

Full Length Research Paper

Inhibition of growth of some phytopathogenic and mycotoxigenic fungi by aqueous extracts of *Combretum imberbe* (Wawra) wood

Peloewetse E. ^{1*}, Thebe M. M. ², Ngila J. C. ³ and Ekosse G. E. ⁴

¹Department of Biology, University of Botswana, Faculty of Science, Private Bag 00704 Gaborone, Botswana.

²Lobatse Secondary School, Private Bag 13 Lobatse, Botswana.

³School of Chemistry, University of KwaZulu-Natal, Westville Campus, Private Bag X54001 Westville, Durban 4000, South Africa.

⁴School of Physical and Mineral Sciences, University of Limpopo, Private Bag X1106, Sovenga 0727, South Africa.

Accepted 26 June, 2008

Fungal attack of crop plants and the associated production of mycotoxins on edible produce from plants is a global concern. This is so because mycotoxins are carcinogenic. This study reports on an investigation of the effects of aqueous extracts of *Combretum imberbe* wood ash on the growth of some phytopathogenic and mycotoxigenic fungi including *Aspergillus flavus*, *Fusarium oxysporum*, *Penicillium italicum*, *Penicillium notatum* as well as *Alternaria alternata*. The aqueous extract of *C. imberbe* wood ash exhibited significant capacity to inhibit growth of all the test fungi, with the exception of *A. alternata*. Arrest of fungal growth by the extract involved inhibition of glucomylase. Investigation of the chemistry of the ash using Fourier-Transform Infrared (FT-IR) spectroscopy and X-ray powder diffraction (XRPD) analysis revealed the presence of aluminium oxide (Al₂O₃), calcium carbonate (CaCO₃), calcium hydroxide (Ca(OH)₂), potassium chloride (KCl), potassium sulphate (K₂SO₄) and cobalt carbonate (CoCO₃). We propose that the inhibition of fungal growth may be related to limited carbon supply as a result of inhibited activity of glucomylase in the presence of *C. imberbe* wood ash extract.

Key words: Adaptive response, alkalization, *Combretum imberbe*, glucomylase, mycotoxins.

INTRODUCTION

Agriculture remains a major means of sustenance for many people around the world. However, Fungi belonging to several genera including *Aspergillus*, *Fusarium*, *Penicillium* as well as *Alternaria* often infest agricultural produce and contaminate them with mycotoxins (Payne, 1992; Pitt et al., 2000; Chelule et al., 2003; Fandohan et al., 2003). Contamination of agricultural products by mycotoxins such as aflatoxins and fumonisins cause particular concern since they are highly toxic and carcinogenic (Shepherd et al., 1995; Chelule et al., 2001). Recent evidence (Oswald et al., 2003) demonstrating that

exposure of pigs to fumonisin B1 predisposes them to infectious diseases adds impetus to concerns about mycotoxins even in the case of other mammalian populations. Altogether, results from these studies underscore the need for effective control of fungal contamination of agricultural products destined for various end uses.

A number of approaches including physicochemical methods (Samarajeewa et al., 1990) as well as fungicide usage have been undertaken to reduce or prevent contamination of food and feed with mycotoxins, especially aflatoxins. However, most physicochemical methods are not feasible at small scale farm level. Fungicide usage is more feasible but the effects of fungicides on non-target organisms coupled with their poor biodegradability are unwelcome. Other ways of reducing human exposure to aflatoxins include consumption of crops with lower

*Corresponding author. E-mail: peloewee@mopipi.ub.bw. Tel: (+267) 3552604.

Aspergillus flavus infestation and aflatoxin level such as rice (Wild and Turner, 2002). However, agroclimatic conditions in different parts of the world preclude production and consumption of rice on a wide scale. This calls for alternative and or additional means of controlling fungal contamination of stored food products and the concomitant production of mycotoxins on such food reserves.

In this study, we have investigated the effect of aqueous extracts from *Combretum imberbe* Wawra wood ash on the growth of some phytopathogenic fungi including *A. flavus*, a toxigenic fungus (Rodriguez and Mahoney, 1994). Demonstration of the efficacy of wood ash against toxigenic fungi could constitute an effective method of reducing human exposure to mycotoxins. To this end, we initiated this study to investigate whether ash derived from *C. imberbe* has any effect on the growth of phytopathogenic fungi including some mycotoxigenic fungi.

MATERIALS AND METHODS

Sample preparation

Dead but erect stem wood of *C. imberbe* Wawra was obtained from Metsimaswaane lands in South East District of Botswana during the dry season (May-August). The wood was placed on a rectangular metal sheet (1 m²) and burnt in open air. Ash produced was collected in a plastic bag and transported to the laboratory at the University of Botswana for analysis. From this, a 100-g weight of the ash was placed in a conical flask to which 250 ml of distilled water was added. The flask was closed with parafilm and placed on an orbital shaker at 120 rpm for 24 h. The ash suspension was filtered on Whatman no 1 filter paper and the filtrate obtained was freeze-dried to complete dryness. The solute was collected into a sample bottle and stored in a dessicator until required for use.

Growth of organisms

All the test fungi used in this study (including toxigenic (*A. flavus*) were obtained from the Department of Biological Sciences at the University of Botswana. The cultures were checked for purity using established techniques. Pure cultures were grown on potato dextrose media at 28°C for 7 days. Conidial suspensions of the test organisms were prepared and adjusted to the same optical density.

Effect of *C. imberbe* ash extract on fungal growth

The disc diffusion assay was used for studying the effects of extract on growth of the test fungi. For this, sterilised Whatman No 1 paper discs of 9 mm diameter were soaked in 500 µl of filter-sterilized extract in a sterile Corning cell well plate (2-ml capacity) under a fume hood. Lawns of test organisms were created by adding suspensions of fungal inocula adjusted to the same optical density into separate Erlenmeyer flasks containing freshly autoclaved media maintained at 40°C. The contents of each flask were thoroughly swirled to mix the contents before plating out in 90 mm Petri dishes under aseptic conditions. Single paper discs that had been preloaded with extract were placed (with sterile forceps) on each plate prior to incubation at 25°C for *Penicillium* spp. and at 28°C for the rest of the test organisms. Effect of ash extract on each test organism was assessed in triplicate. Sizes for zones of inhibition were recorded after incubation for 24, 48 and 72 h, respectively.

Determination of metabolic target of *C. imberbe* ash extract in *A. flavus*

A mycotoxigenic strain of *A. flavus* was cultured on a rotary shaker at 100 rpm as described by Gomes et al. (2005) except that beef extract was not included in the culture medium and the culture period was 7 days. Biomass was separated by centrifugation at 14 000 g for 20 min at 4°C. The supernatant containing the crude enzyme preparation was retained. The crude enzyme was desalted on a sephadex PD10 column prior to use. Amylolytic activity was determined by incubating 0.2 ml of desalted crude enzyme preparation with 2 ml of potato starch (0.8%w/v) for 2 h at 40°C in a buffered medium (pH6.9) comprising 100 Mm Na₂H₂PO₄ and 1 mM CaCl₂. An appropriate control without fungal enzyme extract was also set up. The reduction of NADP⁺ at 340 nm was used to assay glucose released during the hydrolysis of starch (Hill and ap Rees, 1995). To study the effect of *C. imberbe* extract on enzyme activity, assays of enzyme activity were performed in the presence of known concentrations of *C. imberbe* wood ash. Appropriate controls without *C. imberbe* wood ash extract were also set up. Prior to assaying glucose from the hydrolysis of starch, the pH of the reaction mixture was adjusted to pH 7.5 whenever necessary. Amylolytic activity was expressed as a percentage of the activity in the absence of *C. imberbe* wood ash extract.

Analysis of *C. imberbe* wood ash extract by Fourier Transform-Infra Red (FT-IR) spectroscopy

The freeze-dried aqueous ash extract was subjected to FTIR analysis on a Perkin-Elmer 2000 FT-IR instrument set at 7800-370 cm⁻¹ in a single beam mode. For this, a 1.0 mg quantity of the freeze-dried sample was mixed with a 300.0 mg quantity of KBr and then pulverized in an agate mortar and pestle. A sample of the mixture was placed in a sample holder and an absorption scan was obtained. Scans were repeated for each spectrum at 4000-370 cm⁻¹ to obtain detailed information. Compounds contained in the ash were identified according to established criteria of Nyquist et al. (1997) and Socrates et al. (2001), respectively.

Analysis of *C. imberbe* wood ash through X-Ray Powder Diffraction (XRPD)

The freeze-dried sample of the wood ash extract was pulverized in an agate mortar and pestle for textural consistency. The resulting powder was subjected to XRPD analysis using Philips PW 3710 XRPD system with PW1752 a graphite monochromator, a PW1830 generator and a PW 3020 vertical goniometer. The XRPD system was operated at 40 kV and 45 Ma with a Cu-Kα radiation and scanned from 2° 2θ to 70° 2θ using a step size of 2θ = 0.02 and 0.3 s per step. Philips X'PERT graphics software was used for qualitative identification of the minerals and the X-ray data patterns. The data obtained were cross-referenced to database in the Mineral Powder Diffraction File Data books.

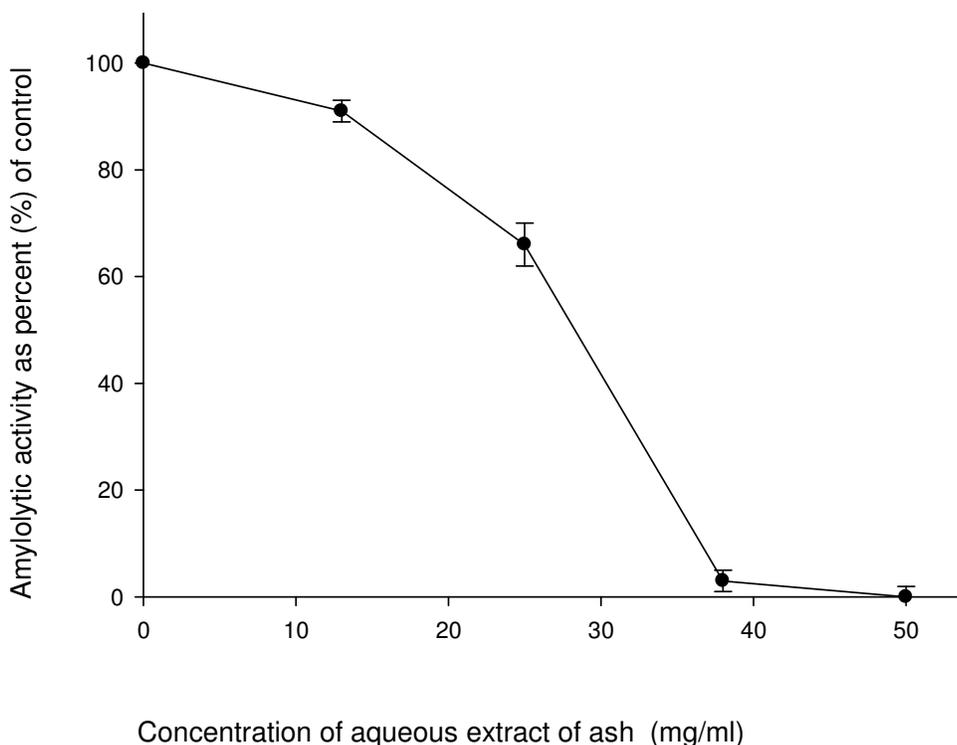
RESULTS

Effect of *C. imberbe* wood ash extract on fungal growth

This study was initiated to investigate whether ash from *C. imberbe* has any effect on the growth of phytopathogenic fungi as well as the mycotoxigenic isolate of *A. flavus*. We used the disc diffusion assay method. Our re-

Table 1. Effect of aqueous ash extracts of *C. imberbe* on growth of phytopathogenic fungi. Data in the table represents mean + S.E.M., n= 3.

Organism	Diameter (mm) of zone of inhibition after		
	24 h	48 h	72 h
<i>Alternaria alternata</i>	20 ± 2	-	-
<i>Aspergillus flavus</i>	30 ± 0.5	20 ± 2	15 ± 2
<i>Fusarium oxysporum</i>	30 ± 2	23 ± 2	16 ± 1
<i>Penicillium italicum</i>	35 ± 3	30 ± 3	20 ± 3
<i>Penicillium notatum</i>	28 ± 3	25 ± 3	18 ± 3

**Figure 1.** Effect of aqueous ash extract of *C. imberbe* on amyolytic activity from *A. flavus*. Each data point in the graph represents mean ± S.E.M., n= 3.

sults (Table 1) show that *C. imberbe* wood ash has growth inhibitory effects against phytopathogenic fungi. However, the inhibitory effects decreased with increasing length of culturing the test fungi in the presence of ash extract (Table 1) and the inhibition was different between the test fungi.

Effect of *C. imberbe* wood ash extract on amylase activity

To gain insight on the metabolic target for the inhibitory effects of the ash extract, we investigated the effects of *C. imberbe* ash extract on a carbohydrate- catabolising enzyme preparation from *A. flavus*. This was important because carbohydrate catabolising enzymes are crucial

for carbon acquisition from any starch-rich substrate. Our results (Figure 1) show dose-dependent inhibition of amyolytic enzyme preparation from *A. flavus*.

Chemistry of *C. imberbe* wood ash extract

To appreciate the chemical nature of the enzyme-inhibiting principles in the extract (Figure 1, we conducted FTIR and XRPD studies the freeze-dried aqueous extracts used in this study. FT-IR analysis (Figure 2) revealed the presence of aluminium oxide (Al_2O_3), calcium oxide (CaO), cobalt carbonate (CoCO_3), potassium chloride (KCl) and potassium sulphate (K_2SO_4) with characteristic absorption at 886.47, 427.59, 1427.41, 706 and 1119.72 cm^{-1} , respectively. XRPD analysis of the

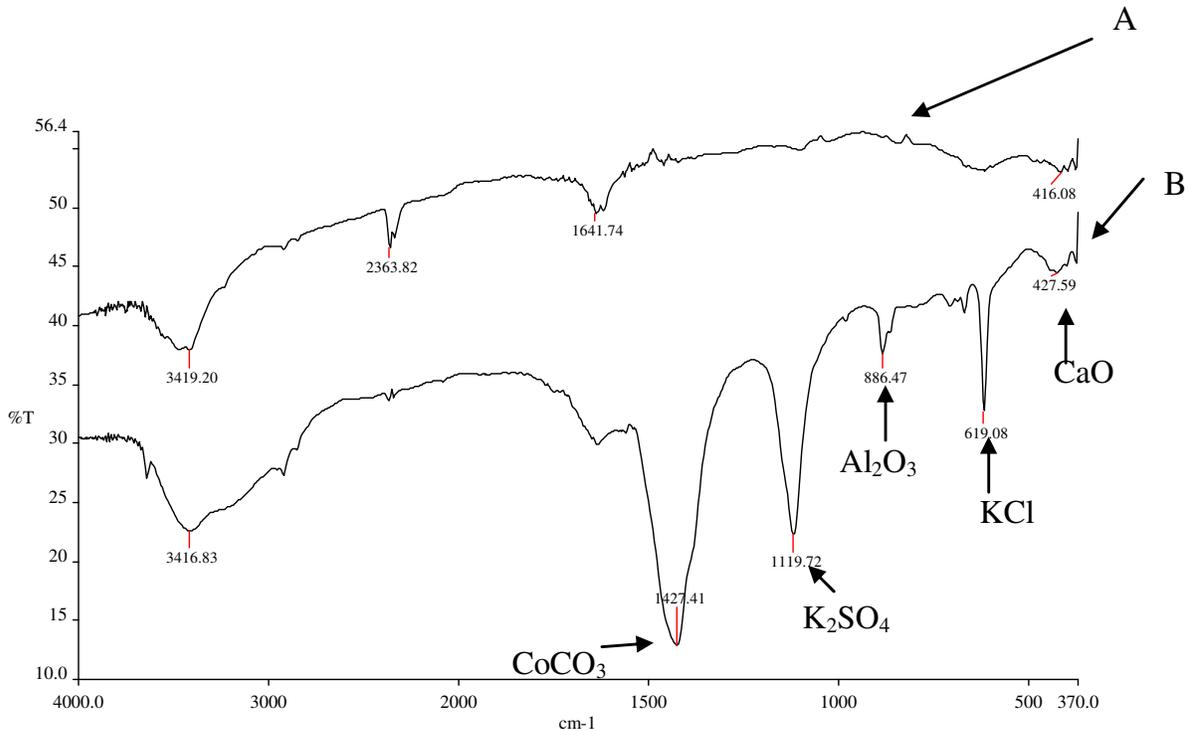


Figure 2. FT-IR spectra of an aqueous extract of *C. imberbe*: (A) KBr alone; (B) sample on KBr (1:300) disc.

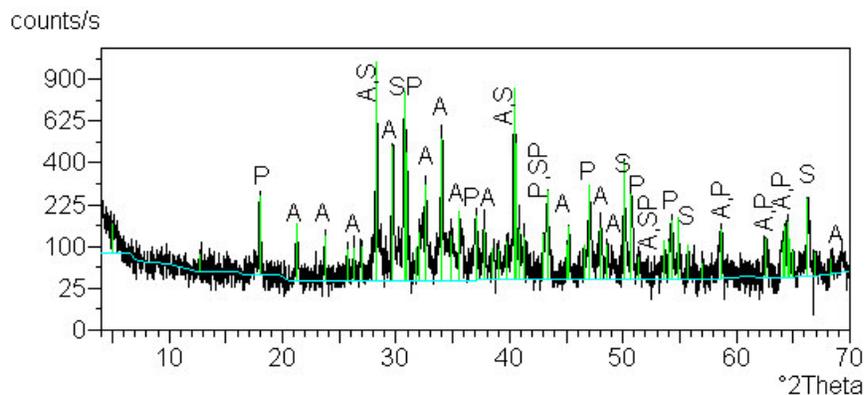


Figure 3. X-Ray diffractogram of aqueous extract of *C. imberbe* wood ash. The letter codes for the peaks are:- A; Arcanite (K_2SO_4), P; Portlandite ($Ca(OH)_2$), SP, spherocobaltite ($CoCO_3$) and S, Sylvite (KCl).

same sample revealed the presence of cobalt carbonate, ($CoCO_3$), potassium sulphate (K_2SO_4), calcium hydroxide, $Ca(OH)_2$ and potassium chloride, (KCl) as the predominant chemical forms (Figure 3).

DISCUSSION

In this report, we present evidence that *C. imberbe* wood ash extracts are effective against *Alternaria alternata*, *A. flavus*, *Penicillium italicum* and *Penicillium notatum*.

These fungi are common causes of damage to agricultural crop products and some of them contaminate affected commodities with mycotoxins (Chelule et al., 2001). Mycotoxins are highly toxic and carcinogenic fungal metabolites.

Wood ash extracts from *C. imberbe* inhibited the growth of all the test fungi used in this study. However, growth inhibition differed according to test organism. Furthermore, the inhibition of fungal growth decreased between 24 and 72 h of culturing being 100, 50, 47, 43 and 36% for *A. alternata*, *A. flavus*, *P. italicum* and *P.*

notatum, respectively. This observation may be an indication that these fungi have differing metabolic abilities to modify the growth conditions created by the extract (Table 1). Such modification of growth conditions (created by the extract) might involve expression of specific genes needed for growth under that environment (Abdollahi and Buchanan, 1981). The metabolic basis of such an adaptive response deserves further investigation.

Nonetheless, we have investigated the metabolic target of the extract by studying the effect of the extract on an amyolytic enzyme preparation from *A. flavus*, an aflatoxigenic fungus. This organism is frequently isolated from aflatoxin-contaminated agricultural products than other fungi (Rodriguez and Mahoney, 1994). In addition, previous separate studies (Woloshuk et al., 1997; Fakhoury and Woloshuk, 1999) have demonstrated that expression of mycotoxin-related genes in *A. flavus* is triggered by products of amyolytic degradation of starch including glucose, maltose and maltotriose (Abdollahi and Buchanan, 1981; Buchanan et al., 1987). In this study, the amyolytic enzyme preparation from *A. flavus* was inhibited by *C. imberbe* wood ash extract in a dose-dependent manner (Figure 1). This enzyme preparation showed optimal activity between pH 5.0 and pH 6.9 and it hydrolysed potato starch to release glucose (results not shown). Therefore, it is likely to be a glucoamylase (Kuek and Kidby, 1985). We hypothesize that the inhibition of fungal growth (Table 1) may be related to limited carbon supply due to inhibited activity of glucoamylase in the presence of *C. imberbe* wood ash extract (Figure 1).

We have used FT-IR analysis to elucidate the chemical nature of the active principles in the extract of *C. imberbe* wood ash (Figure 2). We have corroborated the evidence obtained by subjecting the same sample to XRPD analysis (Figure 3). Both analytical techniques revealed the presence of alkaline compounds in the extract. FT-IR analysis (Figure 2) revealed a strong carbonate signal. These findings lead us to propose that alkalization of the growth medium by the wood ash extract created conditions that were inhibitory to the activity of glucoamylase. This would cause a limitation of carbon supply and fungal growth (Table 1). This hypothesis is consistent with the observations that fungal growth and aflatoxin production require low molecular weight carbohydrates (Abdollahi and Buchanan, 1981; Buchanan et al., 1987).

The fact that some of the test organisms were able to colonize the initial zones of inhibited growth better than others (Table 1) does not detract from our hypothesis that inhibition of fungal growth was due to limited carbon supply in the presence of *C. imberbe* extract. This is more so because other studies with fungi (Rollins and Dickman, 2001; Kolo and Claeys, 2005) have independently shown that some fungi have the capacity to acidify (originally) alkaline growth environments. Consistent with such observations, we propose that the decrease in the respective zones inhibited growth for *A. alternata* and *A. flavus* (Table 1) may be related to their ability to modify

the alkaline condition resulting from the inclusion of the extracts in the growth medium. If this is so, the extract of *C. imberbe* may have served as a cue for the expression of genes necessary for production of acidic compounds for modifying the alkaline condition created by *C. imberbe* extract. This hypothesis deserves further investigation.

The freeze-dried aqueous extract of *C. imberbe* displayed a hygroscopic character and this might have also contributed to the efficacy of the ash extract by reducing the water activity of the treated commodity. Nevertheless, further studies are needed to determine whether the *C. imberbe* ash extract has any direct effect on key enzymes of mycotoxin production. This is more so because aflatoxin production was halted by addition of *C. imberbe* extract to growing cultures of *A. flavus* (results not shown).

Regardless of this, we conclude that inhibition of amyolytic activity by ash extracts of *C. imberbe* is the basis of the fungal growth-inhibiting character of the ash extract. This is in-keeping with the report (Ehrlich et al., 2005) that aflatoxin production requires acidic growth medium. Overall, the inhibitory effect of *C. imberbe* extract on mycotoxigenic fungal growth (as demonstrated herein) has significant positive implications for control of mycotoxin production and preservation of grain quality even at farm level.

ACKNOWLEDGEMENTS

The authors are grateful to the University of Botswana for financial support. In addition, we are grateful to I. Morobe for the fungal isolates used in this study.

REFERENCES

- Abdollahi A, Buchanan RL (1981). Regulation of aflatoxin biosynthesis: induction of aflatoxin production by various carbohydrates. *J. Food Sci.* 46: 633-635.
- Buchanan RL, Jones SB, Gerasimowicz WV, Zaika LLL, Stahl HG, Ocker LA (1987). Regulation of aflatoxin biosynthesis: Assessment of the role of cellular energy status as a regulator of the induction of aflatoxin production. *Appl. Environ. Microbiol.* 53(6): 1224-1231.
- Chelule PK, Gqaleni N, Dutton MF, Chuturgoon AA (2001). Exposure of rural and urban populations in KwaZulu Natal, South Africa to fumonisin B₁ in maize. *Environ. Health Persp.* 109(3) 253-256.
- Ehrlich KC, Montalbano BG, Coity PJ (2005). Divergent regulation of aflatoxin production at acidic pH by two *Aspergillus* strains. *Mycopathologia* 159(4): 579-581.
- Fakhoury AM, Woloshuk CP (1999). *Amy1*, the α -amylase gene of *Aspergillus flavus*: Involvement in aflatoxin biosynthesis in maize kernels. *Phytopathology* 89(10): 908-914.
- Fandohan P, Hell K, Marasas WFO, Wimpfield MJ (2003). Infection of maize by *Fusarium* species and contamination with fumonisin in Africa. *Afr. J. Biotechnol.* 2(12): 570-579.
- Gomes E, de Souza SR, Grandi RP, Da Silva R (2005). Production of thermostable glucoamylase by newly isolated *A. flavus* A. 1.1 and *Thermomyces lanuginosus* A. 13.37. *Braz. J. Microbiol.* 36: 75-82.
- Hill S, ap Rees T (1995). The effect of glucose on the control of carbohydrate metabolism in ripening bananas. *Planta* 196: 335-343.
- Kuek C, Kidby DK (1985). Determination of glucoamylase in culture filtrates containing other amyolytic enzymes. *Starch* 37(5): 161-162.
- Nyquist RA, Kagel RO (1997). *Infrared Spectra of inorganic compounds* 4. Academic Press, Michigan, USA.

- Oswald IP, Desautels C, Laffitte J, Fournout S, Peres SY, Odin M, Le Bars P, Le Bars J, Fairbrother JM (2003). Mycotoxin fumonisin B1 increases intestinal colonization by pathogenic *Escherichia coli* in pigs. *Appl. Environ. Microbiol.* 69(10): 5870-5874.
- Pitt JI, Basilica JC, Abarca ML, Lopez C (2000). Mycotoxins and toxigenic fungi. *Medical Mycology* 38(Supplement1): 41-46.
- Rodriguez SB, Mahoney NE (1994). Inhibition of aflatoxin biosynthesis by surfactants. *Appl. Environ. Microbiol.* 60 (1): 106-110.
- Rollins J, Dickman MB (2001). pH signaling in *Sclerotinia sclerotiorum*: identification of a pac/RIM1 homolog. *Appl. Environ. Microbiol.* 67(1): 75-81.
- Samarajeewa U, Sen AC, Cohen MD, Wei CL (1990). Detoxification of aflatoxins in food and feeds by physical and chemical methods. *J. Food Prot.* 53: 489-501.
- Socrates G (2001). *Infrared and Raman characteristic group Frequencies: Tables and Charts.* 3rd Edition. John Wiley and Sons, Chichester.
- Wild CP, Turner PC (2002). The toxicology of aflatoxins as a basis for public health decisions. *Mutagenesis* 17(6): 471-481.
- Woloshuk CP, Cavaletto JR, Cleveland TE (1997). Inducers of aflatoxin biosynthesis from colonized maize kernels are generated by an amylase activity from *Aspergillus flavus*. *Phytopathology* 87(2): 164-169.