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Subramani Natarajan
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Dananjeyan Balachandar
Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Senthil Natesan
Department of Plant Molecular Biology and Bioinformatics, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Velazhahan Rethinasamy
Department of Crop Sciences, College of Agricultural and Marine Sciences, Sultan Qaboos University, Muscat, Oman

Paranidharan Vaikuntavasan
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Corresponding Author:
Paranidharan Vaikuntavasan
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Occurrence of fungal contaminants and aflatoxin producing potential of *Aspergillus flavus* strains from Indian senna (*Cassia angustifolia*)

Subramani Natarajan, Dananjeyan Balachandar, Senthil Natesan, Velazhahan Rethinasamy and Paranidharan Vaikuntavasan

Abstract
Herbal products are frequently reported with the contamination of mycotoxins. In this present study, we assessed the occurrence of fungal contaminates associated with senna leaves (n=128) which collected from the different time period such as preharvest, drying, processing and storage. The most prevalent fungal genera were isolated as *Aspergillus*, *Penicillium*, *Rhizopus*, *Cladosporium*, *Mucor*, *Fusarium* and *Alternaria*. The maximum fungal count was recorded by *Aspergillus* (11.68 $\times 10^4$ CFU/g) followed by *Rhizopus* (4.65$\times 10^4$ CFU/g). The 18s rDNA analysis was employed to confirm representative *A. flavus* isolates obtained from the senna leaves. The aflatoxin producing ability of *A. flavus* strains were determined by using high performance liquid chromatography (HPLC) coupled with florescence detector. The higher amount of aflatoxin production was showed by strain ASL1 (4824.23 µg/g) followed by ASL7 (3081.67 µg/g). Moreover, the aflatoxin production was significantly varied with in the strains tested in this study ($P< 0.05$). The presence of fungal contaminants and the aflatoxigenic fungi indicated the health risk associated with substrates. Hence, the regular monitoring of herbal drugs is essential to prevent the chronic exposure of aflatoxin contamination.

Keywords: Senna, AFB_1, *Aspergillus flavus*, HPLC-FLD

1. Introduction
Indian senna or Tinnevely senna (*Cassia angustifolia*) is an important medicinal plant belongs to Caesalpiniaceae family. Senna is herbal laxative, wildly utilized in several medicinal systems in India and mainly cultivated in the Southern coastal districts of Tamil Nadu were fall on to the subtropical region. Senna has significant attention in international market due to the greater demand of their active ingredient called sennosides (Tripathy, 1991). Sennosides are the anthraquinone derivatives which is approved as a natural laxative by the world health organization mainly present in the pods and leaves of senna. The essential oils present in the leaves contains terpenes, phenols and aldehydes which give an aromatic odour (Laghari *et al.*, 2011; Majid *et al.*, 2013) [12, 14]. Senna leaves are the important ingredient several herbal drug preparations and used to treat vomiting, dysentery, fever and skin diseases (Aggarwal *et al.*, 2011) [1]. The annual production of senna leaves estimated 2500 tons of leaves annually and exported globally especially Germany, USA, China and France (Revathi *et al.*, 2013; Akerele and Heywood, 1991) [22, 2].
The occurrence of mycotoxin producing fungi in the herbal products reduces the quality of the herbal drug produce (Ałtyn and Twaruzek, 2020) [4]. Mycotoxin contamination in herbal drugs is considered as a serious public health concern throughout the world. The eco physiological conditions especially in subtropical regions were highly prone to toxigenic fungal risk. High temperature and humidity were the major factor that favours the growth of aflatoxigenic fungi mainly during storage (Paterson and Lima, 2010, Jinap *et al.* 2018) [20, 18].
To ensure the quality produce of senna, knowledge about the occurrence of mould contaminants in the raw materials is important. In addition, an idea about the occurrence of aflatoxigenic fungi in senna leaves and their toxigenic potentiality also pave a way to identify critical control points to ensure the aflatoxin free herbal products of senna. This current investigation aimed to explore the fungal contaminants associated with the senna leaves collected from different time period and the aflatoxin producing potentiality *Aspergillus flavus* isolated from senna leaves.

2. Materials and Methods

2.1 Chemicals and reagents

The sterile disposable plates (Himedia, Mumbai) were used for the culture maintenance and autoclaved for 15 mins @ 121 °C twice before disposal. The screw cap amber vials (Agilent technologies, USA) were used for the storage of aflatoxin standards solution. The sodium hypochlorite bleach (NaOCl) (1%) followed by acetone (5%) were used to swab the floor for accidental spill of toxin. All the glass wares including funnel, beaker and amber vials were rinsed by soaking in the 1% NaOCl for 2hrs followed by addition of 5% (total volume) acetone for 30 minutes.

2.2 Collection of samples

Totally 128 leaf samples were collected which include pre harvest (n=32), drying (n=24), processing (n=29), and storage (n=43) from the major senna growing areas of Tamil Nadu, India during 2018 to 2019. All the samples were collected in sterile polythene bags, labelled and brought to the laboratory and stored at 4°C until further analysis. Samples were made into fine powder using hand mill and used for the further analysis.

2.3 Identification of fungal contaminants

To identify the fungal contaminants associated with senna pods, dilution plating technique was used as described by Pitt and Hocking (1997) [21]. Briefly, one gram of powdered sample was transferred to the test tubes containing 10 ml of sterile distilled water and allow it under shaking for 30 mins. After that, this solution was serially diluted with sterile water and one ml of aliquot was plated on the Rose-Bengal Chloramphenicol Agar medium (King *et al.*, 1979) [11]. Then, the plates were allowed it for three days at 30 °C to encourage the fungal growth associated with the samples (Okoth *et al*. 2012) [19]. Fungal colonies were counted and expressed as colony forming units (CFU/g) and the isolation frequency was expressed as percentage (%) as given by Pacin *et al.* (2003)

$$\text{CFU/g} = \frac{\text{Number of colonies} * \text{Reciprocal of the dilution factor}}{\text{Volume of culture on plate}}$$

Further, fifteen representative *Aspergillus flavus* isolates were selected and the pure culture of this isolates were maintained on PDA medium for the further investigation.

2.4 Molecular identification

Genomic DNA of the *A. flavus* isolates were extracted by using CTAB method as per the technique given by Allen *et al*. (2006). The purity and the concentration of the DNA was determined by spectrophotometrically at 260/230 nm. Further, Polymerase Chain Reaction (PCR) was carried out using 18S rDNA universal primers ITS 1 (TCCGTAGCTGAACCTGCCG) and ITS 4 (TCCTCCGCTTATTGATATGC) to confirm the isolates (White *et al.*, 1990) [26]. The amplified PCR products were sequenced using ABI 3730 (48 capillary) electrophoresis instrument by adopting sanger dideoxy sequencing method at Bioserve, Telangana.

2.5. Assessment of toxigenic potentiality of *Aspergillus flavus*

2.5.1. Preparation of Inoculum

Spore suspension was prepared from the seven-day-old fungal cultures grown on potato dextrose agar medium at 28 °C. Ten mL of sterile distilled water (0.05% Tween-20) was added on plate and the mycelial surface was rubbed with sterile glass rod to collect the spores. Further, the concentration was adjusted to 10^7 conidia/mL with sterile distilled water by hemocytometer.

2.5.2 Aflatoxin extraction

To determine the aflatoxin producing ability of the *Aspergillus flavus* isolates, 10 µL of spore suspension was centrally inoculated on the plates containing PDA medium and incubated for 7 days at 28 C. After that, five agar plugs were removed along with mycelium using a cork borer (6 mm diameter) and transferred to a previously weighed microfuge tube. Aflatoxin extraction was carried out by addition of one mL of chloroform (HPLC grade) and shaking for 30 minutes at 150 rpm. Then, the supernatant was transferred to a fresh microfuge tube. Further this procedure was repeated thrice and the supernatant was pooled together and dried under vacuum concentrator at 30 °C. Then, the residue was redissolved in 50% methanol (methanol: water, v/v) and stored at 4 °C for HPLC analysis.

2.5.3 HPLC-FLD conditions

Aflatoxin was determined by reverse-phase (RP) HPLC using an Agilent 1200 HPLC system (Agilent Technologies, USA) equipped with an autosampler and fluorescence detector. A reversed-phase silica-packed C-18 column (150 mm x 4.6 mm, 5 µm particle size; Agilent Technologies, USA) was used for separation, and it was maintained at 40 °C. Postcolumn derivatization was performed through an electrochemical derivatizing unit KOBRA Cell (R-Biophram, Germany). The mobile phase was prepared with a mixture of water and methanol (60:40 v/v) containing 350 µL of 4 M nitric acid and 119 mg of potassium bromide with a 1 mL/min flow rate. The FLD excitation and emission wavelengths were 365/425 nm, and 50 µL of each sample was used as an injection volume. The aflatoxins recovery rate was calculated by manually spiking with three concentrations, *viz.*, 10, 25, and 50 ng/g of total aflatoxin in PDA medium. The mean recoveries (%) for total aflatoxin and aflatoxin B_1 in PDA medium were 80.26 and 83.77, respectively

2.6 Statistical analysis

All the represented data were the means of three replicates and each experiment was repeated thrice. Differences in means were evaluated with ANOVA using Tukey's test at 5% significance (Gomez and Gomez, 1984) [9]. The statistical analysis was performed in SPSS software version 20.0 for Windows (SPSS Inc.)

3. Result and Discussion

In this present investigation, mould fungal count associated with senna leaves were determined in RBC agar medium and expressed in CFU/g (Table 1). Result indicated that seven different fungal genera such as *Aspergillus*, *Penicillium*, *Rhizopus*, *Cladosporium*, *Mucor*, *Fusarium* and *Alternaria* were associated with senna leaves collected from different time periods including pre harvest, drying, processing and storage. The highest fungal population of 11.68 $\times 10^4$ CFU/g was recorded by *Aspergillus* spp with a range of 4.3×10^3-0.7×10^6 CFU/g followed by *Rhizopus* spp showed 4.65×10^4 CFU/g with a range of 3.0×10^3- 0.3×10^5 CFU/g. Many of the

previous researchers were demonstrated that the fungal propagules were the useful indicator to determine the quality of the produce. Our results were in agreement with the previous findings suggested that *Aspergillus* spp and *Penicillium* spp are the most predominant fungal genera associated with the spoilage of agricultural products (Silva *et al.*, 2004; Katsurayama and Taniwaki, 2017; Mauro *et al.*, 2013) [23, 15]. We also demonstrated that *Aspergillus* spp had higher isolation frequency in all the tested stages of senna leaves such as preharvest, drying, processing and storage (Fig 1). Similarly, Palacio and Pan (2020) [7] reported that the high prevalence of *Aspergillus flavus* in wheat and maize silage.

In this current study, we adopted 18S rDNA gene sequences analysis to confirm the representative *A. flavus* strains isolated from the senna leaves. The sequence similarity was compared with the sequences in the NCBI database by using BLAST. All the sequences were submitted in the NCBI database and the accession number were obtained (Table 3). 18srDNA sequence analysis has been widely reported as an optimum tool to discriminate the fungal genera in several mycobiological analysis (White *et al.*,1990) [26].

Aflatoxins are the fungal toxins produced by the members of *Aspergillus* section Flavi in crops at both pre- and post-harvest conditions ((Taye *et al.*, 2016; Kim *et al.*, 2014; Cotty *et al.*, 1994) [24, 13, 5]. Several factors influencing the aflatoxin producing ability of the *A. flavus* such as genotype, substrate, environmental factors and geographic origin (Cotty 1997; Natarajan *et al.*, 2022; Gallo *et al.*, 2016) [6, 17, 8]. In our investigation, aflatoxin producing ability of *A. flavus* was highly variable ($P< 0.05$) with strains with a range of 2.35-4824.23 µg/g. The high amount of aflatoxin was produced by the strain ASL1 (4824.23 µg/g) followed by ASL7 (3081.67 µg/g).

In conclusion, the occurrence of aflatoxigenic fungi and aflatoxin producing potential of *A. flavus* associated with the senna leaves may significantly influence the risk of aflatoxin contamination during herbal drug preparation. The knowledge about the prevalence of fungal contaminants will be useful to develop a good agricultural practice in senna cultivation.

4. Conflict of interest

The authors declare that they have no conflicts of interest.

5. Funding information

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Table 1: Assessment of molds genera from senna leaves collected from Tamil Nadu

Genus	Range (CFU/g)	Mean (CFU/g)
Aspergillus spp	4.3×10^3- 0.7×10^6	11.68×10^4
Penicillium spp	2.3×10^3- 3.3×10^5	3.84×10^4
Rhizopus spp	3.0×10^3- 0.3×10^5	4.65×10^4
Cladosporium spp	0.7×10^3- 0.7×10^4	3.1×10^3
Mucor spp	1.7×10^3- 0.3×10^4	2.7×10^3
fusarium spp	1.0×10^3- 9.3×10^3	3×10^3
Alternaria spp	0.3×10^3- 1.0×10^4	0.6×10^4

Table 2: *Aspergillus flavus* strains isolated from senna leaves

Isolate	Organism	NCBI GenBank accession number	Aflatoxin type
ASL1	*A. flavus*	OM438173	B_1 and B_2
ASL2	*A. flavus*	OM438172	B_1 and B_2
ASL3	*A. flavus*	OM438170	B1
ASL4	*A. flavus*	OM438174	B1
ASL5	*A. flavus*	OM438175	B1
ASL6	*A. flavus*	OM438176	B1
ASL7	*A. flavus*	OM438177	B_1 and B_2
ASL8	*A. flavus*	OM438178	B1
ASL9	*A. flavus*	OM438179	B1
ASL10	*A. flavus*	OM441939	B1
ASL11	*A. flavus*	OM441941	B1
ASL12	*A. flavus*	OM441945	B1
ASL13	*A. flavus*	OM441950	B1
ASL14	*A. flavus*	OM441954	B1
ASL15	*A. flavus*	OM441949	B_1 and B_2

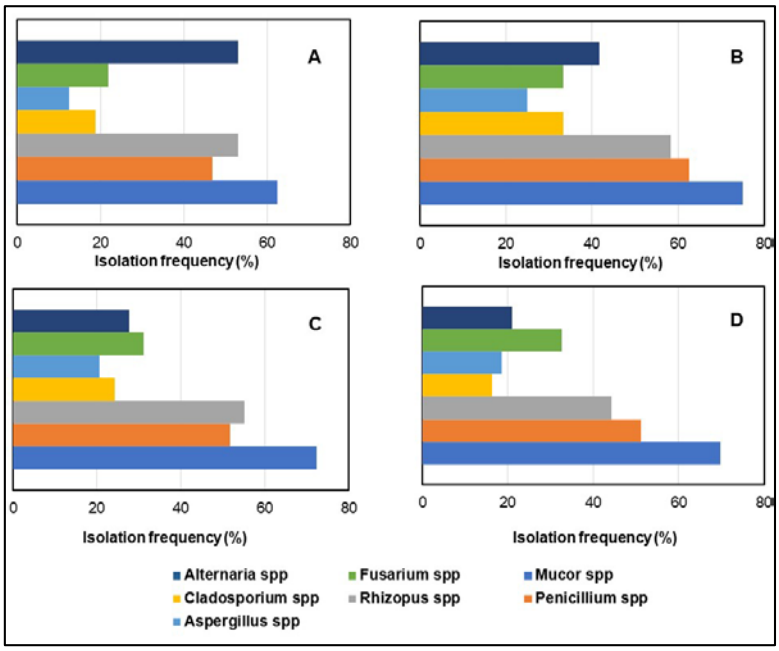

Fig 1: Isolation frequency of fungal contaminants associated in senna leaves collected from different time periods in Rose Bengal Chloramphenicol agar medium at 28 °C. A) Pre-harvest, B) Drying, C) Processing, D) Storage.

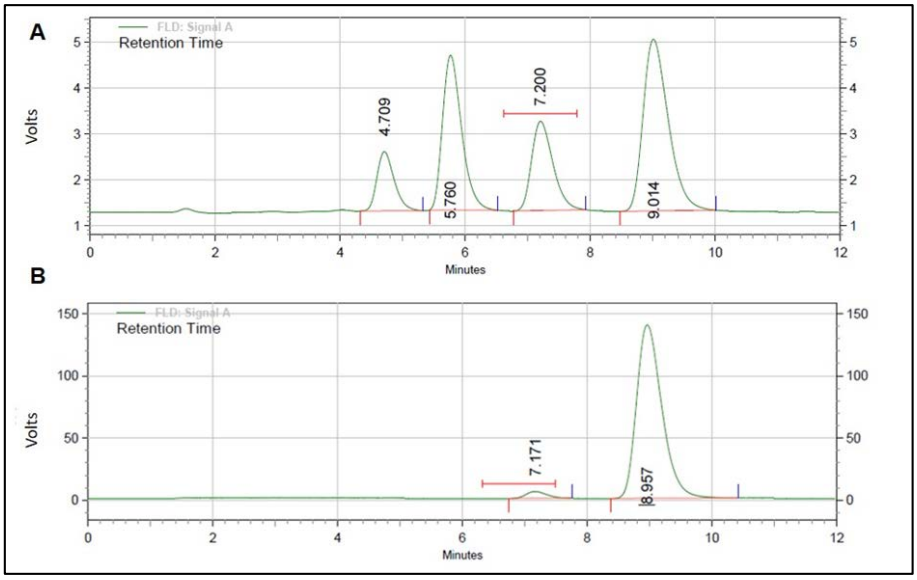

Fig 2: HPLC chromatogram of aflatoxin. 'A' represents aflatoxin reference standard (G_2-4.709 min, G_1-5.760, B_2-7.200, B_1-9.014 min). 'B' represents aflatoxin extracted from ASL1

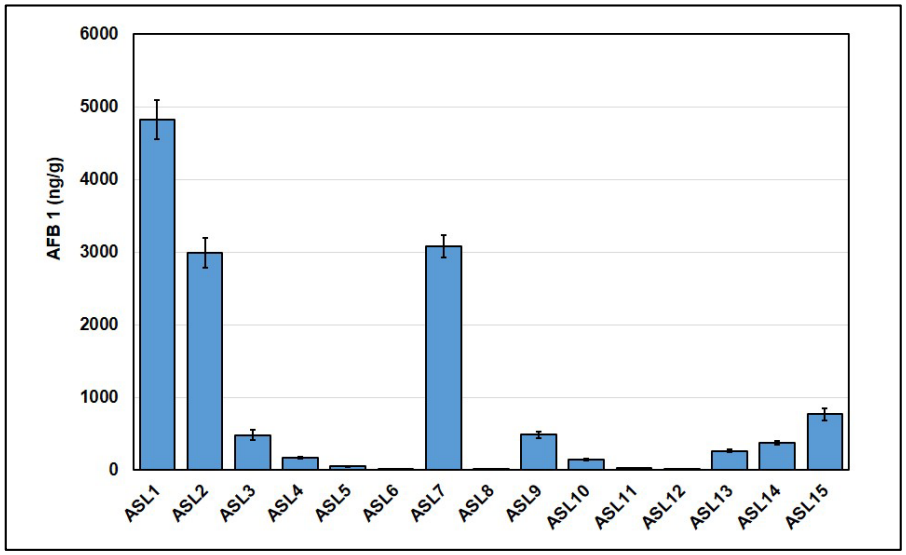

Fig 3: Aflatoxin producing potentiality of *Aspergillus flavus* in potato dextrose agar medium incubated at 28 °C for 7 days. Each value represents mean of three replicates. Error bar indicates standard deviation.

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