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The Use of *Trichoderma harzianum* and *T. viride* as Potential Biocontrol Agents Against Peanut Microflora and Their Effectiveness in Reducing Aflatoxin Contamination of Infected Kernels

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Abstract: The antagonistic mechanism used by four *Trichoderma* isolates against peanut moulds; *Aspergillus flavus*, *A. parasiticus*, *A. niger*, *A. ochraceous* and *Fusarium* species was investigated under laboratory conditions. Two isolates of *T. harzianum* namely Th1 and Th2 and two isolates of *T. viride*, Tv1 and Tv2, were found to efficiently suppress the growth of peanut moulds and to significantly reduce aflatoxins, AFB1 and AFB2, contents in infected peanut kernels. The degree to which *Trichoderma* species suppressed the growth of peanut moulds correlated to their extracellular enzymatic activities. The *Trichoderma* isolates displayed various extracellular enzymatic activities including amylolytic, chitinolytic, pectinolytic, proteolytic, lipolytic and cellulolytic to fight off the other fungal competitors in the media. The more diverse the enzymatic activities, the more effective are the antagonists in suppressing the growth of peanut moulds and aflatoxin production in kernels. Present results suggest that Trichoderma-enzymolomic can be exploited as potential antimicrobial candidates against peanut moulds and the subsequent aflatoxin production in peanut kernels.

Key words: Aflatoxins, *Aspergillus* sp., biocontrol, peanuts, *Trichoderma* sp., enzymatic activity

INTRODUCTION

The genus *Aspergillus* is one of the most widely studied genera of spoilage fungi associated with nuts, grain and seed crop products. Some *Aspergillus* species, predominantly *Aspergillus flavus* and *A. parasiticus* produce aflatoxins as secondary metabolites, which cause harmful effects in humans and animals (Logrieco *et al*., 2003; Gachomo *et al*., 2004). *A. nomius*, *A. tamari* (Goto *et al*., 1997) and *A. pseudotamarii* (Ito *et al*., 2001) are also known to produce aflatoxins. Among several forms of aflatoxin, AFB1 is the most potent and more common in food and feed (Kew, 2003). Plant products that are commonly contaminated by aflatoxins include cereals (maize, rice and wheat), oilseeds (groundnut, soybean, sunflower and cotton), spices (chillies, black pepper, coriander, turmeric and ginger), tree nuts (almonds, pistachio, walnuts and coconut), coffee and most dried fruits (Bankole and Adebanjo, 2003). Almost 40% of crop products are lost yearly due to aflatoxin contamination in developing worlds (Miller, 1996). Moreover aflatoxins are among the most potential mutagenic, carcinogenic and immunosuppressive substances known and represent a major risk factor in the multifactorial etiology of human liver cancer in tropical regions (Montalto *et al*., 2002; Elegbede and Gould, 2002; Bankole and Adebanjo, 2003). Therefore, developing ways of controlling aflatoxin contamination of food commodities is of global importance.

Peanut (*Arachis hypogea* L.) is an important legume grown on 19.3 million ha in about 82 countries worldwide (Reddy *et al*., 2003). It is a high-energy food that contains high amounts of edible oil (44-56%) and proteins (25-30% of the dry seed weight) (Reddy *et al*., 2003; Gachomo *et al*., 2004). Peanuts are rich in niacin and other B vitamins and also contain appreciable amounts of calcium, iron, phosphorus and potassium (McWatters *et al*., 1995). Like other legumes, peanuts possess a unique ability to fix atmospheric nitrogen that enriches soil fertility. Research data show that more than half of the peanut production areas are in warmer arid and semi-arid regions. The major limiting factor in the production and marketing of peanuts remains their contamination with aflatoxins (Gachomo *et al*., 2004).

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Control of peanut infection by aflatoxigenic moulds is still a widely recurring challenge. No single method of control has been found to be completely effective. However, the use of microbial antagonists as biological control agents seems to be a promising way to control the infection of valuable crops by aflatoxigenic moulds.

Trichoderma species are known to be antagonistic to other microorganisms. Application of *T. harzianum* or *T. koningii* to pea seeds reduced the incidence of pre-emergence damping-off caused by *Pythium* sp. (Lifshitz *et al*., 1986). *T. harzianum* was successfully used in the control of fungal soil-borne pathogens (Tronsmo, 1996). Recently, Kanjanamaneesathian *et al.* (2003) reported on the use of *Trichoderma* sp. as seed treatment to control *Pythium aphanidermatum*, the causal agent of damping-off of Chinese kale seedling. However, the mechanisms by which *Trichoderma* species influence and repress growth of other microorganisms are not fully understood. A lack of understanding these mechanisms can limit their use as biocontrol agents.

To investigate the mechanisms by which *Trichoderma* species suppress growth of other microorganisms, we examined in this study the growth pattern of various moulds isolated from fresh peanut kernels (Gachomo *et al*., 2004) in presence of different isolates of *T. viride* and *T. harzianum*. We quantified the amounts of aflatoxins produced by the aflatoxigenic moulds (*A. flavus* and *A. parasiticus*) in peanut kernels in presence of the *Trichoderma* species. In addition the ability of *Trichoderma* species to display various extracellular enzymatic activities is here discussed in an effort to understand the mechanisms underlying the antagonistic action of *Trichoderma* species.

MATERIALS AND METHODS

Peanut samples and fungal species: Peanut kernels variety Homa Bay Local (HbL) sampled from five randomly selected markets within Kenya and the previously described peanut moulds (Gachomo *et al*., 2004) were used in this study. The peanut moulds were used as test pathogens, which included *Fusarium* species, *Aspergillus niger*, *A. flavus*, *A. parasiticus* and *A. ochraceous*. Four isolates of *Trichoderma* species (two isolates of *Trichoderma viride*, Tv1 and Tv2 and two isolates of *T. harzianum*, Th1 and Th2) were used as antagonistic agents in this study. The individual fungal strains were maintained on Potato Dextrose Agar (PDA) and as soil cultures at -20°C. Unless otherwise described, all experiments in this study were done in triplicate and the data represent mean values (±SE) where applicable.

Antagonistic effects of *Trichoderma* species on the test pathogens: To assess the antagonistic effect of *Trichoderma* species on the test pathogens, a co-culture of the test pathogen and the antagonist was established on PDA. A line was drawn diagonally across the bottom of petridishes containing PDA dividing the plates into two equal halves. Points were marked at 2 cm from the edge of the petridish on either side of the line. The middle of each petridish was also marked. A *Trichoderma* isolate was point-inoculated 2 cm from the edge of the petridish on one side of the line and the test pathogen was point inoculated on the other half of the petridish at the second marked point (2 cm). The petridishes were then incubated at 23°C (±2), with light intensity of 300 $\mu E \times m^{-2}$ at day/night cycle of 14/10 h and a relative humidity of 48±5%. For control, each test pathogen alone without the antagonist was inoculated into plates of PDA. Ten petridishes were used per treatment. The colony diameters of the test pathogens and their controls were recorded 7 days after inoculation. The data were expressed in percentage of colony size reduction with the control being taken as reference (100%).

The *Trichoderma* isolates were also tested for the ability to produce volatiles. The effects of volatiles produced by *Trichoderma* species against the test pathogens was investigated using in inverted cultures according to the method of Dick and Hutchinson (1966).

Production of extracellular enzymes by *Trichoderma* species: The production of extracellular enzymes (*cf.* Table 1) by the *Trichoderma* isolates (Tv1, Tv2, Th1 and Th2) was assayed on solid media according to the modified method of Calistru *et al.* (1997). The indicated enzymatic activities were assessed 7 days after incubation.

Chitinase activity of *Trichoderma*-culture filtrates: To test for chitinolytic activity, *Trichoderma* isolates were inoculated into 300 mL of Potato Dextrose Broth (PDB) and incubated (150 rpm, 23±2°C) at constant light for 12 days. The mycelia were filtered out and the filtrate was sterilised by passing it through a 0.45 µm pore filter. The filtrate was used as source of chitinase. Chitinase activity was assayed as described by Vyas and Deshpande (1989). The assay consisted of three replicate reactions of: 10 mg acid swollen chitin (Sigma, St. Louis, MO, USA), 50 µmoles of acetate buffer (pH 5.8) and 1 mL of enzyme source in a total reaction volume of 3 mL. The reaction was done at 50°C for 10 min. The product was estimated by Nelson method (1944). One unit of chitinase activity was defined as the amount of enzyme required to liberate one µmole of N-acetyl-D-glucosamine equivalent per hour at 50°C.

Table 1: Method of assessing extracellular enzymes produced by *Trichoderma* species

Enzyme	Substrate	Method	Positive result
Lipase	Lipid	Flooding of plates with Nile Blue indicator (i.e., pH indicator)	Any change in colour towards blue beyond the colony edges detectable as blue zone
Protease	Gelatine	Flooding of plates with saturated ammonium sulphate solution	The presence of clear zones in the opaque medium
Pectinase	Pectin	Flooding of plates with toluidine blue (aqueous solution)	Presence of pink coloration
Amylase	Starch	Flooding of plates with an iodine solution	A clear zone in an otherwise dark blue surrounding
Cellulase	Cellulose	Zinc chlor-iodide and potassium iodide test	A clear zone in an otherwise dark brown surrounding

Inhibition of aflatoxin production in peanut kernels by *Trichoderma* species: Prior to assessing the ability of *Trichoderma* species to inhibit aflatoxin production by the moulds, the test pathogens were qualitatively assessed for the production of aflatoxins according to Cunnif's method adopted for aflatoxin analysis by the Association of Official Analytical Chemists (AOAC) (Cunnif, 1995). Basically, the method consists of an extraction phase, followed by a column clean-up phase and finally by a qualitative assay via a one-dimensional Thin Layer Chromatography (TLC) (Sorbent Technologies Inc. Atlanta, GA, USA), which uses a silica gel adsorbent and an acidic solvent system as described by Kuiper-Goodman and Scott (1989). For the quantitative estimation of aflatoxins, scanning densimetric analysis was carried out. The TLC plates were scanned according to the instructions of the manufacturer using CD 60 Desaga computer program. The program is set up to analyse the intensity of the spots developed by TLC. The peak areas of the samples were compared to those of the standards to quantify the aflatoxin content in the samples.

Seven-day-old cultures of *Trichoderma* isolates and the selected aflatoxigenic fungi (*Aspergillus flavus* and *A. parasiticus*) were used to make spore suspensions (1×10^5 spores/mL). Two representative *Trichoderma* isolates, *T. harzianum* (Th1) and *T. viride* (Tv1) were used in this experiment. Sterilised peanut kernels were sprayed with *A. flavus* spore suspension and allowed to air-dry before being sprayed with the spore suspension of *T. harzianum* (Th1). The kernels were then incubated on PDA plates for 7 days. After incubation, the kernels were assayed for aflatoxin content as described by Gachomo *et al.* (2004). Three types of control were set up: one in which the kernels were only inoculated with the spore suspension of *A. flavus*, another where the kernels were inoculated with *T. harzianum* and yet another where the kernels were sprayed with sterile water. For the aflatoxin inhibition test, 100 peanut kernels were used per treatment. The same experiment was repeated with *A. parasiticus* as a test pathogen. In another set of experiments *T. viride* (Tv1) was used as the antagonist with *A. flavus* and *A. parasiticus* as test pathogens.

Statistical analyses: Data were expressed as mean values (±SE) of at least three independent experiments where applicable. p-values were determined by Student's t-test analysis. A value of $p<0.05$ was considered statistically significant for the mean differences.

RESULTS

Antagonistic ability of *Trichoderma* species: In our previous report, different microorganisms were isolated from peanut kernels and further characterized with respect to their ability to produce aflatoxins (Gachomo *et al.*, 2004). Here as shown in Fig. 1, it was obvious that the collected kernels, apparently healthy, harboured various moulds. The four isolates of *Trichoderma* species were found to display antagonistic effects against the peanut moulds, by suppressing their growth to varying degrees (Table 2). *T. harzianum* isolates were found to be more effective in suppressing the growth of the test fungi than *T. viride* isolates (Fig. 2). *T. harzianum* isolates overgrew the test pathogens when grown together in a single plate (Fig. 2f, h). The greatest colony size reduction by *Trichoderma* species was observed against *A. niger* and the least was against *A. ochraceous* (Table 2). When the test pathogens were exposed to the trapped atmosphere from cultures of *Trichoderma* species, their growth was inhibited (data not shown). Since *Trichoderma* isolates were able to suppress growth of the peanut moulds, we further examined the probable suppressive mechanisms by which these antagonists operated.

Production of extracellular enzymes by *Trichoderma* species: *Trichoderma* species have been reported to produce various proteins with known antimicrobial effects when co-cultured with other organisms (El-Katatny *et al.*, 2001). We therefore hypothesized that *T. harzianum* and *T. viride* considered in this study probably produced an array of enzymes with antimicrobial effects against the test pathogen(s).

As shown in Table 3, indeed the *Trichoderma* strains showed various extracellular enzymatic activity, including lipolytic, proteolytic, pectinolytic, chitinolytic and cellulolytic in the growth media supplemented with specific substrates. *T. harzianum* isolates displayed five (lipolytic, amylolytic, pectinolytic, chitinolytic and cellulolytic) of the six enzymatic activities examined, while the *T. viride* species produced four (lipolytic, proteolytic,

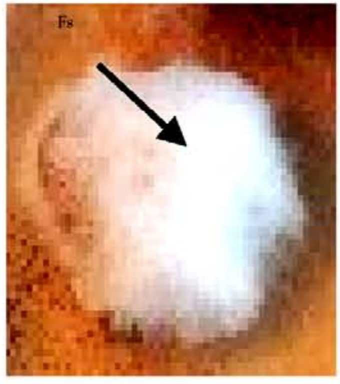
Table 2: Reduction of colony diameters of the test pathogens by *Trichoderma* species. The reduction of the colony size was expressed in percentage reduction compared to the control pathogen grown without the antagonist

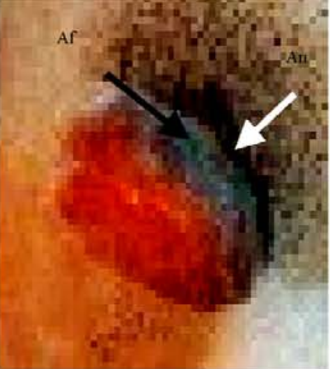
		T. harzianum		*T. viride*	
Test fungi	Control	Th1	Th2	Tv1	Tv2
Fusarium species	100%	48.4%	53.5%	12.40%	15.30%
A. flavus	100%	20.4%	22.8%	1.90%	1.10%
A. parasiticus	100%	16.8%	17.2%	1.20%	1.30%
A. ochraceous	100%	5.2%	3.3%	0.91%	0.89%
A. niger	100%	80.3%	82.8%	38.90%	40.60%

Table 3: Extracellular enzymatic activity produced by *Trichoderma* species

	T. harzianum isolates		*T. viride* isolates	
Extracellular enzyme activity	Th1	Th2	Tv1	Tv2
Lipolytic	+	+	+	-
Proteolytic	+	-	-	+
Amylolytic	+	+	+	+
Pectinolytic	+	+	+	-
Chitinolytic	+	+	-	-
Cellulolytic	+	+	+	+

+: Detection of enzymatic activity, -: No detection of enzymatic activity





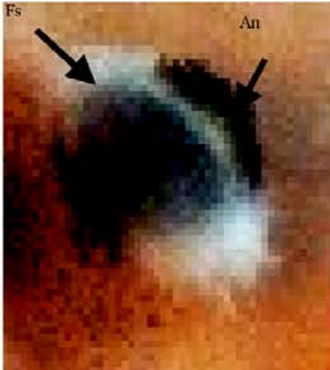


Fig. 1: Visual observation of moulds evolved from apparently healthy peanut kernels. Fs: *Fusarium* species; Af: *Aspergillus flavus*; An: *Aspergillus niger*

amylolytic and cellulolytic) of the six enzymes assayed for. All *Trichoderma* species tested produced amylolytic and cellulolytic enzymes. Both isolates of *T. harzianum* and one isolate of *T. viride*, Tv1, produced pectinolytic enzyme and only one isolate of *T. viride* (Tv2) tested was able to display positive proteolytic activity, while none of the *T. viride* isolates tested showed chitinase activity (Table 3).

Inhibitory effects of the antagonists on aflatoxin production: In our previous study we showed that peanut kernels screened contained high amounts of AFB1 and AFB2 and lower amounts of AFG1 (Gachomo *et al*., 2004). The qualitative assay of aflatoxin production by peanut moulds showed that *A. flavus* and *A. parasiticus* were the producers of aflatoxins, predominantly AFB1 (Fig. 3). Since we observed growth suppression of toxigenic moulds by the *Trichoderma* species (Fig. 2), we therefore hypothesized that the antagonists might probably reduce the aflatoxin contamination of the peanut kernels infected by the aflatoxigenic moulds.

Inoculation of peanut kernels with *Trichoderma* isolates led to significant ($p<0.05$) reduction of aflatoxins, AFB1 and AFB2, produced by *A. parasiticus* and *A. flavus* compared to the control samples, inoculated with aflatoxigenic moulds alone (Fig. 4). In general, the reduction of aflatoxin production was greater when the kernels were inoculated with the antagonists before the test pathogens, while the least reductions were observed when the kernels were inoculated with the aflatoxigenic moulds first (Fig. 4). The same inhibition pattern of aflatoxin production was observed when *T. viride* was used as an antagonist (data not shown).

In summary, these data suggest that *Trichoderma* species can efficiently control aflatoxin contamination in peanut kernels.

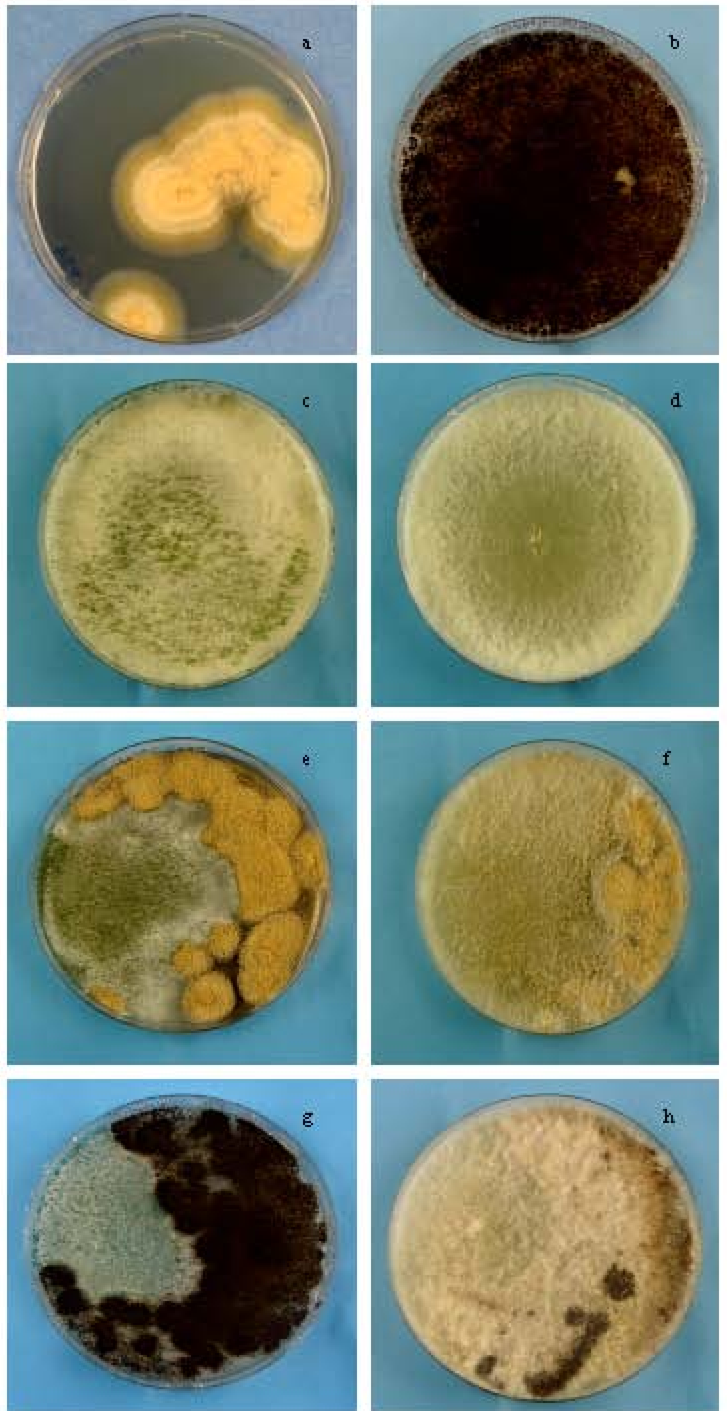


Fig. 2: Antagonism of *Trichoderma* species against *Aspergillus* species. (a) pure cultures of *A. ochraceous*, (b) pure culture of *A. niger*, (c) pure cultures of *T. viride* (Tv1), (d) pure culture of *T. harzianum* (Th1), (e) a culture of *T. viride* (Tv1) and *A. ochraceous*, (f) a culture of *T. harzianum* (Th1) and *A. ochraceous*, (g) a culture of *T. viride* (Tv1) and *A. niger*, (h) a culture of *T. harzianum* (Th1) and *A. niger*

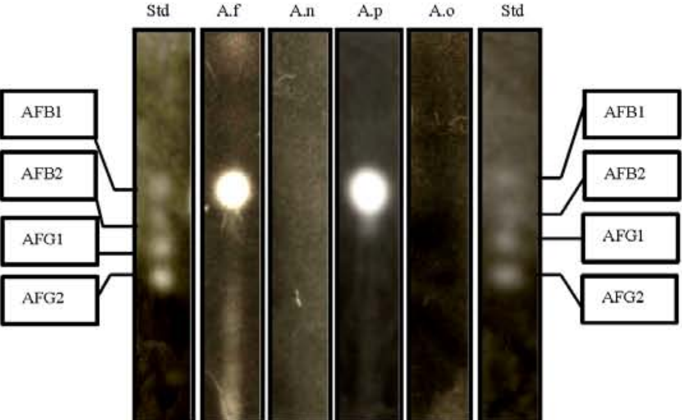


Fig. 3: Detection of aflatoxin AFB1 and AFB2 produced by the genus Aspergillus. TLC analysis of the samples was performed using mixed standards of AFB1, AFB2, AFG1 and AFG2 as control. Representative Data of three independent replicates are depicted. Std: Aflatoxin mixed standards; A.f: *Aspergillus flavus*; A.n: *Aspergillus niger*; A.p: *Aspergillus parasiticus*; A.o: *Aspergillus ochraceous*

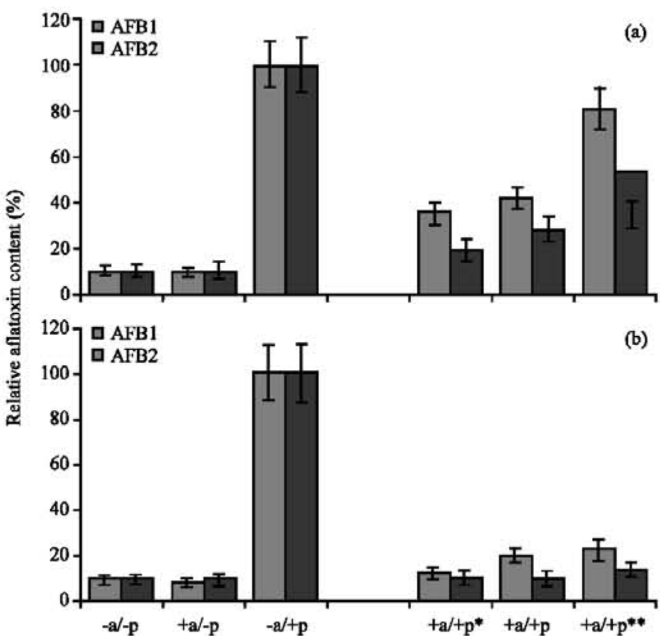


Fig. 4: Aflatoxin content in peanut kernels infected by test pathogens under the control of antagonists. Amounts of aflatoxin produced by *A. flavus* (a) and *A. parasiticus* (b) under the control of *T. harzianum* (Th1). -a/-p, without antagonist/without test pathogen; +a/-p, with antagonist/without test pathogen; -a/+p, without antagonist/with test pathogen; +a/+p, antagonist and test pathogen inoculated the same day; +a/+p*, antagonist inoculated 2 days before the test pathogen was inoculated; +a/+p**, antagonist is inoculated 2 days after the test pathogen has been inoculated

DISCUSSION

Trichoderma species have been successfully used as biocontrol agents due to their high reproductive capacity, efficient utilization of nutrients, strong aggressiveness against other phytopathogens, efficiency in promoting plant growth and defence mechanism and ability to modify the rhizosphere (Kleifeld and Chet, 1992; El-Katatny *et al*., 2001; Benítez *et al*., 2004). However, understanding the antagonistic mechanisms used by *Trichoderma* species on a wide range of pathogens is important in optimizing their use as biocontrol agents. In

this study, we demonstrated that growth of five test pathogens (*Aspergillus niger*, *A. flavus*, *A. parasiticus*, *A. ochraceous* and *Fusarium* species) was significantly inhibited when co-cultured with *Trichoderma* species (Fig. 2). Some of the test pathogens were overgrown by *T. harzianum* isolates but not by *T. viride* isolates suggesting that *T. harzianum* had characteristics that *T. viride* isolates did not have. This could probably be linked to the chitinase activity displayed by *T. harzianum*. Chitinase is believed to be a key enzyme in mycoparasitism (Howell, 2003). Growth of the test pathogens was also inhibited when they were exposed to a trapped atmosphere from *Trichoderma* species, suggesting that the test pathogens were susceptible to the volatiles produced by *Trichoderma* species. *Trichoderma* species used in this study displayed various extracellular enzymatic activities, which included lipolytic, chitinolytic, cellulolytic, amylolytic and pectinolytic. These extracellular enzymatic activities positively correlated with the effectiveness of *Trichoderma* species in suppressing growth of the test pathogens. Chitinase, cellulase and β-glucanse are considered important enzymes against plant pathogens (Chet and Baker, 1981). Based on our findings we concluded that the likely mechanisms by which *Trichoderma* species suppressed peanut moulds might involve production of volatiles and/or production of extracellular enzymes. Mycoparasitism may as well be part of the antagonistic mechanisms, although no experiments were carried out to confirm this. This is an important finding because it opens door to further study the underlying antagonistic mechanisms of the genus *Trichoderma* to efficiently control aflatoxin contamination of crop products.

A study by Calistru *et al.* (1997) revealed that production of volatiles and extracellular enzymes rather than mycoparasitism were the likely means by which *Trichoderma* species suppressed the growth of both *A. flavus* and *Fusarium moniliforme*. These findings were additionally supported by the study of Doi and Mori (1994), which demonstrated that volatiles from *Trichoderma* species were able to arrest the hyphal growth of different fungal pathogens on agar plates. Research has also shown that most *Trichoderma* strains produce volatiles and non-volatile toxic metabolites such as harzianic acid, alamethicins and tricholin that hinder growth of other microorganisms (Vey *et al.*, 2001). Therefore production of volatiles and extracellular enzymes by *Trichoderma* isolates in this study may have been sufficient to suppress the growth of the peanut moulds.

The involvement of enzymes from *Trichoderma* species in antagonism has been further demonstrated using a forward genetic approach by transferring *Trichoderma*-genes encoding cell wall degrading enzymes into plants thereby conferring resistance to various pathogens. Transgenic tobacco (*Nicotiana tabaccum*) and potato (*Solanum tuberosum*) plants overexpressing an endo-chitinase (CHIT42) from *T. harzianum* have been shown to be highly resistant to the foliar pathogens *Alternaria alternata*, *Alternaria solani*, *Botrytis cinerea* and also to the soil-borne pathogen *Rhizoctonia solani* (Lorito *et al.*, 1998). Recently it was demonstrated that overexpression of *T. harzianum*-chitinase (CHIT42) could even enhance resistance to both biotic and abiotic stresses in transgenic tobacco (Dana *et al.*, 2006). *T. harzianum* 1051 is known to produce extracellular enzymes, such as chitinase, endoglucanases, proteases and amylases to repress growth of other organisms (De Marco *et al.*, 2000).

It has been shown that fungi with a broad enzymatic activity possess high eco-nutritional versatility. This concept was defined by Cooke and Whipps (1980) as the ability to survive in a vegetative state in order to overcome environmental changes that might normally be harmful to the antagonists. This wide range of enzymatic activity of the antagonists allows them to have a unique and wide nutritional versatility that increases their survival chances in unfavorable environments (Cooke and Rayner, 1984). This probably explains the array of enzymatic activities detected in the *Trichoderma* species screened (Table 2). These enzymatic activities were presumably used against the other microorganisms competing for the same resources/nutrients in the peanut kernel. Our overall understanding of the mode of action of antagonistic agents against potential pathogens is still limited. However, this study identifies candidate enzymatic activities that are important in antagonistic effects of *Trichoderma* species against peanut moulds.

The antagonists were able to significantly ($p<0.05$) reduce aflatoxins produced by *A. flavus* and *A. parasiticus* in peanut kernels screened. This aflatoxin reduction is thought to be an indirect result of the suppressed growth of the aflatoxigenic moulds by *Trichoderma* species, leading to the assumption that smaller colonies produced less toxins. The reason why the aflatoxin inhibitory effects of the *Trichoderma* species were more pronounced on *A. paraciticus* than on *A. flavus* (Fig. 4) is unknown. This study shows that *Trichoderma*-proteomics/enzymolomics might be a potential source of anti-pathogenic activity that can be used to control crop invaders and mycotoxin contamination in crop products simultaneously.

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