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RESEARCH NOTE

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# PCR-based Identification of Aflatoxigenic Fungi Associated with Iranian Saffron

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**Abstract** Aflatoxins are secondary metabolites produced by the aflatoxigenic fungi in suitable conditions. Saffron, *Crocus sativus*, is the most expensive spice in the world. Saffron is normally contaminated with soil and hand microflora during harvest and post-harvest operations. In this study, rapid assessment of aflatoxigenic fungi in saffron was accomplished using polymerase chain reaction. In total, 37 market samples were assayed in order to isolate aflatoxin-producing fungi. The 18.9% of the total samples were contaminated with aflatoxigenic fungi. Our results also show that most of the isolated fungi were saprophytes which are normally originated from soil during harvest and postharvest process.

**Keywords:** saffron, *Crocus sativus*, spice, aflatoxigenic fungi, polymerase chain reaction

## Introduction

Aflatoxins are secondary metabolites primarily produced by two fungal species, *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin contamination of agricultural commodities has gained global significance as a result of their deleterious effects on human and animal health as well as their importance to international trade. One of the most important requirements for eliminating aflatoxin is identification of mycotoxigenic fungal contamination. Detection of the aflatoxigenic fungi is usually performed by traditional dilution plating, use of diagnostic media or by immunological methods. The traditional methods are time consuming, labor-intensive, costly, and require mycological expertise and facilities. Hence, it is imperative to develop methodologies that are relatively rapid, highly specific, and as an alternative to the existing methods. In this study, rapid assessment of aflatoxigenic fungi in saffron was accomplished using polymerase chain reaction (PCR). The main advantages of PCR over other methods are no need for organism to be cultured, at least not for long time prior to their detection, target DNA can be detected even in a complex mixture, sensitive and highly versatile. The biosynthetic pathway for aflatoxin production by *A. flavus* has been deciphered and genes in the aflatoxin biosynthetic pathway have been identified. The gene *aflR* has been isolated and shown to regulate aflatoxin biosynthesis. In our studies, the PCR reaction was targeted against aflatoxin synthesis regulatory gene, *aflR1*. Since these genes are nearly identical in *A. flavus* and *A. parasiticus*, indicating the possibility of detection of both species with the same PCR system (primers/reaction) (1). Saffron, *Crocus sativus*, is the most expensive spice in the world. Moreover, the flowers have to be individually hand-picked in autumn (upon their opening). It has been reported that cardamon, cayenne pepper, chili, cloves, cumin, curry powder, ginger, mustard, nutmeg, paprika, saffron, and white pepper samples selected from supermarkets and ethnic shops in Lisbon are naturally contaminated with aflatoxins (2). A sampling of commercial spices in Thailand revealed that 18% of samples of herbs and spices were contaminated with aflatoxin $B_1$ ranging between 40-160 ppb (3). In a separate assay on chili all samples contained aflatoxin $B_1$ and high levels were obtained from all ground samples (4). Therefore, the resolutions passed at the 22nd International Organization for Standardization/Technical Committee 37/Sub Committee7 (ISO/TC34/SC7) meeting asked for standardized method for determination of aflatoxin in spices (5). Saffron may be contaminated with soil and hand microflora during the harvest and post-harvest operations and, therefore, it could be one of the contaminated sorts of spices. The aim of this research was to identify aflatoxigenic fungi associated with Iranian saffron using a PCR based method.

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## Materials and Methods

All 37 saffron samples, used here, were supplied from 5 main saffron producers in Iran.

**Isolation and identification of fungi from saffron** Saffron samples (10 g) were homogenized in saline buffer using mortar, serially diluted in peptone water and plated onto yeast extract chloramphenicol agar (Merck, Danstadt, Germany) (6). Strains were isolated by single spore method (7) and subcultured on potato dextrose agar (PDA), allowed to grow at 25°C for 7 days and then stored at 4°C for the final identification to species level (6).

**Isolation of fungal DNA** Template DNA was extracted from the fungal mycelia, harvested from freshly growing cultures in potato dextrose broth (PDB). The mycelium was then transferred to a mortar, containing liquid nitrogen and ground to a fine powder. Lysis buffer [1 M Tris pH 7.5, 0.05 M ethylenediamine tetraacetic acid (EDTA), 1%(w/v) sodium dodecyl sulphate (SDS), 0.9 M sodium choloride (NaCl), 0.1 M sodium sulphite ($Na_2SO_3$)] was added and heat-shock treated at 65°C for 20 min. The suspension was centrifuged (Fisons, Loughborough, England) for 5 min at 2,000×g and the supernatant was transferred to a new microfuge tube. The supernatant was then extracted with chloroform:isoamylalcohol (24:1). The solution was placed on ice for 30 min and then centrifuged for 15 min at 2,000×g (Fisons). After centrifugation, the supernatant was taken to a new microfuge tube. The DNA was precipitated with equal volumes of isopropanol and centrifuged for 5 min at 2,000×g (Fisons). After centrifugation, the supernatant was removed and the precipitate was resuspended in 100 μL distilled water.

**Isolation of fungal DNA directly from saffron samples** *Aspergillus flavus* subspecies (donated by 'Plant Pests and Disease Research Institute of Iran' as the positive control) was inoculated on PDA and cultivated for 7 days at 25°C until they become sporulated. The spores were harvested in a 0.1% Tween 80 solution to make suspension containing 100 spores/mL. A 1,000 μL of the spore suspension was spiked into 1 g of saffron and incubated for 48 hr at 25°C. Uninoculated saffron sample was considered as the control.

**PCR** The PCR was used to amplify a part of the aflatoxin regulatory gene, *aflR1*, from aflatoxigenic fungal genomic DNA. The sequence of the forward and reverse primers are (5AACCGCATCCACAATCTCAT-3) and (5-AGTGCAG TTCGCTCAGAACA-3), respectively. These primers were designed based on the published sequence strand for *A. flavus* and *A. parasiticus* (1). The expected fragment from this amplification was close to the expected size, ca. 798 bp. The PCR was performed, using a commercial kit (Cinagen, Tehran, Iran), in 20 μL reaction mixtures containing 5 μL of genomic DNA. The program included 30 cycles of PCR, 0.3 min at 94°C, 0.45 min at 50°C for annealing, 1.15 min at 72°C for extension, and a 10 min final extension at 72°C was run on a programmable DNA thermal cycle (Bioer, Hangzhou, China). Negative and positive controls were included while performing PCR.

**Nested PCR** Nested PCR was carried out using the primer set *aflR2*. The sequences of the primers were (5-GCACCCTGTCTTCCCTAACA-3) and (5-ACGACCAT GCTCAGCAAGTA-3) with a product size of 400 bp, and were nested to the primer set of *aflR1*. PCR was performed under the above-mentioned conditions (1).

## Results and Discussion

Fungal contamination in saffron reaches to its highest level right after harvesting of saffron and declines from January till December (2001-2004) (Fig. 1A). In other word, the overall fungal contamination decreases during the storage time. Saffron samples used in this research were grouped

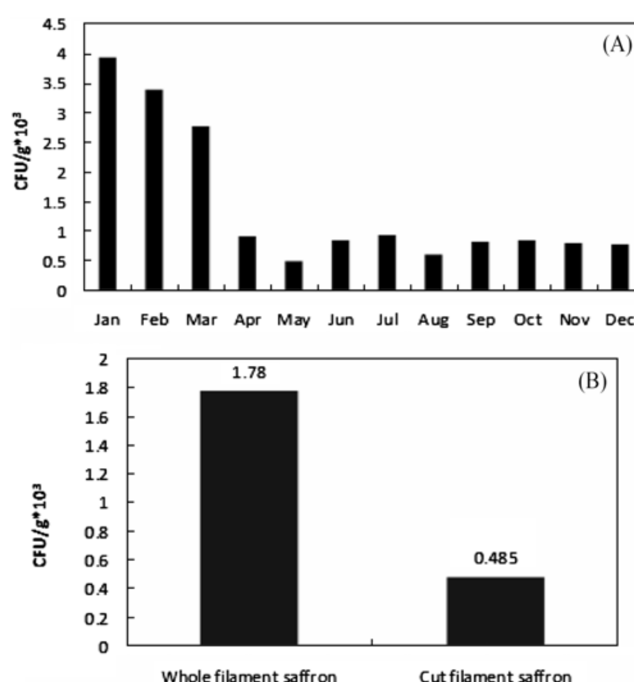


**Fig. 1. Total fungal contamination of saffron samples (A) and fungal contamination of whole and cut filaments of saffron samples (B).** Each column represents the mean value of contamination of 4 years (2001-2004).

in forms of whole filaments, contain stigma and style, and cut filaments, which were free of style. The whole filaments had more fungal contamination compared with the cut filaments (Fig. 1B) that could be related to the higher moisture content of the style which is higher in whole filaments. On the other hand, whole filaments always came as tied bunches of the style part which could be a source of contamination. Saffron is the most expensive spice in the word. The conventional plating techniques for fungi identification in saffron, as a part of hygiene standard for this spice, needs at least 10 g of saffron powder to predict the total fungi and yeast counts in the sample (6). In the present research we developed a PCR-based method for identification of aflatoxigenic fungi associated with saffron using 1 g of sample. The proposed method is less time consuming and more cost effective as well as more reliable and precise in detecting aflatoxigenic fungi in saffron. The PCR analysis of the positive control produced a sharp bound of the expected size (798 bp). The optimized protocol was applied for DNA extraction from 28 different fungal strains isolated from saffron. These included 9 strains of *A. flavus*, 6 strains of other *Aspergillus* spp, 5 strains of *Alternaria* spp, 4 strains of *Penicillium* spp, 3 strains of *Fusarium* spp, and *Rhizopus stolonifer.* The DNA extracts were subjected to PCR analysis to confirm the possible presence of the aflatoxigenic gene. As expected DNA from all aflatoxigenic Aspergilli, produced clear bands upon amplification with *aflR1* set of specific primers. Meanwhile no band was detected on non aflatoxigenic fungi (Fig. 2A). To confirm the specificity of the reaction, all PCR products were subjected to the nested PCR using another set of primers, *aflaR2*. All aflatoxigenic Aspergilli showed positive results (Fig. 2B), with the

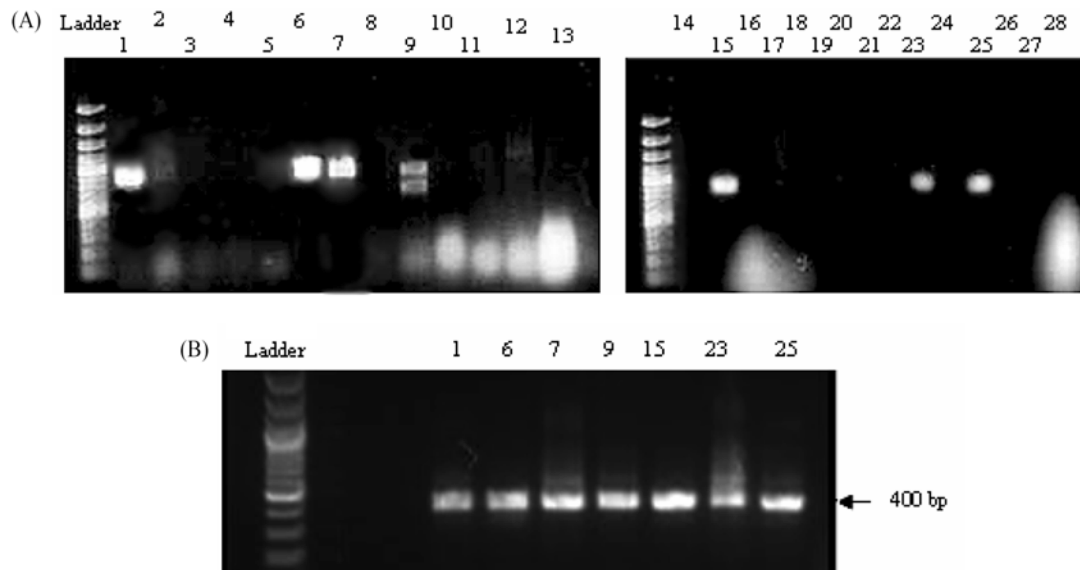


**Fig. 2. PCR products from fungi isolated from saffron (A) and nested PCR products (B).** The numbers show different strains and DNA 100 bp ladder size (Ladder). The bands represent amplified fragments from Aflatoxigenic fungal DNA which were originally isolated from saffron and Ladder.

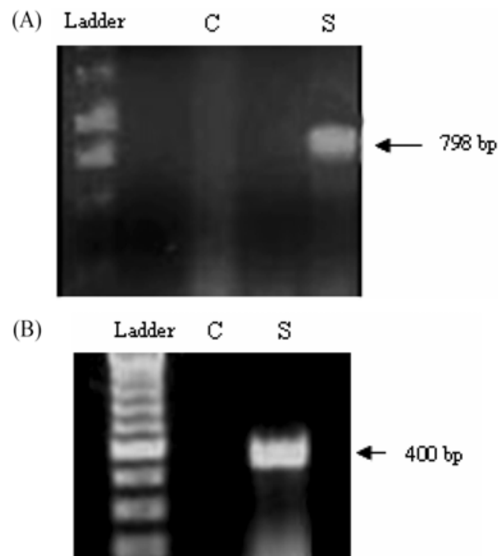


**Fig. 3. (A) PCR products of spiked saffron sample with $10^2$spores/g *A. flavus* and (B) nested PCR products of *aflR* gene from spiked saffron ($10^2$ spores/g) incubation for 48 hr (S), saffron without inoculation with spores, as a control (C), and DNA 100 bp ladder size (Ladder).**

expected size, ca. 400 bp, of the fragment. To develop a direct method for detection of the aflatoxigenic genes in plant material, the DNA was extracted from saffron samples and subjected to the PCR. This would evaluate sensitivity of PCR on the presence of the spores in the samples. The primer pair used for the amplification of *aflR* gene was tested on samples from the enriched cultures after 48 hr of incubation at $10^2$ spores of *A. flavus*/g and positive results were obtained (Fig. 3A and 3B). There was no interference from the DNA of either saffron samples or fungi contaminants. Most of the isolated fungi were saprophytes which are normally originated from soil during harvest and the subsequent stages of drying, transportation, sorting, and storage. The most commonly isolated fungal genera were *Rhizopus stolonifer*, *Aspergillus* spp, *Alternaria* spp, and *Penecilium* spp. In both group of samples, intact and cut filaments, *R. stolonifer*, *Aspergillus* spp, *Alternaria* spp, and *Penicillium* spp seems to be dominant with a presentation of about 40, 36, 30, and 22%, respectively.

The 18.9% of the saffron samples were represented as aflatoxigenic fungus. It is reported that genes involved in the aflatoxin biosynthetic pathway may form the basis for an accurate, sensitive, and specific detection system, using PCR, for aflatoxigenic strains in grains and foods (9). In this study, using primer designed to aflatoxin regulatory pathway gene, *aflR*, the presence of aflatoxigenic fungi was easily detected. This approach presents a rapid method of detecting the aflatoxigenic fungi in saffron, compared to conventional plating techniques. PCR in the present study did not show any false priming results due to the presence of food components or any other contamination. In conclusion, careful hazard analysis and critical control point (HACCP) techniques during harvest and the subsequent stages of drying, transportation, elaboration, and storage are indispensable to prevent the risk of saffron contamination. This technique is able to screen many suspected samples in a time and resource saving manner in fine and expensive products of saffron with the highest possible accuracy.

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