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 hard 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, aflR. 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 hard 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.

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).
PCR-based Identification of Aflatoxigenic Fungi in Saffron

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, 0.9 M sodium chloride (NaCl), 0.1 M sodium sulphite (Na₂SO₃)] was added and heat-shock treated at 65°C for 20 min. The suspension was centrifuged (Fison, 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:isooamylalcohol (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. Disease Research Institute of Iran as the positive controls were included while performing PCR. 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 a sharp band of the expected size (798 bp). The PCR analysis of the positive control produced a sharp band 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, aflR2. All aflatoxigenic Aspergilli showed positive results (Fig. 2B), with the

Results and Discussion

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).
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/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
genra were Rhizopus stolonifer, Aspergillus spp, Alternaria spp, and Penicillium 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
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.

Acknowledgments
This work was supported by a grant from Khorasan Razavi Industries and Mines Organization. The authors appreciate
the important contributions of Novin Saffron and Edman Co. also F. Ghollasee and A. Soleymani.
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