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# QUALITATIVE DETECTION OF FUNGAL CONTAMINATION IN PAPRIKA POWDER

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## ABSTRACT

Dried red pepper is one of the most commonly used spices in many parts of the world. In this study molecular biology methods were applied for detection of contamination in nine samples of paprika powder. Internal Transcribed Spacer (ITS) regions were selected for sequencing as they have a high variability between species and organisms and therefore are an appropriate tool for taxonomic identification. The sequence analysis of the ITS regions were identified by high sequence similarity with the ITS regions of many microscopic fungi, especially representatives of the class *Ascomycota* and several other yeast species. This work was a qualitative rather than a quantitative detection, so the extent to which fungi and yeasts were present in the samples is unknown. However, supplied quality certificates (microbiological data) indicated the overall quality of samples. Harmful microorganisms were identified in the paprika powder samples and the likely mode of contamination was identified.

## PRACTICAL APPLICATION

The presence of undesirable pathogens can be a serious problem for consumers, especially contamination by microscopic fungi that have the potential to produce mycotoxins. Today's conventional methods for determining contamination by microorganisms are time consuming and can be difficult to identify some less common types of pathogens. Fungal DNA (ITS regions) sequences of the class *Ascomycetes* were identified from samples of sweet and hot paprika powder.

## INTRODUCTION

Sweet or spicy fruit from plants of the genus *Capsicum* L. (Solanaceae) is one of the most used spice in the world (Surh 2002). Paprika powder is used to improve the sensory properties of foods, as well as for medicinal purposes (Nieman *et al.* 2012). Paprika retains a high content of compounds with antioxidant activity (e.g., vitamin A, C and carotenoids) after drying and grinding (Daood *et al.* 2014). Because of these this, dried paprika powder has gained a great interest and demand throughout the world. However, it is also perceived as a serious problem for human health due to microbial contamination. The contamination may occur during the growing season such as through improper agrotechnical treatments, improper storage, and inadequate sanitary conditions from the vendor handling the spices. Inadequate quality can impact consumer health and continuing education regarding the safe growing, harvesting, and storage of paprika is important to ensure the quality of the spice is maintained (Keit 2009; Tulu *et al.* 2014).

Common contaminants of dried spices are microbial pathogens that may arise from sources such as the indigenous microflora of the plant, microorganisms present in the processing plant, air, post-harvest contamination from dust, use of contaminated water and from human contact. During cleaning and processing, there is progressive reduction in the number and types of microorganisms; those remaining are usually aerobic spore-forming bacteria and common moulds (Ahene *et al.* 2011).

**TABLE 1.** LIST OF HUNGARIAN AND SPANISH PAPRIKA POWDER SAMPLES ON THE CZECH MARKET USED FOR THE ANALYSIS OF CONTAMINANTS

| No. | Name | Colorimetric index (ASTA) | Moisture content (%) | Source |
|---|---|---|---|---|
| 1. | Sweet paprika powder | 140 | 9.7 | HU |
| 2. | Hot paprika powder | 86 | 9.1 | HU |
| 3. | Hot paprika powder | 65 | 8.8 | HU |
| 4. | Sweet paprika powder | 66 | 8.7 | HU |
| 5. | Hot paprika powder | 48 | 7.4 | HU |
| 6. | Hot paprika powder | 103 | 9.6 | HU |
| 7. | Sweet paprika powder | 102 | 9.2 | HU |
| 8. | Sweet paprika powder | 109 | <12.0 | ES |
| 9. | sweet paprika powder | 83 | <12.0 | ES |

ASTA, American Spice Trade Association; HU, Rubin Paprika Processing, Ltd. (Szeged-Szőreg, Hungary); ES, EVESA Extractos Vegetales, Inc. (Armilla, Spain).

Some fungi are able to produce mycotoxins which can cause illness to animals and humans (Hammami *et al.* 2014). Plants grown for the production of spices, including paprika powder, are often grown in areas characterized by high temperatures and humidity. These conditions also promote the growth of various microorganism that are present (Garbowska *et al.* 2015). In the years between 1973 and 2010, the United States of America, Canada, United Kingdom, France, Germany, Denmark and Norway reported a combined total of almost 2,000 cases of food poisoning, some of which resulted in hospitalization and even death (Van Doren *et al.* 2013). Many studies have reported high levels of microbial contamination from spices. This suggests the need of improvement in quality control measures to prevent possible illnesses due to contamination (McKee 1995).

Various methods, such as Howard's number, specific dyes, fluorescent-based methods, cultivation techniques, chemical, immunochemical and physicochemical methods, have been used for detection of microorganisms in foods. To identify different types of filamentous fungi, is necessary to grow an axenic culture that achieve typical growth and sporulation (Samson *et al.* 2002). Reliable identification using morphological characters alone is difficult due to morphological variations within individual families. This often requires considerable expertise and is the reason DNA based methods have been increasingly used for identification. Molecular biological methods offer many advantages over conventional cultivation methods, as they are faster and more reliable and are highly specific and sensitive. In addition, there is also the automation potential (Bleve *et al.* 2003; Magan and Olsen 2004). Rapid and accurate determination of dangerous microorganisms in food products is crucial to ensure safe products reach consumers (Luque *et al.* 2012a,b). Modern methods for detection of microorganisms in foods are based on molecular biology techniques (e.g., PCR (Polymerase Chain Reaction), RFLP (Restriction Fragment Length Polymorphism), and DNA microarray) which gives qualitative and quantitative information and allows detection of multiple pathogens simultaneously (Mandal *et al.* 2011). In recent years, there has been work done to establish a "DNA barcode" to identify individual species by universal DNA regions (Hebert *et al.* 2003). One of the most important DNA region to identify fungal species is the ITS (Internal Transcribed Spacer) of rDNA (Schocha *et al.* 2012a). Gardes and Bruns (1993) proposed specific selective primers for the ITS region which can be used for amplification of only fungal DNA from comprehensive DNA mixtures. These primers allow for the identification of fungi present in a sample.

The aim of the study was to assess the possibility of sequencing the ITS (Internal Transcribed Spacer) region in fungi for the qualitative detection of contamination in paprika powder samples.

## MATERIAL AND METHODS

There were a total of nine samples of hot and sweet paprika powder delivered by a private company (TRUMF International, Prerov, Czech Republic) that were used for this study (Table 1). DNA was isolated using a DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). The DNA concentration and purity was assessed using a Picopet 1.0 (Picodrop, Cambridge, United Kingdom) with the ITS region used for DNA analysis. The PCR reaction mixture had a total volume of 25 μL and contained 0.5 U *Taq* polymerase (Promega, Madison, WI), 1× aliquot buffer, 0.1 mmol/L of each deoxynucleotide (Promega), 0.3 mol/L of each primer (ITS1-F 5′-CTTGGTCATTTAGAGGAAGTAA-3′ (Gardes and Bruns 1993) and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ (White *et al.* 1990)) and 20 ng of template DNA. The reaction conditions for PCR using a T3 thermocycler (Biometra, Götingen, Germany) were as follows: 3 min incubation at 94°C, then 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and the cycle repeated 40 times. Finally, the reaction was held at 72°C for 10 min, before a final 10°C hold.

**TABLE 2.** MICROSCOPIC FUNGI WITH THE HIGHEST SEQUENCE SIMILARITY TO THE SEQUENCES OBTAINED FROM SAMPLES OF PAPRIKA POWDER

| No. | Species of the genus and species | GenBank accession number |
|---|---|---|
| 1 | Species of the genus *Alternaria* and *Eurotium*, *Cladosporium cladosporioides*, *Pichia kudriavzevii** | KT722341, KT722342, KT722343, KT722344, KT722345, KT722346, KT722347 |
| 2 | *Aspergillus fumigatus, Cladosporium cladosporioides, Xeromyces bisporus* | KT722348, KT722349, KT722350, KT722351, KT722352, KT722353 |
| 3 | Species of the genus *Eurotium*, *Cladosporium cladosporioides, Verticillium dahlie* | KT722354, KT722355, KT722356, KT722357 |
| 4 | *Aspergillus terreus*, *Cladosporium cladosporioides, Epicoccum nigrum, Plectosphaerella cucumerina* | KT722358, KT722359, KT722360, KT722361, KT722362 |
| 5 | Species of the genus *Eurotium* and *Fusarium* | KT722363, KT722364, KT722365, KT722366, KT722367, KT722368 |
| 6 | *Cryptococcus psychrotolerans**, *Pichia kudriavzevii** | KT722369, KT722370, KT722371 |
| 7 | *Cladosporium cladosporioides, Colletotrichum coccodes, Gibellulopsis nigrescens* | KT722372, KT722373, KT722374, KT722375 |
| 8 | *Alternaria alternata, Cladosporium cladosporioides, Epiccocum nigrum* | KT722376, KT722377, KT722378, KT722379 |
| 9 | *Alternaria* spp. | KT722380, KT722381 |

*Yeast.

The PCR products were run on a 1.5% agarose gel in 1× standard Tris Acetate-EDTA buffer and then cloned using a pGEM-T Vector System (Promega 2010). This was done either directly after purification of PCR product using an Invisorb Fragment CleanUp kit (Stratec Molecular, Berlin, Germany) or, to limit the presence of nonspecific products, by excising from the gel and subsequently purified (Invisorb Fragment CleanUp kit, Stratec Molecular). Ligated pGEM-T plasmids were transferred into competent *E. coli* cells via electroporation using an Easyjet (EquiBio, Ashford, United Kingdom) at 12.5 kV/cm, 25 μF and 400 Ω. *E. coli* cells with the recombinant plasmids were plated on Petri dishes containing solid lysogeny broth (LB) medium with carbenicillin (100 mg/L) as a selection agents and the components for blue/white selection (20 mg/L 5-bromo-4-chloro-3-indolyl *beta*-D-galactopyranoside/X-gal/and 100 μM isopropyl *beta*-D-1-thiogalactopyranoside/IPTG/). After blue-white screening, clones with potentially recombinant plasmids (white in color, transformation efficiency $2.10^8$ CFU/μg DNA) were transferred into solution. A total of 1 μL of this solution was used for PCR using primers T7 5′-TAATACGACTCACTATAGGG-3′ and SP6 5′-ATTTAGGTGACACTATAG-3′ to confirm of presence of insert (Promega 2010). Products of 160 bp length were identified as plasmids without insert with longer products (160 bp + length of original PCR product) identified as recombinant products. The PCR products containing recombinant plasmids were treated with Exonuclease I and Alkaline Phosphatase to remove the remaining primers and deoxynucleotides and were sequenced (Macrogen, Seoul, Republic of Korea). The DNA sequences were analysed and the similarity of sequences were evaluated using the National Center for Biotechnology Information (NCBI) and BLAST (Basic Local Alignment Search Tool) algorithms (blast.ncbi.nlm.nih.gov/Blast.cgi).

## RESULTS AND DISCUSSION

Nine samples of sweet and hot paprika powder were analysed by PCR using primers for amplification of fungi-specific spacers, ITS1 and ITS2. ITS regions enable successful interspecific and intraspecific identification of a wide range of eukaryotic organisms and in recent years, are also used for fungal DNA barcoding (Schocha *et al.* 2012b). Of the nine samples obtained, a total of 41 sequences were obtained and analysed using the NCBI database and the BLAST algorithm. These sequences were recorded in the NCBI database and assigned a GenBank accession number (KT722341 - KT722381). Each sequence matched a known fungal genus and/or species with a 99 to 100% identity (Table 2). Two samples (1 and 6), had a consensus sequence representative of yeast, which are systematically classified into the taxonomic group of *Ascomycota* (Weiss *et al.* 2013).

One of the biggest problems of food contamination by fungi is the potential of some species to produce mycotoxins that can have significant adverse effects on the human body in both acute and chronic form (Zain 2011). Some agents are also potent allergens and cause deadly lung disease in human (Gibbons *et al.* 2012; Dang and Lawrence 2014). This study identified several fungi, *Alternaria* spp., *Aspergillus fumigatus*, *A. terreus*, *Eurotium* spp. and *Fusarium* spp., which are able to produce mycotoxins and therefore can be detrimental to human health. The remaining identified fungi

**TABLE 3.** THE LIST OF IDENTIFIED CONTAMINANTS IN PAPRIKA POWDER AND ANTICIPATED CONTAMINATION SOURCE

| Period of contamination | | | |
|---|---|---|---|
| During vegetation | During storage | During processing | Unable to determine |
| *Alternatia* spp. | *Alternaria* spp. | *Aspergillus* spp. | *Cryptoccocus* spp. |
| *Eurotium* spp. | *Eurotium* spp. | *Xeromyces* spp. | *Pichia* spp. |
| *Aspergillus* spp. | *Aspergillus* spp. | | |
| *Verticillium* spp. | *Xeromyces* spp. | | |
| *Plectosphaerella* spp. | *Epicoccum* spp | | |
| *Epicoccum* spp. | | | |
| *Fusarium* spp. | | | |
| *Colletotrichum* spp. | | | |
| *Gibellulopsis* spp. | | | |

from the paprika powder samples are not able to produce mycotoxins.

Preventing the successful colonization of unwanted food pathogens is the most important thing to ensure a safe food supply. Based on the characteristics of each fungi, the likely route of contamination was determined to help eliminate contamination of the paprika powder. Distribution of fungi based on the type of contamination was evaluated from previous studies (Lacey and West 2006). The genus and likelihood of when contamination took place during the various steps of paprika production are outlined in Table 3. However, most of the identified representatives could contaminate peppers in various stages of production of paprika powder.

Members of the genus *Alternaria* were identified in the samples (1, 8 and 9). The genus *Alternaria* are saprophytic or parasitic fungi commonly occurring on plants before or after harvest and cause many plant diseases (leaf blotch, fruit rot, etc.). Black rot infection of *A. alternata* occurs on pepper fruits when they are over-ripened or injured by sunlight (Bottalico and Logrieco 1998). Some *Alternaria* species can produce mycotoxins that persist in food. Danger of toxins is still debated (Lee *et al.* 2015). *A. alternata* AAL (Alternaria Alternata Lycopersici) toxins such as tenuazonic acid (TA), alternariol (AOH), and alternariol methyl ether (AME) that have also been isolated from grapes and tomatoes (Trinidad *et al.* 2015; Van de Perre *et al.* 2015). TA has also been isolated from samples of spices (Asam *et al.* 2012) and its carcinogenic effect was demonstrated in laboratory animals (Lacey and West 2006). Bottalico and Logrieco (1998) detected samples of pepper with black spot (*A. alternata*) that were collected in southern Italy and had low concentrations of TA (up to 54 μg/kg), AME (49 μg/kg), and AOH (640 μg/kg). In addition, representatives of the genus *Alternaria* are also potent allergens that cause exacerbations of asthma (Dang and Lawrence 2014).

Members of the genus *Aspergillus* were also identified from the paprika powder (samples 2 and 4). The introduction of *Aspergillus* can occur during growing, harvest and post-harvest with the degree of colonization depending on the conditions during harvest, transport and storage. These fungi are able to adapt to environmental conditions without free water available and grow at low humidities (Amaike and Keller 2011). They live on various substrates such as soil, grains, nuts and numerous other food products (Gorran *et al.* 2013; Matsuzawa *et al.* 2014). *Aspergillus* spp. is among the most serious contaminants of food and some representatives produce mycotoxins called aflatoxins (Rezaei *et al.* 2014). Aflatoxins are well known to be carcinogenic to the human body (Khlangwiset *et al.* 2011). El Mahgubi *et al.* (2013) found widespread *Aspergillus* contamination in 80 samples of spices, including paprika powder, with 57% still capable of producing toxigenic substance. Hammami *et al.* (2014) used a similar method as described in this study to identify *Aspergillus* spp. in samples of paprika powder. *Aspergillus fumigatus* was identified in sample 2 which is the world's most common fungal contaminate that cause fatal lung disease (Gibbons *et al.* 2012). In addition, *Aspergillus terreus* was identified in sample 4 which often causes sickness and death in patients with hematological malignancies (Hachem *et al.* 2014).

*Fusarium* is a fungal species that is a common plant pathogens with great inter-species diversity. Currently, the estimated number of *Fusarium* species is 300 representatives. A recent report of the plant disease sheet maintained by the American Phytopathological Society (www.apsnet.org/online/common/search.asp) revealed that over 80 of the 101 economically important plants on the list are associated with *Fusarium* disease (Aoki *et al.* 2014). *Plectosphaerella*, most frequently encountered in its *Plectosporium* state, is well known as a pathogen of several plant species causing fruit, root and collar rot, as well as collapse. It is considered to pose a serious threat to melon (*Cucumis melo*) production in Italy (Carlucci *et al.* 2012). The genus *Plectosporium* was introduced by Palm *et al.* (1995) for the species previously known as *Fusarium tabacinum* (*Cephalosporium tabacinum*), the anamorph of *Plectosphaerella cucumerina*. *Fusarium* spp. were identified samples 4 and 5 with some species able to produce several mycotoxins, of which fumonisin, deoxynivalenol (DON) and zearalenone (ZON) are the most

important due to their negative impact on human and animal health. Exposure to these mycotoxins always result in gastrointestinal effects (fumonisin and DON) or growth retardation in animals (RFQ). Fumonisins are associated with carcinogenic effects of the esophagus and liver (Shephard 2011). Van Poucke et al. (2012) isolated several *Fusarium* spp. from paprika powder.

The genus *Verticillium* (samples 3 and 7) comprises a small group of plant pathogenic fungi that cause billions of dollars of damage annually to a variety of agricultural crops in many parts of the world. *Verticillium* species are soilborne and cause *Verticillium* wilt, a plant disease that affects the vasculature of many different hosts (Pegg and Brady 2002), and can cause significant crop losses (Subbarao et al. 1997). The advent of molecular systematics confirmed that *Verticillium* was composed of several distantly related and ecologically diverse groups which were subsequently removed from *Verticillium* (Rehner and Samuels 1995), and placed in another genera. These include *Lecanicillium*, containing insect and fungus pathogens (Zare and Gams 2008), Pochonia and *Haptocillium* that comprise nematode parasites (Zare et al. 2001), and *Gibellulopsis* and *Musicillium* containing plant pathogens (Zare et al. 2007). *Verticillium* (Plectosphaerellaceae) is closely related to *Colletotrichum* (Glomerellaceae), another important group of plant pathogens.

*Colletotrichum* (sample 7) is a genus of fungi that are symbionts to plants as endophytes or phytopathogens. Many species in this genus are plant pathogens, but some species may have a mutualistic relationship with hosts. Anthracnose, or fruit rot, caused by *Colletotrichum gloeosporioides* and, to a lesser degree, *Colletotrichum capsici*, is a major problem of ripe fruits especially in humid lowlands that are responsible for losses of up to 90% (Grubben and Denton 2004). The occurrence of *C. capsici* may by based more on opportunistic effect, such as bruises and injuries caused by weather or pre- or post-harvest handling in storage (Swinburne 1983).

The genus *Xeromyces* (sample 2) has a single species, X. bisporus, that has the lowest requirement for available water of any known organism. *X. bisporus* has been isolated from honey in Israel, tobacco in the Netherlands, and chocolate in the United Kingdom (Pitt and Hocking 2009).

In addtion to filamentous fungi, yeasts have also been identified from paprika samples in this study. Yeasts of the genus *Cryptococcus* (in sample 6) and *Pichia* (in samples 1 and 6) are contaminants of various organic and inorganic materials. They are also found in many different parts of the world. *Cryptococcus* has been isolated from soil samples in Antarctica, vineyards in Hungary, pastures in New Zealand, and from quinces in Germany. *Cryptococcus* can also be found in the air, water, insects and humans. Similarly, representatives of the genus *Pichia* can be found on plants, in maize flour, juice, beer, silage, insects, water, and as human pathogens (Barnett et al. 2007). Given their ubiquitous nature, it is difficult to pinpoint the time of yeast contamination of a given product (Martín et al. 2005).

Knowing that fungi are present does not mean that the product is necessarily harmful to consumers. The level of fugal contamination of paprika samples is typically around 1,500 CFU/g (e. g. red chilli 1,580 CFU/g (Mandeel 2005) and cayenne pepper 1,010–2,300 CFU/g (Hashem and Alamri 2010)). This study did not quantify the microorganisms present, however, examined the different species that were present. In addition, determining if mycotoxins are present is critical to determine the safety of the product to consumers. Additional analysis of the samples for the amount of mycotoxin present would further be needed to determine the safety of the product.

## CONCLUSIONS

Spices are susceptible to microbial contamination so it is important that the growing conditions, harvesting and processing methods, storage conditions, and post-harvest treatments are carefully controlled to prevent potential food spoilage and foodborne illnesses due to contaminated spices. Fungal DNA sequences were identified from samples of sweet and hot paprika powder and compared with the NCBI database. The sequence showed a high overlap with the sequences of representatives of the class *Ascomycetes*. This was a qualitative determination of the contamination, which does not provide information as to the overall quality and safety of the paprika samples. However, fungi that are able to produce mycotoxins (particularly known toxigenic species *Aspergillus*, *Fusarium* and *Penicillium*) were found in the samples indicating that additional care for the processing and storage of the product are needed so that mycotoxins are not produced at levels that can be harmful to human and animal health.

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