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Identification and characterization of toxigenic Fusaria associated with sorghum grain mold complex in India

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Abstract *Fusarium* species are dominant within the sorghum grain mold complex. Some species of *Fusarium* involved in grain mold complex produce mycotoxins, such as fumonisins. An attempt was made to identify *Fusarium* spp. associated with grain mold complex in major sorghum growing areas in India through AFLP based grouping of the isolates and further confirmation of the species by sequencing part of *α-Elongation factor* gene and comparing the sequences with that available in NCBI database. The dendrogram generated from the AFLP data clustered the isolates into 5 groups. Five species of *Fusarium* – *F. proliferatum*, *F. thapsinum, F. equiseti*, *F. andiyazi* and *F. sacchari* were identified based on sequence similarity of *α-Elongation factor* gene of the test isolates with those in NCBI database. *Fusarium thapsinum* was identified as predominant species in *Fusarium* – grain mold complex in India and *F. proliferatum* as highly toxigenic for fumonisins production. Analysis of molecular variance (AMOVA) revealed 54% of the variation in the AFLP patterns of 63 isolates was due to the differences among *Fusarium* species and 46% was due to differences among the strains within a species.

Keywords AFLP · *α-Elongation factor* · *Fusarium* · Mycotoxin · Fumonisin

Introduction

Grain mold is a major biotic constraint to production, marketing and utilization of grain sorghum. The term "grain mold" is used to describe the diseased appearance of sorghum grains resulting from infection by one or more pathogenic/saprophytic fungi. The disease is particularly important on improved, short and medium duration sorghum cultivars that mature during the rainy season in humid, tropical and subtropical climates. Several fungal species of the genera *Fusarium, Curvularia, Alternaria, Phoma, Bipolaris* and *Colletotrichum* have been reported to be associated with grain mold [1]. Of these, *Fusarium* spp. are dominant within the grain mold complex. Some species of *Fusarium* (*F. andiyazi, F. proliferatum, F. thapsinum* and *F. sacchari*) involved in grain mold complex produce mycotoxins, such as fumonisins, moniliformin, trichothecenes, and fusaproliferin [2]. The secondary invading *Aspergillus* spp. may produce aflatoxins in the contaminated grains as well. These toxins reduce the quality of grains as food/feed sources as well as the value of grain in the market. These toxins are associated with a variety of human and animal health problems including acute toxicity, increased incidence of cancer, inhibition of normal growth and development, immunity suppression and increased disease susceptibility, increased risks of birth defects and reduced nutritional quality of resulting grain. Risk analysis studies have indicated that when these molds affect staple food grains such as sorghum and maize, the significance to human and animal health is considerable because their quantum of consumption is higher than that of other foods. The first report of fumonisins toxicity to human and poultry came in 1998 from the Deccan plateau of India [3]. An outbreak of food poisoning, characterized by abdominal pain and diarrhea, attributed to the ingestion of fumonisins-contaminated maize and sorghum, had been reported from several villages in India [4]. The disease was observed only in adults who were consuming the molded grains. The affected people mostly belonged to lower socioeconomic groups, such as marginal farmers and landless agricultural labourers who did not have access to other staples. Similarly, an outbreak of trichothecene mycotoxicosis associated with the consumption of mold-damaged wheat had been reported from Kashmir Valley in India [5]. To reduce levels of fumonisin in grain, efforts have been made to identify sources of maize with increased resistance to fungal infection and fumonisin contamination [6]. Although mycotoxins have been detected from various food grains infected with *Fusarium*, information regarding the profile of toxin-producing species of

Fusarium involved in sorghum grain mold complex in India is not yet available. Since several species of *Fusarium* have been reported to be associated with this disease, it is essential to identify more precisely the toxigenic species of *Fusarium* and genetic resistance in sorghum against potential toxigenic strains.

Materials and methods

Identification of *Fusarium* spp. through DNA markers

Six hundred seventy-two isolates of *Fusarium* collected from naturally molded sorghum grains from five locations (Akola, Parbhani, Patancheru, Palem and Surat) in India during 2003-05 were established on potato dextrose agar (PDA) plates. Morphological/cultural characters of these 672 isolates were studied on PDA and Banana leaf agar [7]. From these, 63 isolates from different regions representing different species of *Fusarium* (initial identification based on morphological/cultural characters) were selected to study genetic relatedness/diversity through amplified fragment length polymorphism (AFLP) and sequence based identification, and their fumonisins production levels. Three to four day-old mycelia of the *Fusarium* isolates grown in potato dextrose broth were harvested by filtration and washed in sterile deionized water. The mycelia were ground using liquid nitrogen and genomic DNA of the test isolates was extracted as per Kim et al. [8].

AFLP analysis

AFLPs were generated based on selective amplification of DNA restriction fragments [9]. Five EcoR1-Mse1 AFLP® primer combinations (Etg + Mcag, Eaa + Mctt, Eac + Mcag, Etc + Mctt and Etg + Mcag), were examined in the 63 isolates. The analysis was carried out using the commercial kit (Life Technologies, USA) following the manufacturer's protocols with slight modifications. Primary template DNA was prepared in a one-step restriction-ligation reaction. Fungal genomic DNA (400 ng) was digested with *Eco*R1 and *Mse*l at 37 °C for 2 h and heated at 70 °C for 15 min to inactivate the enzyme. The DNA fragments were ligated to *Eco*R1 and *Mse*l adapters at 20 °C for 2 h. After terminating the reaction, the

ligation mixture was diluted 10-fold with TE buffer and the fragments were pre-amplified in a thermal cycler (MJ Research, USA) using a temperature cycle of 94 °C for 30 sec, 56 °C for 1 min and 72 °C for 1 min, in a total of 30 cycles. Five EcoR1-Mse1 AFLP primer combinations, each with two-base extension in EcoR1 and three-base extension in MSe1 primer, were examined in the 63 isolates. The *Eco*R1 primer was labeled with [γ-^{32}P]-ATP (3000 Ci/mmol) and the selective amplification was carried out according to the manufacturer's protocol. After selective amplification, the PCR products in 3 µl sub-samples were separated by electrophoresis on 6 % denaturing polyacrylamide DNA sequencing gel containing 7.5 M urea. Autoradiograms were obtained using Kodak X-Omat film.

AFLP profiles of 63 isolates were used to construct a binary matrix. Each band was scored as present (1) or absent (0) across the isolates. The data were then analyzed using Numerical Taxonomy System Version 2.2 (NTSYSpc). The proximity matrix was computed using Dice similarity coefficient and a dendrogram was constructed by unweighted pair group method of arithmetic averages (UPGMA) using the SAHN (Sequential Agglomerative Hierarical Nested) cluster analysis module [10]. Analysis of Molecular Variance version 1.55 (AMOVA) was used to estimate variance components for the AFLP patterns and to partition the total variance into 'within species' and 'among species' [11]. To see the significance of variance components 1000 permutations were used.

Phylogenetic analyses

DNA sequence variation was assessed by sequencing part of *α-Elongation factor* (EF-1α) using the gene-specific primer pair using BigDye Terminator cycle sequencing kit on ABI3130XL (Applied Biosystems, California, USA). EF1 (5'-ATGGGTAAGGARGACAAGAC-3') and EF2 (5'-GGARGTACCAGTSATCATGTT-3') primers [12] were used to amplify ~650 bp fragment, a part of EF-1α gene of *Fusarium*. PCR was performed in a 25 µl final reaction mixture containing 2.5 µl 10 X buffer, 1 U of XT-Taq, 0.5 µl dNTP mix (2.5 mM each), 2 µl template DNA (20-50 ng) and 1 µl each of both forward and reverse primers (10 nM). DNA amplification was performed in a PCR thermocycler (Gene Amp PCR system 9700, Perkin-Elmer, USA) using an initial 5 min denaturation at 95 °C followed by 34 cycles of 1 min denaturation at 95 °C, 50 sec annealing at 50 °C and 1 min extension at 72 °C followed by

a final extension of 10 min at 72 °C. The PCR amplification products were checked on 1.5 % agarose gel electrophoresis. The EF-1α PCR amplified products were sequenced using specific primers. Sequences of the EF1-PCR fragments were searched against those in the NCBI database using BLAST [13] for species identification. The sequences were aligned through CLUSTAL W. The dissimilarity index was calculated from the aligned sequences of different isolates using 'Simple matching' option in DARwin and a weighted neighbor joining tree was constructed.

Detection of Fumonisins B_1 (FB_1)

All the single-spore cultures of test isolates were inoculated into Erlenmeyer flasks containing 50 g sterilized sorghum grains. The flasks were incubated for 4 weeks at 25 ^{o}C for fumonisin production. The *Fusarium* colonized sorghum grains were removed from the flasks and made into powder using blender and 20 g sub-sample was drawn for toxin extraction. Each 20 g sample was extracted with 100 ml of solvent containing 70 ml methanol + 30 ml water + 0.5 g KCl and blended for 2 minutes. The extracts were transferred to conical flask and shaken for 30 min. at 300 rpm before filtering through whatman No. 4. Then the extracts were diluted 1:10 with 0.2% bovine serum albumin prepared in PBS-tween, pH 7.4 [14].

All the methanol extracts were subjected to indirect competitive enzyme-linked immuno-sorbant assay (cELISA) as per method described by Yu and Chu [15] and Waliar et al. [16] with little modifications. In general, ELISA plates (Maxi-sorp, Nunc A/S, DK-4000, Rorkilde, Denmark) were coated with FB_1-BSA conjugate prepared in carbonate buffer at a concentration of 200 ng/ml and dispensed 150 µl /well. At each step plates were incubated at 37 ^{o}C for one hour followed by three washes with PBS-Tween. Then the plates were treated with 0.2% bovine serum albumin (BSA) prepared in PBS-tween (PBST-BSA), followed by competition step in which standard or samples and specific antibodies were added in the plate. The FB_1 concentrations of the standards starting from 100.00, 50.00, 25.00, 12.50, 6.25, 3.12, 1.56, and 0.78 ng/ml was used and each concentration was duplicated in two wells. Similarly, duplicates of each test sample in two wells were maintained. To the diluted sample extract or standards 50 µl of FB_1 antiserum diluted to 1:5,000 in 0.2% PBST-BSA was added. Then alkaline phosphatase labeled goat antirabbit IgG conjugate diluted to 1:4000 in PBST-BSA was added to the plate, followed by substrate p-nitrophenyl

phosphate prepared in 10% diethanolamine. After 30 min incubation at room temperature plates were read at 405 nm in an ELISA reader (Multiskan plus Labsystems). Regression curve was drawn using Log_{10} values of toxin concentration plotted on the Y-axis and optical density values plotted on the X-axis and using the regression equation FB_1 concentration was determined.

Results

Genetic variation

A high level of polymorphism was observed among isolates following selective amplification with 5 AFLP primer combinations (Etg + Mcag, Eaa + Mctt, Eac + Mcag, Etc + Mctt and Etg + Mcag). A total of 473 bands were amplified, of which 470 were polymorphic. The dendrogram generated from the AFLP data revealed genetic diversity among the isolates by clustering them into 5 groups. Of the 63 isolates, 31 were clustered in one major group (group IV) with ~70% genetic similarity among isolates (Fig. 1). The remaining 32 isolates were clustered in 4 major groups and many sub-groups within main clusters indicating the involvement of many species of *Fusarium* in the grain mold complex. Group I clustered 12 isolates, group II 3, group III 10 and group V 6 isolates.

α-Elongation factor sequence based identification

The sequences of EF-1α gene from each of the 63 *Fusarium* isolates (query) were compared to those from various known species of *Fusarium* (subject) in the NCBI database. Five species of *Fusarium* – *F. proliferatum*, *F. thapsinum, F. equiseti*, *F. andiyazi* and *F. sacchari* were identified based on sequence similarity. Neighbor-joining tree constructed using *α-Elongation factor* gene sequence of test isolates clustered the isolates of same species in the distinct groups (Fig. 2).

Analysis of molecular variance

Analysis of molecular variance (AMOVA) revealed 54% of the variance in the AFLP patterns of 63 isolates was due to the differences among different species of *Fusarium* and 46% was due to differences among the isolates within a species (Table 1). The variance component exhibited almost 50% variation because of genetic variation between and within *Fusarium* species which indicate large variation between strains/isolates of the same species.

Identification of toxigenic *Fusarium* spp.

Strains of *F. proliferatum* were identified as highly toxigenic for fumonisins production followed by *F. andiyazi* (Fig. 3). Isolate F 242 (*F. proliferatum*) was the highest fumonisin (B_1) producing strain (476539 µg kg^{-1} seed). Ten of the 12 *F. proliferatum* isolates produced high levels (>28000 µg kg^{-1} seed) of fumonisins, whereas two isolates F316 and F 953 produced less (<300 µg kg^{-1} seed) fumonisin. Two isolates of *F. andiyazi* F 409 and F 943 also produced high levels (>100000 µg kg^{-1} seed) of fumonisin. Strains of *F. thapsinum, F. equiseti*, and *F. sacchari* were non-fumonisin producers, however, one isolate of *F. thapsinum* (F88) produced good amount of fumonisins. One isolate of *F. sacchari* (F 22) and two isolates of *F. thapsinum* (F 241 and F 329) produced about 1500 µg fumonisin kg^{-1} seed.

Prevalence of *Fusarium* spp. in major sorghum growing areas in India

Based on *α-Elongation factor* sequence similarity, 63 *Fusarium* isolates were identified as five species of *Fusarium* – *F. proliferatum*, *F. thapsinum, F. equiseti*, *F. andiyazi*, and *F. sacchari*. Frequency of occurrence of these species varied across locations (Table 2). *Fusarium thapsinum* was identified as predominant (32 of the 63 isolates tested) species in *Fusarium* – grain mold complex. *F. thapsinum*, *F. proliferatum* and *F. andiyazi* were present at all the five locations, whereas *F. equiseti* was not detected at Patancheru and Surat and *F. sacchari* was present only at Patancheru and Palem.

Discussion

There is a general consensus that *Fusarium* growing on sorghum belongs mainly to *Giberella fujikuroi* species complex, which comprises of *Fusarium* species included in section Liseola [2]. Since several species of *Fusarium* have been reported to be associated with sorghum grain mold [17], and most of them are morphological similar, it is essential to identify species of *Fusarium* causing grain mold more precisely. Therefore, an attempt was made to identify *Fusarium* spp. associated with grain mold complex from five locations (Patancheru, Parbhani, Palem, Surat and Akola) representing major sorghum growing areas in India using AFLP based grouping of the isolates and further confirmation of the species by sequencing part of *α-Elongation factor* gene and comparing the sequences with that available in NCBI database.

Dendrogram generated from AFLP data obtained from selective amplification with 5 primer combinations (Etg + Mcag, Eaa + Mctt, Eac + Mcag, Etc + Mctt and Etg + Mcag) clustered the isolates in 5 distinct groups. Since isolates of the same species of *Fusarium* have been reported to have >65% genetic similarity [2], it was expected that the isolates representing each group with more than 70% similarity to be the same species. The grouping of each species in distinct cluster was confirmed by *α-Elongation factor* sequence based species identification. Neighbor-joining tree constructed using *α-Elongation factor* gene sequence of test isolates clustered the isolates of same species in the distinct groups. A very high level of correspondence was observed between AFLP and *α-Elongation factor* sequence based grouping with only one exception. Isolate F 311 was identified as *F. thapsinum*, but it clustered along with *F. equiseti* group in the AFLP generated dendrogram. AMOVA results showed that both within and between species variance was high indicating that strains variation also exist in *Fusarium* species associated with sorghum grain mold.

Frequency of occurrence of different species also varied across different locations. Of the 63 isolates analyzed, 32 were identified as *F. thapsinum*, followed by *F. proliferatum* (12 isolates) and *F. andiyazi* (10 isolates) and were present at all the five locations from where the grain mold samples were collected, whereas *F. equiseti* (6 isolates) and *F. sacchari* (3 isolates) were present in small proportion. *Fusarium equiseti* was not detected at Patancheru and Surat, whereas *F. sacchari* was present only at Patancheru and Palem. *Fusarium thapsinum* has been reported to be common in sorghum grain mold [2]. Other species identified in this study, such as *F. proliferatum*, *F. andiyazi* and *F. sacchari* have been reported to be associated with sorghum grain mold [17]. At some locations *F. andiyazi* may be the

dominant *Fusarium* species on sorghum. However, it has not been searched for extensively, but probably has a much broader distribution with sorghum [18].

Members of *Fusarium* species included in section Liseola are generally known to produce fumonisins. However, fumonisin production levels vary among the species. Strains of *F. proliferatum* were identified as highly toxigenic for fumonisin production followed by those of *F. andiyazi*. Fumonisins are produced by *F. proliferatum*, often at high levels [19, 20], and the *FUM* gene cluster encoding for fumonisin biosynthesis has been sequenced and characterized in some detail [21]. *Fusarium thapsinum*, although identified most commonly associated with sorghum grain mold complex in India, it was atoxigenic for fumonisin production except one isolate F 88. *Fusarium thapsinum* has been reported to produce high levels of moniliformin, but only traces of fumonisins [2, 22, 23].

Genetic resistance to ear rot and mycotoxin content has been found in European maize inoculated with *F. graminearum* and *F. verticillioides* [24]. Maize genetics and breeding studies have guided strategies to improve resistance to fumonisin accumulation [6]. In this study, we have been able to identify toxigenic strains of *F. proliferatum* (F 242 or F 234), *F. andiyazi* (F 943) and *F. thapsinum* (F 88) which can be used in greenhouse for evaluating sorghum lines for resistance to toxigenic *Fusarium* associated with grain mold complex. Such resistance sources could be used in breeding fumonisin tolerant sorghum varieties and hybrids.

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Table 1 AMOVA for partitioning AFLP variation within and between *Fusarium* species

Source of variation	df	Sum of squares	Variance component	% total
Among species	4	1829.35	40.97 Va	54.41
Within species	58	1990.71	34.32 Vb	45.59
Total	62	3820.06		
Fixation index (Fst) = 0.544				

Table 2 Prevalence of different *Fusarium* spp. associated with sorghum grain mold complex in major sorghum growing areas in India

Location/State	Total samples	*Fusarium* spp.*				
		F. andiyazi	*F. proliferatum*	*F. equiseti*	*F. sacchari*	*F. thapsinum*
Patancheru/Andhra Pradesh	17	2	6	-	2	7
Palem/Andhra Pradesh	13	4	1	1	1	6
Surat/Gujarat	10	1	1	-	-	8
Parbhani/Maharashtra	10	2	2	3	-	3
Akola/Maharashtra	13	1	2	2	-	8
Total	63	10	12	6	3	32

*Species identified based on *α-Elongation factor* sequence similarity

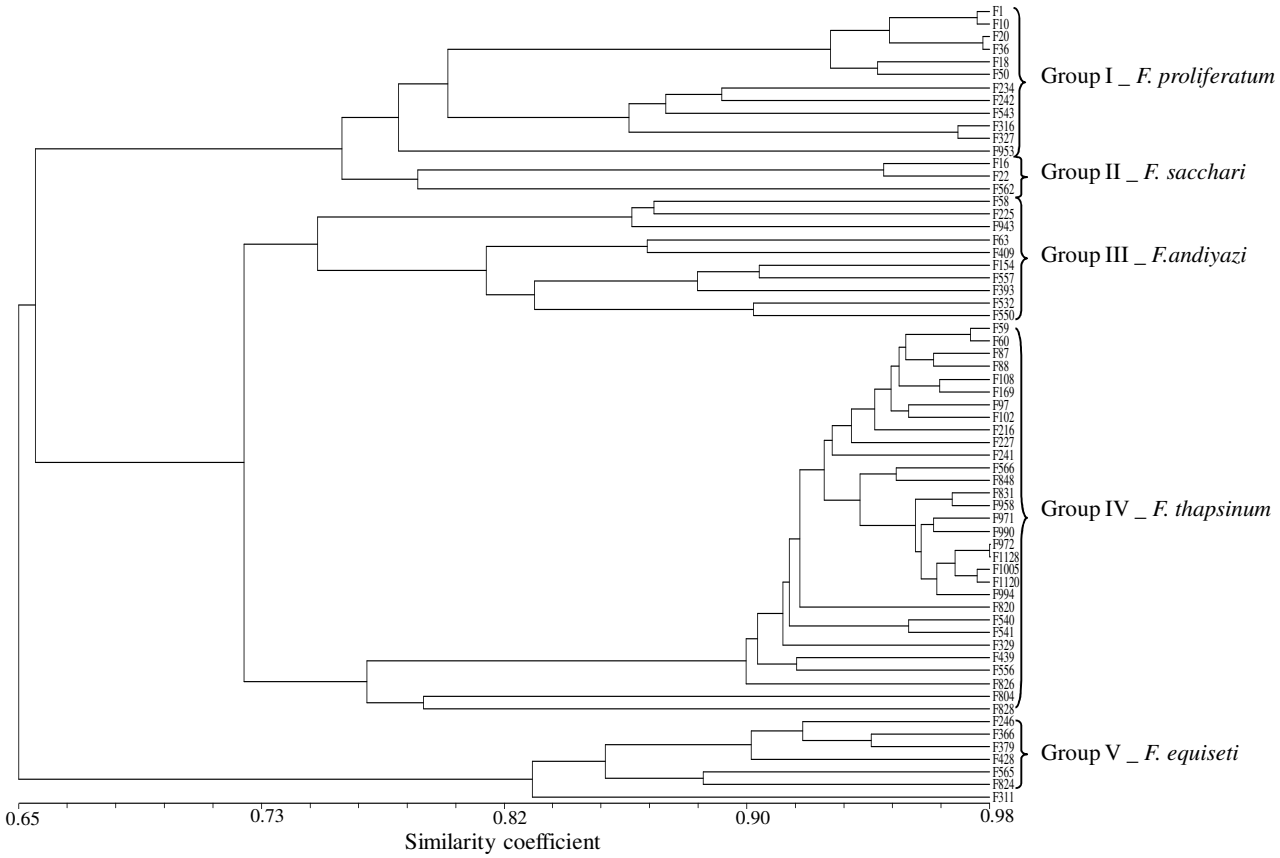

Fig. 1 Dendrogram depicting genetic relatedness among 63 isolates of *Fusarium* based on similarity coefficient calculated from AFLP fingerprints

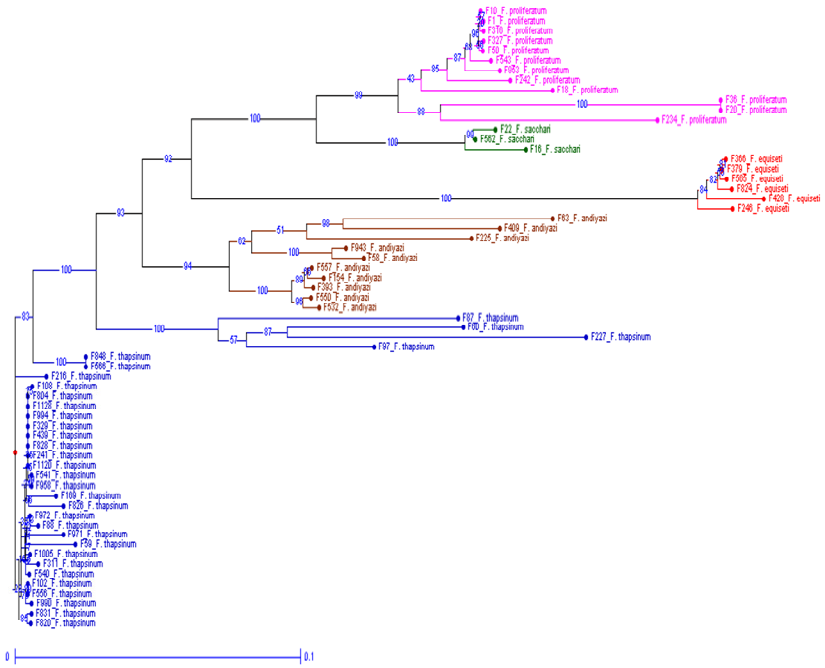

Fig. 2 Neighbor joining consensus tree for translation elongation factor -1α (EF-1α) sequences of *Fusarium* spp.

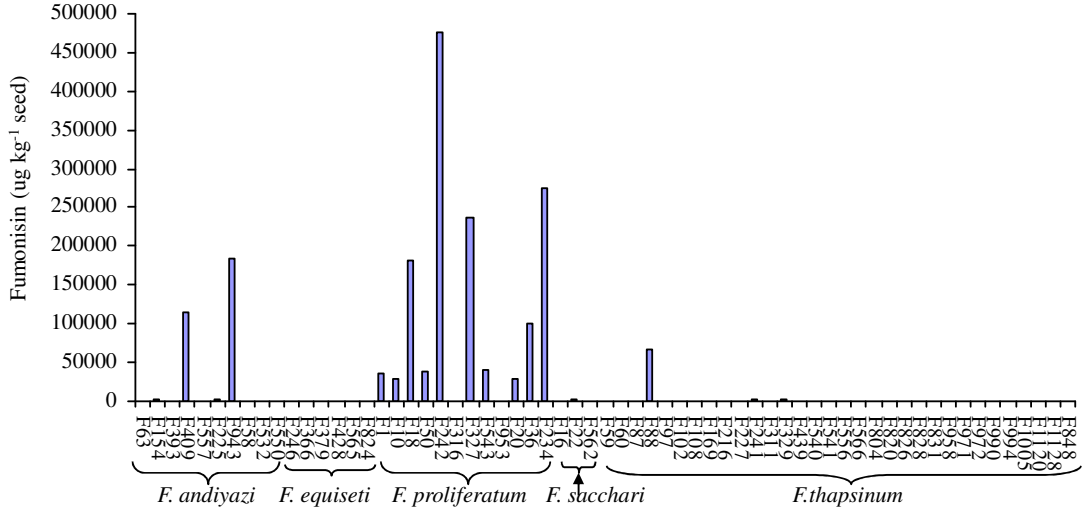

Fig. 3 Fumonisin (ug kg^{-1} seed) produced by strains of *Fusarium* spp.