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ORIGINAL ARTICLE

Molecular identification of isolated fungi from stored apples in Riyadh, Saudi Arabia

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Abstract Fungi causes most plant disease. When fruits are stored at suboptimal conditions, fungi grows, and some produce mycotoxin which can be dangerous for human consumption. Studies have shown that the *Penicillium* and *Monilinia* species commonly cause spoilage of fruits, especially apples. Several other genera and species were reported to grow to spoil fruits. This study was conducted to isolate and identify fruit spoilage by fungi on apples collected in Riyadh, Saudi Arabia and conduct a molecular identification of the fungal isolates. Thus, we collected 30 samples of red delicious and Granny Smith apples with obvious spoilage from different supermarkets between February and March of 2012 in Riyadh, Saudi Arabia. Each apple was placed in a sterile plastic bag in room temperature (25–30 °C) for six days or until fungal growth was evident all over the sample. Growth of fungal colonies on PDA was counted and sent for molecular confirmation by PCR. Six fruit spoilage fungi were isolated, including *Penicillium chrysogenum*, *Penicillium adametzi*i, *Penicillium chrysogenum*, *Penicillium steckii*, *Penicillium chrysogenum*, and *Aspergillus oryzae*. *P. chrysogenum* was the most frequent isolate which was seen in 14 of a total of 34 isolates (41.2%), followed by *P. adametzii* and *A. oryzae* with seven isolates each (20.6%) and the least was *P. steckii* with six isolates (17.6%). Penicillium species comprised 27 of the total 34 (79.4%) isolates. Sequence analysis of the ITS regions of the nuclear encoded rDNA showed significant alignments for *P. chrysogenum*, *P. adametzii* and *A. oryzae*. Most of these fungal isolates are useful and are rarely pathogenic; however they can still produce severe illness in immune-compromised individuals, and sometimes otherwise healthy people may also become infected. It is therefore necessary to evaluate the possible production of mycotoxins by these fungi to determine a potential danger and to establish its epidemiology in order to develop adequate methods of control.

1. Introduction

Yeasts play a significant role in nutrition, medicine, and biocontrol of plant pathogens. Certain yeasts act as antagonists in postharvest infections on fruits such as apples and citrus (Blevea et al., 2006; Janisiewicz et al., 2001). However, fungi cause most plant disease, accounting for perhaps 70% of all

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major crop diseases (Doores and Splittstoesser, 1983; Janisiewicz et al., 2001). Apart from the effects of high temperature and relative humidity, fungi produce pectic enzymes that break down apple pectin to expose the nutrients of the cells to the fungi (Blevea et al., 2006; Garcia et al., 2011).

Fruits such as apples contain high levels of sugars and nutrients, making them desirable for fungal growth (Prasad, 2007). Fungi can take hold of apples, particularly through a puncture or other wound that breaks the skin of the apple. Toxigenic fungi have been isolated from spoiling fruits (Pose et al., 2004). When fruits are stored at suboptimal conditions, it promotes fungal growth and mycotoxin production (Tournas and Memon, 2009).

The most common causes of apple rot are from the fungi *Penicillium expansum* and *Monilinia fructigena* (Fiori et al., 2008; Holb and Scherm, 2008). Other fungal genera that were isolated from apples include *Colletotrichum, Xylaria, Botryosphaeria* (Camatti-Sartori et al., 2005) and *Rhizopus oryzae* (Kwon et al., 2011). *Aspergillus* spp. has also been isolated and known to cause infections or allergies (Mons, 2004). In some studies, *Cladosporium* spp. was found to be a frequent fungus found in stored apples, and also *Penicillium, Acremonium, Aspergillus, Aureobasidium, Cryptococcus, Sporobolomyces* and *Alternaria* spp. (Robiglio and Lopez, 1995; Watanabe, 2008).

Traditional practices for studies of fungi include conventional cultivation and microscopic identification (Advances in Food Mycology, 2005). Identification of the fungi species is based on mycelia (color, size and shape) and morphological characteristics (morphology, conidia size and morphology conidiophore) (Al-Hindi et al., 2011; Pitt and Hocking, 2009). These techniques require skilled taxonomists. Minor differences in medium composition can impair effective comparison of mycelia characters (Larone, 1995). Molecular techniques have been demonstrated as an effective and easy way to identify fungi. DNA-based assays are reliable to detect a variety of fungi. Various molecular approaches have been used for the detection of *Aspergillus* from environmental and clinical samples (Einsele et al., 1997; Leinberger et al., 2005; Liu, 2011). Targets for the genus level detection of *Aspergillus* have included the 18S rRNA gene, mitochondrial DNA, the intergenic spacer region and the internal transcribed spacer (ITS) regions The ITS regions are located between the 18S and 28S rRNA genes and offer distinct advantages over other molecular targets including sensitivity due to the existence of approximately 100 copies per genome. The sequency variation of the ITS regions has led to their use in phylogenetic studies of many different organisms (Anaissie et al., 2009). Other studies used ITS 1 and 2 nucleotide sequences of the clinically important *Aspergillus* species and determined whether sufficient variability existed for identification to species level (Henry et al., 2000; Hinrikson et al., 2005). We conducted this study to isolate and identify fruit spoilage by fungi on apples and conduct a molecular identification of the fungal isolates.

2. Materials and methods

2.1. Sample source

2.1.1. Red delicious apples

This apple variety has a deep red color and thick skin. The apple skin is bitter in taste and its edible portion has a crispy texture. Farmers prefer this popular cultivar over local varieties. Unlike the rounder varieties, the red delicious apples are thinner.

2.1.2. Granny Smith apples

The sweetness and tartness of Granny Smith apples are well-balanced. These green colored apples are durable and retain their freshness throughout the shipping journey. It is possible to store the Granny Smith apples for about 6 months in cold storage.

2.2. Sample collection

Thirty samples of apples were collected from different supermarkets between February and March of 2012 in Riyadh, Saudi Arabia. The samples were collected six at a time over five visits to various supermarkets. A total of fifteen apples of the red delicious variety and fifteen of the Granny Smith variety were collected. The collected apples had some obvious lesion or spoilage. Each apple was placed in a sterile plastic bag in room temperature (25–30 °C) for six days or until fungal growth was evident all over the sample (after approximately six days). Spoiled or diseased apples were identified by morphological examination using the method of Bukar et al. (2009).

2.3. Fungal isolation and purification

The apple fruits were divided into species. The samples, which were apparently diseased, were cut from the advancing edges of the lesion with a sterilized knife. The cut portion of the lesion was disinfected with ethanol of 85% concentration for 2 minutes. These were then rinsed in three different changes of distilled water. Each portion was then homogenized using a sterile glass rod and a test tube containing 10 ml of the homogenate (1 g + 9 ml) (101) was made and serially diluted down to 104. Plates of already prepared Potato Dextrose Agar (PDA) containing Chloramphenicol (30 mg/l) to prevent the growth of bacteria were inoculated with 0.1 ml aliquots of the serially diluted samples and incubated at ambient room temperature (25–30 °C) for seven days. After seven days, growth of fungal colonies on PDA was counted and recorded in a colony forming unit per milliliter (cfu/ml). Isolated species were sent for molecular confirmation.

2.4. DNA extraction and sequence analyses

DNA was extracted from isolates using the CTAB (N-cetyl-N,N,Ntrimethyl-ammonium bromide) method (Murray and Thompson, 1980). Small subunit ribosomal RNA (mtSSU rRNA) and β-tubulin were then amplified by PCR using primer pairs ITS1/ITS2 and the conditions described by O'Donnell et al. (1998), Borneman and Hartin (2000) and Glass and Donaldson (1995), respectively. PCR products were purified using the QIA quick PCR purification kit (QIAGEN, GmbH, Germany), and sequenced in both directions using the respective PCR primers. For this purpose, the Big Dye terminator sequencing kit (Version 3.1, Applied Biosystems) and an ABI PRISMTM 3100 DNA sequencer (Applied Biosystems) were used. All PCRs and sequencing reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems). Gene sequences were assembled using Sequence Navigator (Version 1.0.1, Applied Biosystems), and aligned using ClustalX; Version 1.8 (Thompson et al., 2002), after which the alignments were manually corrected where needed. The predicted

Table 1 Frequency of occurrence of various fungal isolates and frequency percentage from 30 samples (total count: tfc/30 apples) on Potato Dextrose Agar (pda) containing Chloramphenicol (30 mg/l).

Kinds of apples	TFC (cfu/g)	Fungi	TC
Red delicious apples	4×10	*Penicillium steckii*	40
	9×10	*Penicillium steckii*	90
	12×10	*Penicillium steckii*	120
	5×10	*Penicillium chrysogenum*	50
	64×10^2	*Penicillium chrysogenum*	6400
	12×10	*Penicillium chrysogenum*	120
	18×10	*Penicillium chrysogenum*	180
	25×10	*Penicillium chrysogenum*	250
	57×10^2	*Penicillium adametzii*	5700
	1×10^4	*Penicillium adametzii*	40
	4×10	*Penicillium adametzii*	40
	3×10	*Aspergillus oryzae*	30
	21×10^3	*Aspergillus oryzae*	21000
	50×10	*Aspergillus oryzaePenicillium adametzii*	500
	64×10^2	*Aspergillus oryzae/ Penicillium chrysogenum*	6400
Granny Smith apples	20×10^2	*Penicillium steckii*	2000
	23×10^3	*Penicillium steckii*	23000
	17×10^2	*Penicillium chrysogenum*	1700
	9×10^4	*Penicillium chrysogenum*	90000
	21×10	*Penicillium chrysogenum*	210
	23×10	*Penicillium chrysogenum*	230
	13×10	*Penicillium chrysogenum*	130
	15×10	*Penicillium chrysogenum*	150
	13×10	*Penicillium adametzii*	130
	23×10	*Penicillium adametzii*	230
	18×10^2	*Aspergillus oryzae*	1800
	24×10^3	*Aspergillus oryzae*	24000
	23×10^3	*Aspergillus oryzae/Penicillium chrysogenum*	2300
	34×10^3	*Penicillium chrysogenumPenicillium adametzii*	34000

sequences were then compared with the corresponding sequences in GenBank to determine the possible positions of introns. All characters were weighted equally and alignment gaps were treated as missing data. Bootstrap analysis was based on replications (Makarenkov et al., 2010).

3. Results

3.1. Fungal isolates

Six fruit spoilage fungi were isolated and identified as follows: *P*. chrysogenum, P. adametzii, P. chrysogenum, P. steckii, P. chrysogenum, and *A*. *oryzae*. Among red delicious apples, *P*. chrysogenum was isolated in six samples, *P*. adametzii and *A*. oryzae were isolated in four samples each and *P*. steckii was isolated in three samples. Among Granny Smith apples, *P*. chrysogenum was isolated in eight samples, whereas *P*. adametzii, A. oryzae and P. steckii were isolated in three samples each of Granny Smith apples (Table 1).

P. chrysogenum was the most frequent isolate, seen in 14 of a total of 34 isolates (41.2%), followed by *P*. *adametzii* and *A*. *oryzae* with seven isolates each (20.6%), and the least common was *P*. *steckii* with six isolates (17.6%). *Penicillium* species consisted of 27 of the total of 34 (79.4%) isolates. (Table 2)

Table 2 Total count, frequency of occurrence of various fungal and frequency percentage from 30 samples on Potato Dextrose Agar (PDA) containing chloramphenicol (30 mg/l).

Genera and species	Total count	Frequency	%
Penicillium chrysogenum	4180	14	41.2
Penicillium adametzii	1195	7	20.6
Penicillium steckii	747	6	17.6
Aspergillus oryzae	1648	7	20.6
Total count	379	34	100

3.2. PCR identification

Sequence analysis of the ITS regions of the nuclear encoded rDNA showed significant alignments of 97–99% in sample 1 for *P*. *chrysogenum* with 550/557 (99%) identities, 96–100% alignment in sample 2 for *P*. *adametzii* with 710/715 (99%) identities, and 99% alignments in all 10 accessions for *A*. *oryzae* in sample 3 with 519/525 (99%) identities (Table 3). Similarities within each morphotype were relatively high, within the range of 96% to 100%. Inter-morphotype score was considerably less than this range (52–95). (Table 4) Fig. 1 shows the dendogram generated based on the similarity values of the three strains as well as reference strains obtained from GenBank. Isolates within each morphotype were clustered together and distinctly separated from each other. The generated phylogenetic tree (Fig. 1) shows that the studied isolates were clustered in three groups. The first group includes 3 isolates: numbers 1, 6 and 5. The second group includes the 2 isolates: numbers 2 and 3. The third group includes isolate number 4. Many authors have reported similar results. In this concern,

Table 3 Sequence and alignment of three isolates.

Isolate no (MSA1)

1 gaatccgagt gaggctctgg gtccacctcc cacccgtgtt tattttacct tgttgcttcg
61 gcgggcccgc cttttctggc ccccgggggg cttacccccc cgggcccgcc ccccccgaag
121 acaccctcga actctgtctg aagattgtag tctgagtgta aatataaatt atttaaaact
181 ttcaacaacg gatctcttgg ttccggcatc gatgaagaac gcagcgaaat gcgatacgta
241 atgtgaattg caaattcagt gaatcatcga gtctttgaac gcacattgcc ccccctggta
301 ttccggccgg catgcctgtc cgagcgtcat ttctgccctc aagcacggct tgtgtgttgg
361 gccccgtcct ccgatcccgg gggacgggcc cgaaaggcag cggcggcacc gcgtccggtc
421 ctcgagcgta tggggctttg tcacccgctc tgtaggcccg gccggcgctt gccgatcaac
481 ccaaattttt atccaggttg acctcggatc aggtaccgat acccgctgaa cttaagcata541 tcaataggac ggaggaag

Isolate no (MSA2)

1 tcggccacgt cgattccggc aagtccacca ccaccggtaa gcttcaatcc ccccaacctt
61 cgcatcccat cgaacaacag aactgattca tcaatttagg tcacttgatc tacaagtgtg
121 gtggtatcga ctctcgtacc atcgagatgt tcgagaagga ggctgctgag ctcggtaagg
181 gttccttcaa gtacgcttgg gttcttgaca agctcaaggc cgagcgtgag cgtggtatca
241 ccatcgatat cgccctctgg aagttccaga cctccaagta tgaggtcacc gtcattggta
301 agctttatcc ctgagccctc tttattctct ttcagccatt aacccttta tttcagatgc
361 ccccggtcac cgtgacttca tcaagaacat gatcactggt acctcccagg ctgactgcgc
421 cattctcatc attgcctccg gtactggtga gttcgaggct ggtatctcca aggatggcct
481 gacccgtgag cacgctctgc ttgctttcac ccttggtgtc cgccagctca tcgtcgccct
541 gaacaagatg gatacctgca agtgggctga ggagcgttac atcgagattg tcaaggagac
601 ctccaacttc atcaagaagg tcggctacaa ccccaagggt gtccccttcg tccccatctg
661 cggtttcaac ggtgacaaca tgcttgagcc ctcccccaac tgcccctggt acaagc

Isolate no (MSA3)

1 acggtgctgc tttctggtat gtctcaatgc cttcgagtta gtatgctttg gaccaaggaa
61 ctcctcaaaa ccatgatctc ggatgtgtcc tgttatatct gccacatgtt cgctaacaac
121 tttgcaggca aaccatctct ggcgagcacg gccttgacgg ctccggtgtg taagtacagc
181 ctgtatacac ctcgaacgaa cgacgaccat atggcattag aagttggaat ggatctgacg
241 gcaaggatag ttacaatggc tcctccgatc tccagctgga gcgtatgaac gtctacttca
301 acgaggtgcg tacctcaaaa tttcagcatc tatgaaaacg ctttgcaact cgtgaccgct
361 tctccaggcc agcggaaaca agtatgtccc tcgtgccgtc ctcgttgatc ttgagcctgg
421 taccatggac gccgtccgtg gcggtccctt cggtcagctc ttccgtcccg acaacttcgt
481 taacggccag tccggtgctg gtaacaactg ggccaagggt cactc

Isolate no (MSA4)

1 cagcgctgcg ttgagaccaa ccgtgagatt tacttgaaca ttggtctgaa ggctgccact
61 ttgactagtg gtcttaaata tgctcttgct acaggtaact ggggtgagca aaagaaggca
121 gccagcgcca aggccggtgt ttctcaagtg ctgagtcgct atacatttgc ttcttcgtta
181 tctcacttgc gtcggactaa tacgcccatt ggtcgtgatg gtaagattgc caaacctcgc
241 cagctacata atacccactg gggtctggtt tgtccagccg aaacacctga aggacaggct
301 tgtggtctcg tcaagaactt ggcactcatg tgctacatca ctgtcggtac cccaagcgag
361 cctatcattg atttcatgat ccaaagaaac atggaagttc tggaagagtt cgagccccaa
421 gtcacaccaa atgccacgaa ggtcttcgtc aatggtgtct gggttggtat ccaccgtgat
481 ccatcgcatc tggtgaacac tatgcaatcg ctgcgtcgac gcaacatgat ttcccacgaa
541 gtcagcttaa ttcgtgatat ccgtgagcga gagttcaaga tcttcaccga tactggacgt
601 gtctgccgtc cattgttcgt cgttgacaat gatcctaaga gcgaaaatgc cggatctttg
661 attctcaaca aggaacatat tcacaagctt gaacaagata aggacttgcc acttgatatg
721 gatgtggaag agcgccggga gcgttacttc ggatgggatg gtcttgttcg atcaggtgcc
781 gttgaatacg tcgatgctga agaagaagag acaattatga ttgtcatgac accagaagat
841 cttgagatct ccaaacagct tcaggctggc tatgcactgc ccgaggagga gaccaatgat
901 ccaaacaagc gagtc

Isolate no (MSA5)

1 ttaccaagct tcgtagtgac tgcggaggac attaccgagt gagggccctc tgggtcctac
61 ctcccacccg tgtttatttt accttgttgc ttcggcgggc ccgccttaac tggccgcggg
121 ggggcttacg cccccgggcc cgcgcccgcc gaagacaccc tcgttctctg tctgaagatt
181 ctagtctgag tgaaaatata aattatttaa aactttcaac aacgggtctc ttggttccgg
241 gatcgatgaa gaacgcagcg aaatgcgata cgtaatgtga attgcaaatt cagtgaatca
301 tcgagtcttt gaacgcacat tgcgcccccc ggtattccgg ggggcatgcc tgtccgatcg
361 tcatttctgc cctcaagcac ggcttgggtg tggggccccg ccctcggggg gggggggggg
421 gggggcagaa cgccccgggg aaacatcggg gggccctccc aaagagtgat aattaaaaca
481 aagggggggg gggggggggg ggccccccag ccccccccgc ttttttagt gcctcccgcc
541 cgggggggca aaaggaaaaa ccctttttt ttttttttt tttggagggg gggggggggag
601 aaggg

Table 3 Sequence and alignment of three isolates.

Isolate no (MSA6)
1 ggtttcgagt gagcccctct gggtccacct cccacccgtg tttattttac cttgttgctt
61 cggcgggccc gccttaactg gccgccgggg ggcttacgcc cccgggcccg cgcccgccga
121 agacaccctc gaactctgtc tgaagattgt agtctgagtg aaaatataaa ttatttaaaa
181 ctttcaacaa cggatctctt ggttccggca tcgatgaaga acgcagcgaa atgcgatacg
241 taatgtgaat tgcaaattca gtgaatcatc gagtctttga acgcacattg cgcccctgg
301 tattccgggg ggcatgcctg tccgagcgtc atttctgccc tcatgcacgg cttgtgtgtt
361 gggccccgtc ctccgatccc gggggacggg cccgaaaggc agcggcggca ccgcgtccgg
421 tccacgagcg tatggggctt tgtcacccgc tctgtaggcc cggccggcgc ttgccgatca
481 acccaaattt ttatccaggt tgtcctcgga tcaggtaggg atacccgctg aacttaagga
541 tatcaaaggc cggggaggaa t

Table 4 The similarity between the sequences of isolates.

SeqA	Name	Length	SeqB	Name	Length	Score
1	Isolate1	558	2	Isolate2	716	54.0
1	Isolate1	558	3	Isolate3	525	52.0
1	Isolate1	558	4	Isolate4	915	60.0
1	Isolate1	558	5	Isolate5	605	78.0
1	Isolate1	558	6	Isolate6	561	95.0
2	Isolate2	716	3	Isolate3	525	58.0
2	Isolate2	716	4	Isolate4	915	54.0
2	Isolate2	716	5	Isolate5	605	46.0
2	Isolate2	716	6	Isolate6	561	53.0
3	Isolate3	525	4	Isolate4	915	66.0
3	Isolate3	525	5	Isolate5	605	52.0
3	Isolate3	525	6	Isolate6	561	53.0
4	Isolate4	915	5	Isolate5	605	55.0
4	Isolate4	915	6	Isolate6	561	60.0
5	Isolate5	605	6	Isolate6	561	80.0

Mansfield showed that ITS regions' analyses detected a greater number of species then selective plating (Mansfield and Kuldau, 2007). Peterson also isolated 2 new *Penicillin* species from peanut field soils and used ITS to identify the novel species (Peterson and Horn, 2009).

4. Discussion

Penicillin and *Aspergillus* species have been reported before as pathogens of fruit spoilage (Fiori et al., 2008; Holb and Scherm, 2008; Mons, 2004; Robiglio and Lopez, 1995; Watanabe, 2008). However, in this study, we found a different species specifically *P. chrysogenum*, *P. adametzii* and *A. oryzae*. *P. chrysogenum*, previously known as *P. notatum* can be found on salted food products and in damp buildings (Andersen et al., 2011; Houbraken et al., 2012). It rarely causes human disease; in fact it is the source of β-lactam antibiotics, most significantly penicillin (Anaissie et al., 2009). Transcription of genes involved in the biosynthesis of valine, cysteine and α-aminoadipic acid—precursors for penicillin biosynthesis—as well as of genes encoding microbody proteins, was increased in the high-producing strain of *P. chrysogenum* (Berg et al., 2008). However, despite its highly useful traits, *P. chrysogenum* has been implicated in several diseases. There have been reports of Intestinal invasion and disseminated disease associated with *P. chrysogenum* identified in immunosuppressed patients, either due to human immunodeficiency virus or from immunosuppressant medications post-transplantation (Barcus et al., 2005). In other reports, *P. chrysogenum* was identified to cause invasive pulmonary mycosis in transplant patients (Geltner et al., 2013).

P. adametzii on the other hand, is a glucose oxidase producing fungi (Mikhailova et al., 2007). Glucose oxidase per se, acts a natural preservative acting as bactericide in many cells. It has become commercially important in the last few years, gaining a multitude of different uses in the chemical, pharmaceutical, food, beverage, and other industries (Guimara˜es et al., 2006).

The genus *Aspergillus*, particularly *A. oryzae,* is a filamentous fungus used in Chinese and Japanese cuisine to ferment soybeans, saccharify rice and potatoes. Its most popular use is in making the alcoholic Japanese beverage sake and the production of rice vinegars (Rokas, 2008). Pathogenecity for *A. oryzae* is quite rare, although there are reported cases of fungal

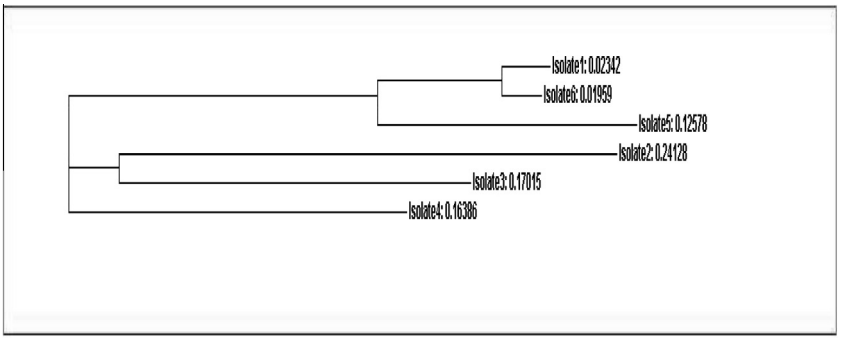

Figure 1 The cluster analysis and distance of tested isolates.

peritonitis caused by *A. oryzae* reported in the literature (Schwetz et al., 2007). Other reports showed cultures of *A. oryzae* in nasal mucosa and sinuses of patients undergoing chemotherapy (Pagella et al., 2007).

Although most of these fungal isolates are useful, whether in the production of penicillin or in the food industry, they can still produce severe illness in immune-compromised individuals, and sometimes otherwise healthy people may also become infected. They can be an occupational hazard, especially among people who work on farms. It is therefore necessary to evaluate the possible production of mycotoxins by these funguses to determine a potential danger and to establish its epidemiology in order to develop adequate methods of control.

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