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Isolation of *Hortaea werneckii* for the First Time, and Fungi Related to Producing Aflatoxins from Livestock Feeds in Ghemins, Libya

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Abstract: ***This research paper includes two studies simultaneously. First, the detection of the fungi associated with livestock feed samples in Ghemins stores and mills, as well as to verify the presence of aflatoxigenic Aspergillus flavus. There are seven genera found, Penicillium, Aspergillus, Scedosporium, Mucor, Fusarium, Alternaria and Rhizopus. On the other hand, the researchers aim to provide some important scientific information about Hortaea werneckii, which was isolated for the first time from the hay in Libya.***

Keywords: *Hortaea werneckii*, Tinea Nigra, halotolerant, Melanin pigment, *Aspergillus flavus*, Aflatoxins

1. Introduction

Hortaea werneckii is the causative agent of Tinea nigra in humans (Mok, 1982), which is common in tropical and subtropical countries (Chen *et al.,* 2012). Tinea nigra is described as brown to black spots that usually occur on the palms of hands and sometimes on other parts of the body, such as blood or splenic abscess (Bonifaz *et al.,* 2008 & Ng *et al.,* 2005).

This genus Hortaea was established in 1984, and it is classified as the Ascomycota from the order Capnodiales (Lenassi *et al.,* 2013), and currently includes three species (Holker *et al.,* 2004). *H. werneckii* is among the black yeasts that have been known since the end of the nineteenth century (De Hoog *et al.,* 2000). It was previously called *Exophiala werneckii* and *Cladosporium werneckii (*McGinnis, Schell and Carson, 1985 & Perez *et al.,* 2005). Their colonies are described as slow growing, initially mucous, yeast-like, and shiny black. After several days, they develop abundant aerial mycelia and become dark olivaceous in colour. The conidia are one to two-celled, cylindrical, or spindle-shaped (Chen *et al.,* 2012).

H. werneckii is a saprophytic fungus (Zalar, De Hoog and Gund-Cimerman, 1999), and is distinguished by its great ability to tolerate salinity, so it has been considered one of the largest halotolerant eukaryotes (Gund-Cimerman and Plemenitas, 2006 & Lenassi *et al.,* 2013 & Marchetta *et al.,* 2018). This is clearly demonstrated by living in many saline habitats, for instance, seawater and beach soil (Chen *et al.,* 2012 & Cabanes, Bragulat and Castella, 2012). The black colour of this fungus is due to the melanin pigment that is secreted from it (Elsayis *et al.,* 2022).

The importance of this study is due to highlighting the contamination of animal feed with storage fungi and its risks that affect the health of livestock and humans due to their production the carcinogenic mycotoxins, in addition to their economic losses. After appearance the growth of *H. werneckii* on hay, the researchers decided to shed light on this fungus, as the first simplified study of it in Libya.

2. Materials and Methods

Sample Collection

Livestock feed samples were collected from animal feed stores in Ghemins, according to the following types: barley, wheat, maize, and hay. Approximately 150 g were collected for three varieties of barley, wheat, and maize, and approximately 50 g of hay was collected. These samples were placed in sterile bags and kept in the refrigerator until using them.

Fungal Isolation

Samples were isolated using blotting paper and nutrient agar medium (Neergaard, 1983). The samples were superficially sterilized with 4% sodium hypochlorite for 1 minute and then washed with sterile distilled water. They were then placed in Petri dishes containing filter papers about 9 cm in diameter slightly moistened with sterile distilled water (blotting paper technique). In the same previous sterilization way, the tested samples were placed in Petri dishes containing Potato Dextrose Agar PDA (agar plate technique). All dishes were placed in the incubator for one week at room temperature.

Fungal Identification

In the two previous methods, the fungi that grew on the tested samples were isolated in the subculture to identify them morphologically by using microscopically, where a part of the growing fungus is placed on a glass slide and stained using Lactophenol Blue Dye.

Investigating the capability of *Aspergillus flavus* to produce aflatoxins

Aspergillus flavus was isolated and was grown in potato dextrose broth (PDB) at 25ºC for 20 days. The Test Procedure was applied to the media after removing the

fungus by using Alfacard total test (Image 1) (R. Biopharm Rhone LTD, 2005).

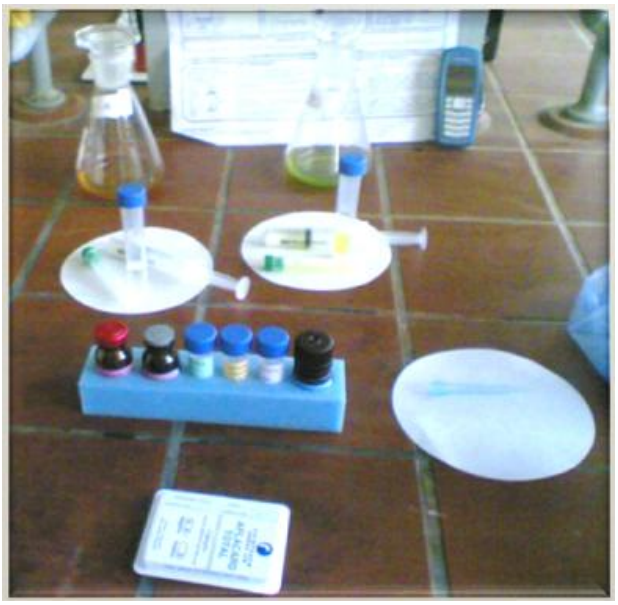

Image 1: Aflacard Total Test reagents

3. Results

The results of the study conducted on samples of hay, corn and wheat showed the presence of storage fungi (seed-borne fungi) contamination. The approximate contamination rates in the hay sample were more than in the maize and wheat samples. *Penicillium canescens* reoccurred in all hay sample plates (Image 2) by both methods that were used. Growth of *Aspergillus Flavus*, *Aspergillus clavatus (*Image 2) and *Aspergillus Candidus (*Image 3) appeared in a very small percentage in the hay sample, in addition to *Scedosporium aurantiacum (*Images 3 & 4) and *Hortaea werneckii (*Images 5 & 6). As for the maize sample, it was contaminated quite slightly with the fungi, *Mucor sp*., *Fusarium graminearum (*Image 7), *Alternaria sp*. and *Rhizopus sp* (Image 8).

Although all the wheat sample plates showed no significant contamination in the first week, they all showed perfect germination naturally. However, some grains showed growth of *Fusarium gramine arum* at the end of the second week, in a very small percentage. But the barley sample showed no significant contamination in all testing dishes (Image 9).

The experiment of Alfacard Total Test found out that the isolated *Aspergillus flavus* from hay is from aflatoxin producer group.

Image 2: Isolates of *Penicillium canescens*, *Aspergillus flavus* and *Aspergillus clavatus*

Image 3: Isolates of *Aspergillus candidus* and *Scedosporium aurantiacum*

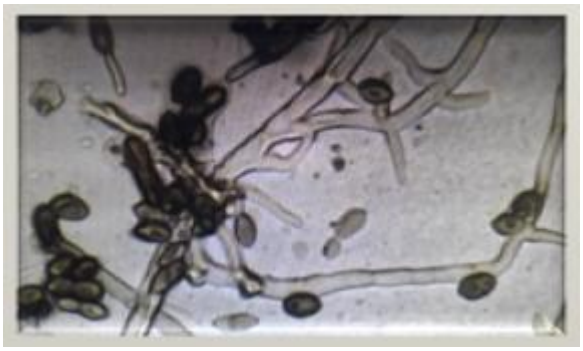

Image 4: *Scedosporium aurantiacum* under light microscope

Image 5: Isolates of *Hortaea werneckii*

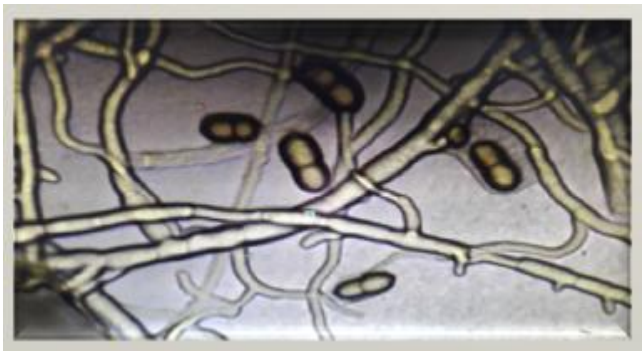

Image 6: *Hortaea werneckii* under light microscope

Image 8: Isolates of *Alternaria sp*. and *Rhizopus sp*.

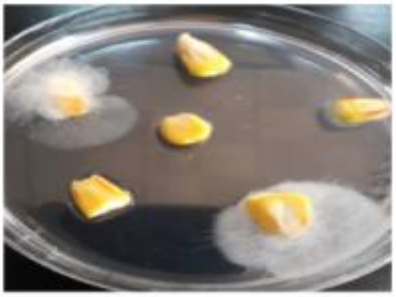

Image 7: Isolates of *Mucor sp*. and *Fusarium graminearum*

Image 9: Natural germination and free from fungal contamination of barley grains

4. Discussion

Growth of *Fusarium graminearum* at the end of the second week, which is known for its slow growth. it appears that the spores are presented in the embryos of grains that grew on them after the beginning of their germination.

The living of fungi such as *Aspergillus flavus* in any animal feed is a source of enormous concern as it is a hazardous factor for farm animals, in addition to their dangerous impact on milk and dairy products, due to the secretion of mycotoxins, which are known to be carcinogenic factors. These toxins are possibly transmitted to people through the food chain by eating meat, milk, and dairy products, which are contaminated with these toxins. Buckley, Creighton, Fogarty, 2007 claimed that Fungi, especially, *Aspergillus* and *Fusarium*, and their mycotoxins are a major cause of respiratory infections; recurrent airway obstruction RAO and exercise-induced pulmonary haemorrhage EIPH in horses. Their study was conducted on feed fodder used in Irish racing yards for one year. The results proved that 50% of Irish hay, 37% of haylage and 13% of Canadian hay were contaminated with pathogenic fungi and their mycotoxins, T2 and zearalenone.

Aspergillus flavus, *A. niger* and *F. verticillioides* were the prevalent isolates from raw materials and finished feed intended for sows at different reproductive stages in Argentina. The samples were contaminated with Aflatoxin; in 80% samples, and Fumonisin B1, in addition to a low level of ochratoxin A in non-pregnant gilt samples. The mycotoxins were detected by high-pressure liquid chromatography (HPLC) (Pereyra *et al.*, 2010). Fifty-one samples of animal feeds, cereals and grains were collected from farms and retail shops in the intense farming areas of Tamil Nadu, they were analyzed for the presence of toxicogenic fungi by conventional mycological identification methods. The predominant fungal contaminant was aspergillus spp. (83%), followed by *Penicillium spp*. and *Fusarium* spp. *A. flavus* was the most species additionally to presence of *A. fumigatus* and *A. niger*. The researchers revealed that poultry feed seems to be the more predominant animal feed that is infected with such mycotoxigenic fungi (Sivakumar, Singaravelu and Sivamani, 2014).

Naseer *et al.*, 2018 reported that 67 specimens out of 74 concentrate feed specimens were collected from different

livestock farms and commercial feed mills in Pakistan. These specimens were contaminated with six fungal genera and aflatoxin B1. The fungal genera were *Aspergillus (*43.3%), *Fusaram (*38.8%), *Mucor (*8.9%), *Penicillium (*5.9%), *Rhizopus (*1.5%) and *Alternaria (*1.5%), and their species were *A. flavis*, *A. parasiticus*, *A. fumigates*, *A. niger*, *F. chlamydosporum*, *F. oxysporum*, *F. solani*, *R. arrhizus*, *M. amphibiorum*, *M. circinelloides*, *M. indicus*, *P. crustosum*, *P. palmae* and *Alternaria solani*. Silage and concentrated feed samples were collected from 21 dairy farms in the Western part of Paraná state in Southern Brazil. These samples were analyzed to check for the presence of aflatoxigenic Aspergillus. Two silage samples and four concentrated feed samples were contaminated with *Aspergillus parasiticus* and *Aspergillus nomius*. Four out of seven isolates, two from the silage samples and two from the concentrated feed samples, produced the aflatoxins B1, B2, G1, and G2 in culture media (Variane *et al.,* 2018). Aspergillus, Penicillium and Fusarium were isolated from 40 feed samples in a feed mill near Palermo (Sicily, Italy). SAB and PDA were used in the isolation process. The species isolates of Aspergillus were *A. amstelodami*, *A. awamori*, *A. flavus*, *A. niger*, *A. oryzae* and *A. tubingensis*. Furthermore, aspergillus spp, isolates that are likely to be able to produce mycotoxins emerged (Mirabile *et al.,* 2019).

Sukmawati *et al.,* isolated *Aspergillus flavus* from maize that is used in livestock feed in Bogor, West Java. A total of 33 various fungal species were isolated from 55 samples collected from 20 dairy farms in South Italy. *Cladosporium, cladosporioides* was the most representative fungus that was isolated, followed by *Alternaria alternata* and *Rhizopus stolonifer*. Further, *Aspergillus flavus* that known to be the most aflatoxigenic fungus was isolated (Ceniti *et al.,* 2021). 79 samples out of 90 different samples of animal feed that were collected from 90 sites in Egypt were recorded to be contaminated with fungi, including 16 species of Aspergillus. The highest percentage of which was *Aspergillus flavus*, followed by *Aspergillus niger*. In addition, *Penicillium chrysogenum* was isolated from 43 samples. Moreover, the results displayed that two samples were contaminated with aflatoxin B1, and only one sample was contaminated with aflatoxin B2. These results were obtained by performing the High-performance liquid chromatography (HPLC) (Khalifa *et al.,* 2022).

Isolation of *Penicillium canescens*, *Aspergillus flavus*, *Aspergillus clavatus*, *Aspergillus candidus*, *Fusarium graminearum* and *Alternaria sp*, from the tested samples in this study, may lead to the explanation of the cause of the death of large numbers of livestock in July 2022. Significant contamination was observed in samples of hay, which is the most widely used livestock feed in Libya. Abubakr, 2017 isolated of *Alternaria raphani*, *A. tenusinae*, *Aspergillus Flavus, A. niger*, *Fusarium graminearum*, *F. moniliforme*, *F. solani*, *Rhizopus stolonifer*, *Penicillium digitatum* and *P. notatum* from four cereal grains; wheat, barley, rice, and maize collected from three Libyan cities, Al-Zawia, Subratah and Tripoli. Dextrose-Czapkes agar medium was used in the fungal isolation. The fungus, which is observed in the current research is like those isolated from soils in the same town of this study, Ghemins (Jwieli *et al.,* 2021). This may indicate that these fungi are common in Ghemins environment. The aforementioned study recorded an increase in salinity in one of the study regions, which may be the same region in which the hay, contaminated by *H. werneckii*, was collected after harvest, and before it was transferred to livestock feeds stores.

Since *H.* werneckii is commonly found in the depths of the Mediterranean (De Leo *et al.,* 2018), this may explain its growth in environmental sources of the beach town of Ghemins, such as hay. *H.* werneckii does not grow on PDA only. Mok, 1982 isolated 44 isolates, humans and environmental sources that caused tinea nigra by using Sabouraud Dextrose Agar (SAB) and Czapek-Dox Agar. Mok identified the fungus by its old name *Exophiala werneckii*. The first isolation of *H. werneckii* from a domestic guinea pig in Japan was by Sharmin *et al.,* 2002. It was isolated for the first time from diving equipment, such as silicone masks and snorkel mouthpieces in Spain (Cabanes, Bragulat and Castella, 2012). Chen *et al.,* 2012 were the first to isolate *H. werneckii* from the medicinal plant mangrove in the South Sea of China. Elsayed *et al.,* 2016 recorded the first report in which three strains of *H. werneckii* were isolated from the salt marshes of Egypt.

Although Tinea nigra is rare in Libya, the purpose of mentioning its causative agent *H. werneckii* in this scientific paper is due to the usefulness of this fungus in the production of melanin pigment and several enzymes. Leitgeb *et al.,* 2013 considered that *H. werneckii* is among the most technically and economically important microorganisms due to its ability to secrete enzymes, intracellular enzymes α-amylase and extracellular enzymes cellulase. Consequently, they made a suspension culture from *H. werneckii*, and incubated it in supercritical carbon dioxide (SC CO2) to use their enzymes for biotransformation and compare their activity with the activity of purified commercial enzymes at the same conditions, α-amylase from *Aspergillus oryzae* in the powder form and cellulase from *Trichoderma reesei*, Cellusoft L, in the liquid form. Five strains of *H. werneckii* were isolated from a solar salter in Alexandria and stimulated to produce an optimized melanin pigment. Therefore, it may facilitate the incorporation of a variety of pharmaceutical and environmental applications (Elsayis *et al.,* 2022).

H. werneckii is possibly used in genetic applications because it is considered a model for intraspecific hybridization in clonal fungi (Zalar *et al.,* 2019). According to Hodhod *et al.,* 2020, *H. werneckii* can be used as an antibacterial agent against both gram-positive and gram-negative pathogenic strains. Three new strains of *H. werneckii* were isolated. The ethyl acetate crude extract active fractions of these strains possessed significant activities against Methicillin-resistant *Staphylococcus aureus* (MRSA), *Campylobacter jejuni* and *Salmonella typhimurium*. On contrary, Selbmann *et al.,* 2008 considered the biggest obstacle to studying *H. werneckii* in its inability to prohibit the invasion of other competing microorganisms that is like the behavior of the acidophilic fungi.

5. Conclusion

Fungi accompanying animal feeds in general, have come to be seen as a double-edged sword. From the first side, their huge hazards that result from its secretion of carcinogenic toxins to both animals and people. Also, their spores when they contaminate the feed stores air; are likely to be inhaled by warehouse workers, and lead to the occurrence of numerous respiratory diseases. On the other hand, some strains of these fungi are now used in varied valuable industries. Based on this, firstly, it became necessary to carry out a periodic check of the feed samples to ensure that they are free of toxin-producing fungi. As well as performing several studies related to them, for instance, the appropriate environmental conditions for their growth, and thus improving storage conditions and access to a safe health situation. In addition to all that, developing research that helps as much as possible to benefit from the beneficial strains of these fungi and use them in industry, such as melanin and enzymes.

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