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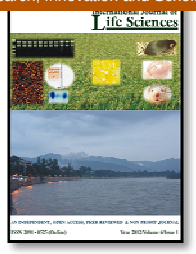

Research Article

Determination of toxigenic potential of *Fusarium* species occurring on sorghum and maize grains produced in Karnataka, India by using Thin Layer Chromatography

M Y Sreenivasa [1], B T Diwakar [2], Adkar Purushothama Charith Raj [3], Regina Sharmila Dass [3], K A Naidu [2] and G R Janardhana [3,*]

[1] Department of studies in Microbiology, University of Mysore, Manasagangotri, Mysore- 570 006, Karnataka, India; [2] Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore- 570 020, Karnataka, India; [3] Mycology and Phytopathology Laboratory, Department of studies in Botany and Microbiology, University of Mysore, Manasagangotri, Mysore- 570 006, Karnataka, India..

Corresponding author
Email:
grjbelur@gmail.com

Phone No:
091-821-2419763

Fax No:
091-821-2419759

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Ganesh Kumar Agrawal

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ABSTRACT

In the present study, the fumonisin producing ability of *Fusarium* species from different geographic regions of Karnataka was investigated. Sorghum and maize samples were examined for the presence of *Fusarium* species. Mycological studies confirmed the occurrence of *Fusarium* species such as *Fusarium verticillioides* (18), *F. proliferatum* (2) and *F. anthophilum* (2) isolates. Efficacy to produce fumonisins on maize patties was carried out by using thin-layer chromatography. Fumonisin B_1 and B_2 were detected as a red-purple spots with an Rf value of 6.1 and 6.3 respectively. The TLC analysis showed that 17 of 18 *F. verticillioides* isolates, 02 of 02 *F. proliferatum* isolates and 01 of 02 *F. anthophilum* isolates produced fumonisins. On the chromatograms, identical colored spots for standards and samples with same Rf value were detected. Early detection of toxigenic *Fusarium* species is important to prevent the entry of these toxic substances into the food chains.

Key words: **Fumonisin; *Fusarium sp.*; Maize; mycotoxin; Sorghum**

INTRODUCTION

The FAO estimated that each year, 25% to 50% of the world's food crops are contaminated by mycotoxins (Fandohan *et al.* 2003). *Fusarium* species are known to produce a chemically diverse array of mycotoxins such as diacetoxyscirpenol, deoxynivalenol, nivalenol, T-2 toxin, zearalenone, fumonisins, fusarin C, beauvericin, moniliformin, and fusaproliferin (Glenn, 2007; Logrieco *et al.* 2002). Fumonisins are a family of mycotoxins produced primarily by *Fusarium verticillioides* (Saccardo) Nirenberg (=*F. moniliformae*), *F. proliferatum* (Matsushima) Nirenberg, *F. anthophilum* (A. Braun) Wollenweber and others. Although 28 structurally related fumonisin analogues have been identified, only fumonisin B_1 (FB_1) and B_2 (FB_2) occur in abundant levels. Generally FB_1 represents 70-80% of the total fumonisin levels and FB_2 accounts for 15-25% when cultured on maize, rice or in liquid medium (Rheeder *et al.* 2002). *F. verticillioides* and *F. proliferatum* are the main source of fumonisins, a health risk mycotoxin, contaminating the agro-products (Morales-Rodriguez *et al.* 2007).

Contamination of food commodities by fumonisins has become a serious food safety problem throughout the world (Munkovold and Desjardins 1997). Fumonisins have emerged as highly visible animal and human health safety concern since they have been associated with many animal diseases (Gelderblom *et al.* 2001). Consumption of corn highly contaminated with fumonisins has been associated with increased risk of human oesophageal cancer in some regions of South Africa (Rheeder *et al.* 1992) and China (Chu and Li 1994). In India, field surveys revealed the occurrence of FB_1 in many samples collected from markets and households in maize and sorghum-growing regions of Karnataka (Sreenivasa *et al.* 2008a; Nayaka *et al.* 2010), Andra Pradesh (Bhat *et al.* 1997) and Harayana (Jindal *et al.* 1999).

Alarmingly high levels of fumonisins have also been detected in maize and sorghum (Chhatterjee and Mukherjee, 1994; Jindal *et al.* 1999). An epidemiological survey conducted in Karnataka and Andra Pradesh during 1997 revealed that consumption of moudly grains affected 1424 people from 27 villages (Shetty and Bhat, 1997).

Some of the existing chromatographic methods for detecting fumonisin includes thin-layer chromatography, liquid chromatography, gas chromatography-mass spectroscopy and high-performance liquid chromatography with an electrospray mass spectrometer. Among the available methods, most commonly used method is thin layer chromatographic technique (TLC) as initial screening method. The main objectives of the present investigation were to screen the cultures of *F. verticillioides*, *F. proliferatum* and *F. anthophilum* species isolated from maize and sorghum for their ability to produce fumonisin B_1 and B_2.

MATERIALS AND METHODS

Fusarium strains for fumonisin production

F. verticillioides (18), *F. proliferatum* (2) and *F. anthophilum* (2) isolates were originally isolated from maize and sorghum samples. These isolates were identified up to the species level based on the micro-morphological features using fungal keys and manuals (Leslie and Summerell 2006) and confirmed with molecular markers (Sreenivasa *et al.* 2006, Sreenivasa *et al.* 2008b). Further, all the species were evaluated for their ability to produce fumonisins B_1 and B_2 in maize patty cultures.

Preparation of maize patty culture

Preparation of maize patty cultures, inoculation and incubation was performed as per the protocol described by Vismer *et al.* (2004). Whole maize kernels were ground in a fire proof mixer grinder to a fine powder and 30g was taken in Pyrex Petri dishes (100mm x 18mm) and 30ml of distilled water was added. The preparation was autoclaved at 121 ^{o}C for 1 h and allowed to stand overnight. The sterilization was repeated next day for 1 h (Figure-1A). All *Fusarium* species were inoculated on PDA and incubated at 26$\pm$2 ^{o}C for 4 days. One ml ($1x10^6$conidia/ml) of conidial suspension of each fungal isolate was prepared in sterile distilled water and was used to inoculate maize patty in a culture plates. Patties were incubated in dark at 28 ± 2 ^{o}C for 4 weeks (Figure-1B), after which they were dried in an hot air oven at 60 ^{o}C for 8-10hrs. Harvested dry patties were ground in a mixer grinder (Kenstar, Classique, MG-9605A) to a fine meal and used for fumonisin analysis.

Clean up with Sep-Pak C_{18} column

10g of maize patty culture sample was weighed and transferred into a 250ml beaker and mixed with 50ml of acetonitrile/water (50/50, v/v). The beaker was covered with aluminum foil and shaken for 30min. The mixture was filtered through Whatman No. 4 filter paper. The Sep-Pak C_{18} column was connected to peristatic pump and was preconditioned with by rinsing with 2ml ACN followed by 1% KCl. Two ml of filtered extract was added to 6ml of 1% KCl and loaded into a C_{18} clean-up column. The solution was then forced through the Sep-Pak C_{18} column at a flow rate of 1ml/min. The column was rinsed with 2ml of 1% KCl followed by 2ml of ACN/ H_2O (15+85, v/v). The rinses were discarded, and air was forced through the column to expel all the rinse solution. Finally FB_1 and FB_2 were eluted from the column with 2ml of ACN/ H_2O (70+30, v/v). These elutants were evaporated to complete dryness under a gentle stream of nitrogen and dissolved in 100µl of ACN/ H_2O (50+50, v/v) for TLC analysis.

Thin layer chromatographic analysis

TLC was performed as per the protocol described by (Bially *et al.* 2005) with some modifications. The samples (5-10µl) was spotted on a thin-layer chromatographic (TLC) plates (20 x 20cm, normal phase) (ALUGRAM® SIL G/UV_{254}, Macherey-Nagel, Germany) pre-heated at 110 °C for 10min. The FB_I and FB_2 (100ng/ml) standards from the stock solutions were spotted on TLC plate. Separation was carried out in 1-butanol-acetic acid-water (20+10+10, v/v/v) as mobile phase. After separation, the plates were dried at room temperature, sprayed with p-anisaldehyde (0.5% in MeOH/sulfuric acid/acetic acid, 90/5/l, v/v/v) and heated at 100 °C for 5min. The Rf value is the ratio the spot moved compared to the distance the solvent moved from the starting point. The detection limit of fumonisins in samples on the TLC plate was 0.1µg/ml.

RESULTS AND DISCUSSION

Fusarium species are among the most studied phytopathogenic fungi, with several species causing diseases on maize, wheat, barley, and other food and feed grains (Glenn, 2007). Wheat, maize and other grain crop residues are generally regarded as the

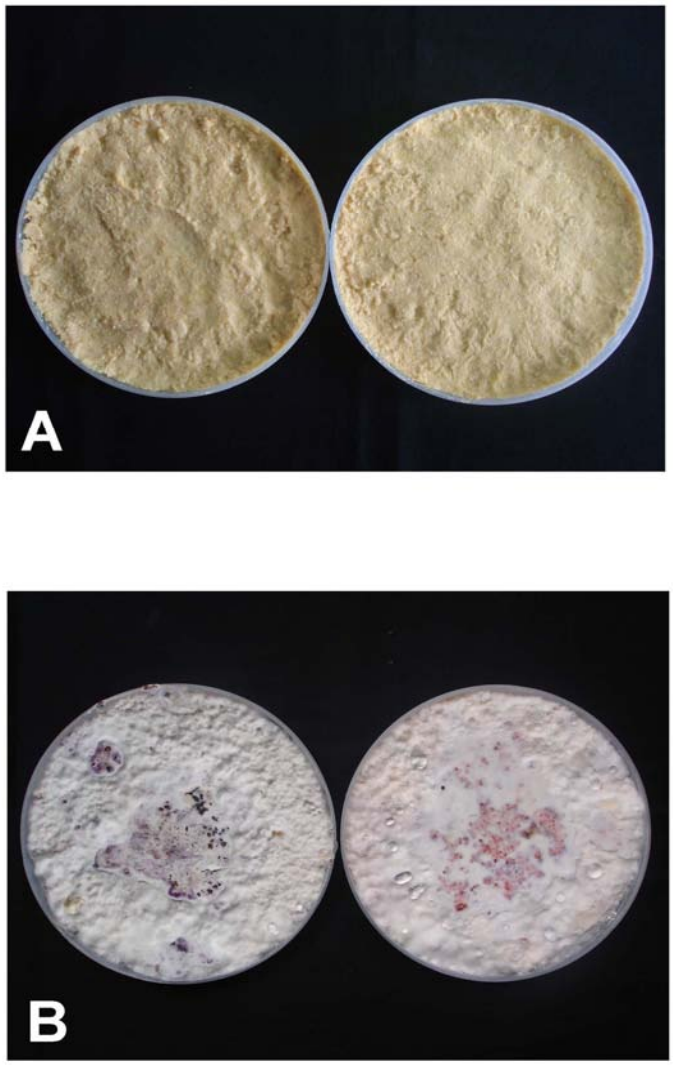

Figure 1. Maize patty cultures. A: Pyrex petri dishes with maize patty (before inoculation), B: Fusarial growth on maize patties after 4 weeks of incubation.

primary source of inoculum for *Fusarium* species (Shaner, 2003). Decreased yield, as well as diminished quality and value of the grain, results in significant worldwide economic losses. Although fumonisins are a relatively novel class of *Fusarium* toxins, they have attracted both social experts and scientist's attention because of their high health risk potentials (Nelson *et al.* 1992). In some regions of Africa, Asia and other parts of the world, high levels of fumonisins have been constantly detected and recorded on maize (Shephard *et al.* 1996). Many isolates of *F. verticillioides* and *F. proliferatum* isolated from maize and sorghum and other substrates from different geographic locations of North America, Africa, Asia and Australia (Ghiasian *et al.* 2005). As identification of *Fusarium* species is critical to predict the potential mycotoxigenic risk of the isolates, there is a need for accurate and complementary tools which permit a rapid, sensitive and reliable specific diagnosis of potential fumonisin producing *Fusarium* species.

The TLC analysis showed that 17 of 18 *F. verticillioides* isolates, 02 of 02 *F. proliferatum* isolates and 01 of 02 *F. anthophilum* isolates produced fumonisins. Fumonisin B_1 and B_2 were detected as a red-purple spots with an Rf value of 6.1 and 6.3 respectively (Figure 2). On the chromatograms, identical colored spots for standards and samples with same Rf value were detected. The TLC method failed to measure toxin concentrations as accurately as HPLC because the TLC method estimates sample concentration by comparison to standards. The concentration determined by TLC can only be as precise as the number of standards used, and therefore the measured concentration is dependent upon the concentration difference between standards. In view of this, the same samples were confirmed for the presence and quantification of FB1 and FB2 with

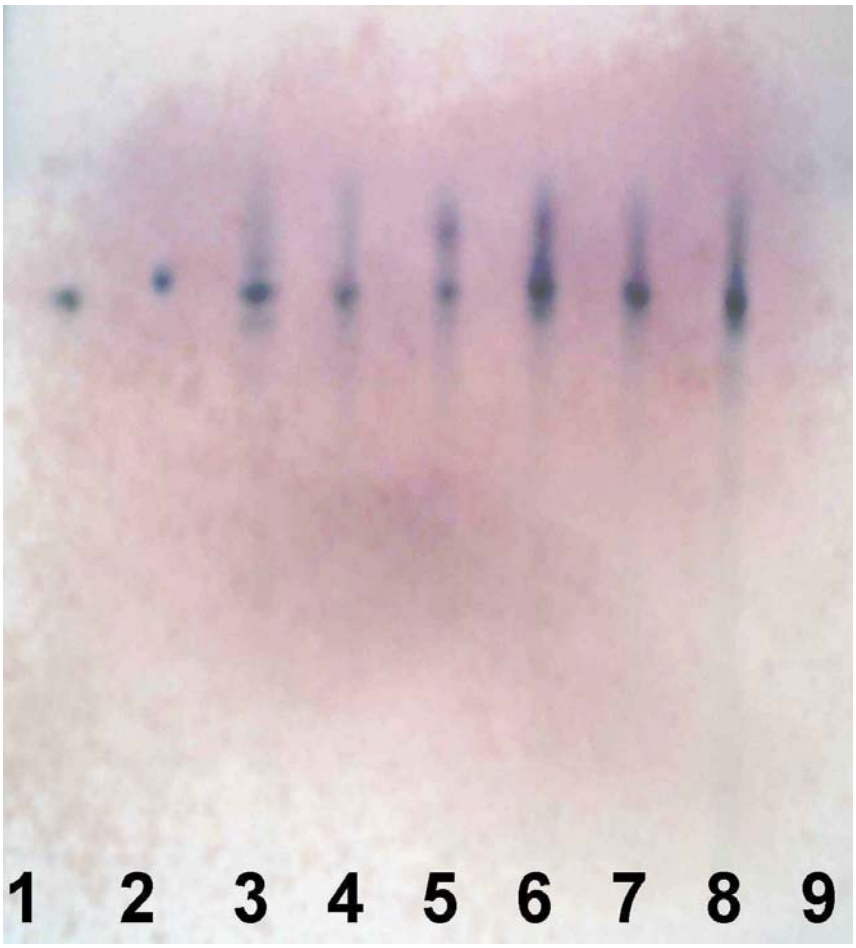

Figure 2. Thin-layer chromatographic plate showing the red-purple spots of fumonisins; Lane 1: Standard fumonisin B_1 (Rf - 6.1); Lane 2: Standard fumonisin B_2 (Rf - 6.3); Lane 3 – 8: Positive maize patty samples inoculated with testing *Fusarium* species for Fumonisin; Lane 9:Negative control.

HPLC technique (Sreenivasa *et al.* unpublished data). The two species of *Fusarium* viz., *F. verticillioides* and *F. proliferatum* are the most prolific fumonisin producers and are known to produce fumonisins from 17,900µg/g to 31,000µg/g of FB_1 (Rheeder *et al.* 2002). The production of fumonisins by different strains of *F. proliferatum* varies widely. Some studies have shown that only low or even no fumonisin production (Tseng *et al.* 1995; Da silva *et al.* 2004), while other study has shown that, *F. verticillioides* can produce high levels of fumonisins (Nelson *et al.* 1992). The natural occurrence of fumonisin B_1 in Indian sorghum, where the contamination levels ranging from 0.01-5.0 mg/kg and 0.15-0.51 mg/kg has been reported (Shetty and Bhat 1997). (Da silva *et al.* 2004) reported that production of fumonisins by *Fusarium* species in Brazilian sorghum. Fumonisins have also been detected at lower levels in sorghum (Shetty and Bhat, 1997; Leslie and Marasas, 2001).

Consumption of cereals contaminated with fumonisins involves high health risks to animals and humans. Recent studies also revealed that, fumonisins are associated with human oesophageal cancer. The International Agency for Research on Cancer (IARC) evaluated these toxins as human carcinogens (Fandohan *et al.* 2003). In view of their toxic properties, the FDA has fixed 2-4ppm/Kg of food as the permissible limit for fumonisins in foods. In view of all these, accurate detection and quantification of fumonisins in cereal samples is very important. It is also essential for a reliable evaluation of human exposure to these carcinogenic mycotoxins. Such detection systems set the realistic tolerance levels of fumonisins in food products for human and animal consumption. The data on the natural occurrence of fumonisins in cereals is also very much required for food and nutrition specialists for an appropriate toxicological evaluation. The study revealed the occurrence fumonisin producing strains of *Fusarium* species in many samples of maize and sorghum, warrants the need for systematic investigations of foods that are routinely channeled for human and animal consumption.

In conclusion, the research presented here demonstrates that TLC could reliably be used as an alternative to the more expensive methods of analyzing grain samples for fumonisins. Diagnostically, this TLC procedure can be used to initially screen sorghum and maize samples intended for human and animal consumption for the possible contamination with fumonisins, thus reducing the number of samples requiring quantitative analysis for the fumonisins by HPLC analysis in the laboratory. The present study is a sample investigation and further efforts are very much needed to know the epidemiological implications of these toxins on humans and animals. More studies are required to address this problem in other states of India.

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