aspergillus fumigatus* and its detection in tissue. *Journal of Medical Microbiology*, *33*, 243–251.

Rhodes, J. C., Amlung, T. W., & Muller, M. S. (1990). Isolation and characterization of an elastinolytic proteinase from *Aspergillus flavus*. *Infection and Immunity*, *58*, 2529–2534.

Roncero, M. I. G., Hera, C., Ruiz-Rubio, M., García Maceira, F. I., Madrid, M. P., Caracuel, Z., et al. (2003). Fusarium as a model for studying virulence in soilborne plant pathogens. *Physiological and Molecular Plant Pathology*, *62*, 87–98. doi:10.1016/S0885-5765(03)00043-2.

Sarath, G., De La Motte, R. S., & Wagner, F. W. (1989). Proteolytic Enzymes: A Practical Approach. In (Ed.), *Protease Assay Methods* (pp. 25–55). Oxford: IRL Press.

Sieber, P., Schorderet, M., Ryser, U., Buchala, A., Kolattukudy, P., Metraux, J. P., et al. (2000). Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. *The Plant Cell*, *12*, 721–738.

Sreedhar, L., Kobayashi, D. Y., Bunting, T. E., Hillman, B. I., & Belanger, F. C. (1999). Fungal proteinase expression in the interaction of the plant pathogen *Magnaporthe poae* with its host. *Gene*, *235*, 121–129. doi:10.1016/S0378-1119(99)00201-2.

St Leger, R. J., Joshi, L., & Roberts, D. W. (1997). Adaptation of proteases and carbohydrates of saprophytic, phytopathogenic and entomopathogenic fungi to the requirements of their ecological niches. *Microbiology*, *143*, 1983–1992.

Tubajika, K. M., & Damann, K. E. (2001). Sources of resistance to aflatoxin production in maize. *Journal of Agricultural and Food Chemistry*, *49*, 2652–2656. doi:10.1021/jf001333i.

Williams, J. H., Phillips, T. D., Jolly, P. E., Stiles, J. K., Jolly, C. M., & Aggarwal, D. (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *The American Journal of Clinical Nutrition*, *80*, 1106–1122.

Xu, H., Annis, S., Linz, J., & Trail, F. (2000). Infection and colonization of peanut pods by *Aspergillus parasiticus* and the expression of the aflatoxin biosynthetic gene, *nor-1*, in infection hyphae. *Physiological and Molecular Plant Pathology*, *56*, 185–196. doi:10.1006/pmpp.2000.0265.