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Transport via xylem and accumulation of aflatoxin in seeds of groundnut plant

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HIGHLIGHTS

- Natural field soils from Karnataka, India were contaminated with aflatoxin, esp. AFB_1.
- *In vitro* experiments proved that the plant roots can uptake aflatoxin via xylem.
- AFB_1 uptake was affected by initial toxin concentration, pH of the medium.
- Uptake varied considerably in different groundnut varieties.
- Aflatoxin was gradually taken up by the roots and later accumulated in seeds.

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ABSTRACT

Aflatoxin contamination in groundnut seeds in the absence of any aflatoxigenic fungi leads to a hypothesis that aflatoxins are present naturally in soil and is transferred to seeds through uptake by roots. A survey was conducted on the natural occurrence of aflatoxins in agricultural soils, among nine main groundnut-growing regions of Karnataka state, India. All 71 soil samples collected in this survey were contaminated with aflatoxins esp. AFB_1. An *in vitro* xylem sap experiment proved the ability of groundnut plant roots to absorb AFB_1, and transport to aerial plant parts via the xylem. Hydroponics experiment also proved the uptake of AFB_1 by the roots and their translocation to shoot. Uptake was affected by the initial concentration of toxin and pH of the medium. Among the 14 varieties screened, GPBD4 and MLT.K.107 (III) recorded highest and least AFB_1 uptake, respectively. The above results were validated using a greenhouse experiment. Here, the aflatoxin absorbed by root gradually transferred to shoot that was later found in seeds towards the end of experiment. Thus, the groundnut seeds can also get contaminated with aflatoxin by direct uptake of aflatoxin through conducting tissue in addition to fungal infection. The present study revealed the novel mode of aflatoxin contamination in groundnut seeds without fungal infection.

1. Introduction

Mycotoxins, mainly aflatoxins play a leading role in contamination of agriculturally important crops (Hedayati et al., 2007). It is reported that aflatoxigenic fungi are common soil inhabitants, occurs all over the world (Bennett and Klich, 2003; Razzaghi-Abyaneh et al., 2005; Hedayati et al., 2007; Sepahvand et al., 2011). Among 22 closely related species in the genus *Aspergillus*, *Aspergillus flavus* is the most common fungi responsible for the majority of aflatoxin contamination (Varga et al., 2011). These strains, which are abundant in agricultural/farmland top soil act as a primary inoculum source for colonization on crops (Starr and Selim, 2008).

Aflatoxins, especially aflatoxin B_1 (AFB_1), is the most potent toxic metabolite that shows hepatotoxic, mutagenic and teratogenic properties that results in hepatic carcinoma, immunosuppression and toxic hepatitis in human and animals (Speijers and Speijers, 2004). AFB_1 is classified under Class 1 human carcinogen by the International Agency for Research on Cancer (IARC, 2002). Moreover, economic losses resulting from poor crop quality, reduction in animal productivity and its impact on public health and trade are the foremost concern due to aflatoxin contamination of agricultural crops. In a global context, aflatoxin

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contamination is an everlasting concern among the developing countries between the 35°N and 35°S latitude where people often rely on susceptible crops for their daily nutrition and income (Shams-Ghahfarokhi et al., 2013). Dietary exposure to aflatoxin and human aflatoxicosis is now became a threat in developing countries that previously were not concerned about this issue (Williams et al., 2004).

Among the aflatoxigenic fungi, *Aspergillus flavus* is the most common and abundant species associated with groundnut (*Arachis hypogaea* L.) (Joffe, 1969). Since the fungus and the metabolites gain access to the plant under field conditions, the potential impact on seed/grain is of interest. Several prevention techniques, including physical, chemical and biocontrol, did not solve this problem completely, and aflatoxin issues continued to be a threat to producers and consumers worldwide, till date.

Mycotoxins are absorbed by plants from contaminated water or soils and is distributed to aerial plant parts via conducting tissues. Uptake of aflatoxin by maize (Mertz et al., 1980, 1981), citrinin, patulin and terreic acid by rice seedlings (Rao et al., 1982), ochratoxin A by coffee plants (Mantle, 2000), and fumonisin by wheat plants (Zitomer et al., 2010) were the major studies where mycotoxin uptake was successfully proved. Moreover, earlier work conducted in our laboratory revealed the aflatoxins uptake ability of green leafy vegetables (Hariprasad et al., 2013).

In spite of numerous previous reports, efforts to investigate the chances of aflatoxin absorption by groundnut plants through roots and accumulation in aerial plant parts, including seeds were not undertaken. So this study hypothesized that groundnut seedlings can uptake aflatoxin from the soil in which they grow and translocate to aerial plant parts including seeds. The objectives include (i) a screening of agricultural soil samples from main groundnut growing districts of Karnataka state, India for aflatoxin contamination, (ii) evaluation of aflatoxin uptake ability of groundnut seedlings under laboratory conditions and (iii) validating the uptake of aflatoxin and accumulation in seeds using greenhouse experiment.

2. Materials and methods

2.1. Seed samples

Certified groundnut varieties were procured from the National Seed Corporation, Karnataka and Department of Biotechnology, University of Mysore, Manasagangotri, Mysore, India. Seeds were surface sterilized with 0.1% mercuric chloride for 5 min followed by thorough washing with distilled water and used throughout the experiment. Groundnut variety TMV-2 was used for all laboratory and greenhouse experiments. Hybrid varieties (MLT.K.101 (RI), MLT.K.102 (RII), MLT.K.103 (RII), MLT.K.104 (RI), MLT.K.105 (RI), MLT.K.106 (RII), MLT.K.107 (RIII), MLT.K.103 (RIII), MLT.K.101 (RI)) and local varieties (ICGV-91114, GPBD4, K-6, Local variety-65 and TMV-2) were used for to analyze the differential uptake ability of aflatoxin. Furthermore, these seeds were tested to confirm the absence of aflatoxigenic fungi using blotter paper test (Bewley et al., 2006). Presence of aflatoxins was tested based on standard method mentioned below.

2.2. Collection of soil samples

Soil samples were collected from nine groundnut-growing regions of Karnataka state, India during January–May, 2011. Plots were of varying size and mostly rain fed. Samples were collected as described by Jones (2001). Five samples from each field were analyzed separately.

2.3. Chemicals

All chemicals were of analytical grade purchased from M/S Sisco Research laboratories (Mumbai, India). Solvents used for HPLC and HPTLC were of HPLC grade. TLC silica gel plates (20 × 20) were obtained from Merck, Germany. Aflatoxin standards were procured from Sigma, USA.

2.4. Aflatoxin uptake studies

Desired amount of aflatoxin standards (AFB_1) from stock solution prepared in benzene/acetonitrile (98:2) (10 μg mL^{-1}) were dried using nitrogen gas and re-dissolved in 0.5% aqueous acetone and used in all uptake studies.

2.4.1. Xylem sap experiment

Surface sterilized groundnut seeds of variety TMV-2 were soaked in aerated distilled water for 8–12 h. Seedlings were raised in a growth chamber, by sowing soaked seeds in sterile coir pith, and maintained on 16/8 h light/dark at 28 ± 2 °C and 60% relative humidity. Seven day-old-seedlings were treated with 10 mL distilled water spiked with 500 ng of AFB_1 dissolved in 500 μL of 0.5% aqueous acetone, twice at intervals of 12 h. Xylem sap was collected as described by Su et al. (2010). Three h after the final treatment, seedlings were cut just above a cotyledonary leaf with a sterile blade. Decapitated seedlings were kept in a moist chamber for 10 min and xylem exudates were collected from the cut end of both aflatoxin-treated and control seedlings, which later analyzed for the presence of aflatoxin through HPLC described below.

2.4.2. Hydroponics' experiments

A modified method of Mertz et al. (1980) was followed here. Fifteen-day-old seedlings were transferred to glass bottles (7 × 10 cm) containing 50 mL Hoagland's solution (Hoagland et al., 1950), pH 6.6 amended with known concentration of aflatoxin dissolved in 500 μL 0.5% aqueous acetone. Control plants were grown in Hoagland's solution, without aflatoxin. To induce proper mixing and supply of oxygen to the roots, the solution was aerated continuously by an air pump. The experimental setup was maintained in the controlled-environment chamber with 26/22 °C day/night temperature cycles, 13/11 h light/dark cycles and 60% relative humidity. The light intensity was 200 W/m^2 at the plant level. All experiments were performed thrice using five glass bottles each with twelve seedlings. At the end of respective growth period, seedlings were removed without damaging the root system, rinsed with 0.5% aqueous acetone thrice to remove surface adhered aflatoxin. Further, control and treated seedlings were dried at 40 °C until a constant weight was achieved. Aflatoxin was extracted separately from the root and shoot according to procedure mentioned below.

To analyze the effect of initial aflatoxin concentration on uptake ability of groundnut. Initially; seedlings were incubated in 50 mL Hoagland's solution amended with varying concentrations of AFB_1 (150, 300 and 450 ng mL^{-1}) for three and seven days. After the growth period, seedlings were separated into root and shoot to extract and quantify aflatoxin taken up by plant. To check the effect of varying pH in uptake, seedlings were incubated for three days in 10 mL Hoagland's solution of three different pH (5.6, 6.6, 7.6), each amended with 50 ng mL^{-1} AFB_1. Hoagland's solution was prepared using potassium phosphate and sodium acetate buffer to maintain pH during the growth period. The pH was adjusted using 1 M NaOH throughout experimental period. After the growth period, seedlings were separated into root and shoot to extract aflatoxin taken up by plant.

Hydroponic experiment continued for 14 different cultivars of groundnut to compare the differential uptake ability of AFB_1.