



Molecular diagnosis and possible control of the pathogenic *Aspergillus flavus* isolates associated with cultivated mushroom *Agaricus bisporus* in Iraq

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Received: Sept. 20, 2022	Abstract Five isolates of <i>Aspergillus flavus</i> were isolated from casing soils, compost and fruiting bodies of <i>Agaricus bisporus</i> from several culture stations in Iraq. According to the pathogenicity test, the highest percentage of inhibition was recorded for isolate A.flavusB1 .It was molecularly confirmed to the level of A.flavus on the basis of the nucleotide sequence analysis of the ITS genome region and it was recorded globally on the NCBI data base under the accession number ON738705.1. The effect of the hot aqueous extract of Cloves and Conocarpus, and the efficacy of the biocide VEROX on the production and phenotypic characteristics of the white edible fungus A. bisporus was studied in presence of the pathogenic fungus. The results showed that the treatment of the combination of plant extracts + biocide + pathogenic fungus was recorded the highest rate of growth inhibition of the pathogenic fungus compared with other treatments ,and it was increased the number of fruiting bodies up to 5 compared with the control treatment of pathogenic fungus was 195. The total yield of the same treatment was 19.894 kg compared to 4.933 kg in the control treatment, and the biological efficiency was recorded 33.7% compared to 12.6% in the control treatment. In general, this combination increased production and improved the qualitative characteristics of the fruiting bodies, as the average diameter of the cap was 43 mm and the stalk length was 39.1 mm compared to 28.5 mm and 23 mm in the control treatment, respectively. Keywords: <i>Agaricus bisporus</i> , <i>Eugenia caryophyllata</i> ,VEROX, , <i>Aspergillus flavus</i>
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Introduction

The white edible mushroom, *Agaricus bisporus* is a desirable edible fungus consumed worldwide because of its economic, nutritional and medicinal importance [1]. It is rich in minerals, proteins and vitamins [2]. Global production is estimated to be approximately 40 million tons [3], which is cultivated in special controlled growth rooms using different agricultural materials [4]. White edible mushrooms are exposed to many fungal, bacterial and viral diseases that affect production [5]. One of the most important diseases that infect the edible white mushroom is the fungal diseases, which accompany the cultivation of the fungus causing pathogenic infection with increased

competition for nutrients [6]. Several fungi competing with the white mushroom were isolated, including *Aspergillus* spp., found in casing soils or composted media [7]. Infection with the pathogenic fungus was also recorded on the inoculum of the white edible *A.bisporus* on wheat grains [8]. It was found that it increased competition with the edible mushroom and caused an infestation causing green molds [9].

Several studies have been conducted to control diseases that affect food mushrooms by several chemical, biological methods and plant extracts [10]. Due to the importance of diseases caused by competing organisms to edible mushrooms, especially the pathogenic fungus *A.flavus*, and the damage and economic losses it causes in production, and its danger as it produces a group of highly toxic mycotoxins, therefor, *A. flavus* was isolated, diagnosed and studied for its effects on edible white mushroom and the possibility of controlling the pathogen using different combination methods.

Materials and Methods

Isolation of *A. flavus*

Samples of infected fruiting bodies of the white edible fungus, casing soils, and compost were collected from several farms in in different governorates of Iraq (Baghdad ,Erbil ,Diwaniyah ,Kirkuk and Muthanna) and gave them the symbols (B1,E1,D1,K1 and M1) Straight. *A. flavus* was isolated from mulching soils and compost by dilution method by taking 1 gm of soil, after mixing it well, it was placed in series sterile test tubes containing 9 ml of sterile distilled water. The suspension was shaken for 30 seconds, then a series of dilutions were made. A fifth dilution was taken for all soil samples 1 ml of the last dilution and added to Petri dishes containing PDA medium with a slight stir to ensure homogeneous distribution and incubated at 25 ± 2 °C for 3 days[11]. The colonies growing on the PDA nutrient medium were purified using the Streak-plate method on a number of Petri dishes by (loop) and incubated at a temperature of 25 ± 2 °C for two days, after which the germinated colony was taken and transferred to new dishes containing the same medium and incubated for five days and kept in the refrigerator until use[12] As for the fruiting bodies, samples that showed symptoms of fungal infection were taken, washed well, then cut to 0.5 cm pices, and superficially sterilized by sodium hypochlorite at a concentration of 2% for two minutes, then washed with sterile distilled water and dried using sterile filter paper. Then, the sterilized parts were transferred by sterile forceps to Petri dishes containing PDA, and the same previous steps were performed. The different isolates were distinguished according to the phenotypic characteristics depending on the color, shape and microscopic properties of the fungal hyphae and the shape of the conidia and conidio-phore [13].

Laboratory pathogenicity test

Pathogenicity was tested by dual culture method. A petri dish was divided into two parts inoculated, one with the pathogenic fungus (treatment) and the other with the mushroom *A. bisporus* (control). The colony diameter was measured with a ruler and the percentage of inhibition growth was calculated using the following equation [14].

$$\text{Inhibition rate \%} = \frac{\text{Colony radius of the control} - \text{colony radius of a treatment}}{\text{Colony radius of the control}} \times 100$$

Molecular diagnosis

The molecular diagnosis of the most pathogenic fungal isolate was sent to the Korean Macrogen Company to determine the nucleotide sequence of the ITS genetic region. After receiving the molecular diagnosis, the BLAST program was used to compare the results with the data available at the National Center for Biotechnology Information (NCBI) within the electronic gene bank, which belong to the same fungal isolates diagnosed globally. Nucleotide analyzes were also conducted using the MEGA program to draw a relationship tree between the isolate under study and similar isolates registered at NCBI, where the genetic tree was built based on the molecular nucleotide sequence of the ITS region of the isolate.

Preparation of plant extracts

A hot aqueous extract of the plants under study (Cloves and conocarpus leaf) was prepared with a weight of 20 g of dried plant powder mixed with 100 ml of distilled water at 60 °C, and then placed in a magnetic stirrer for 24 hours. Then, the extract was filtered using filter paper and concentrated in an evaporator, then the extract was dried center at 45 °C [15].

Preparation of the fungal inoculum

Rice was used as a carrier of the fungal inoculum for *A. flavus*. Rice seeds were moistened and sterilized, and after cooling, inoculated with 3 pieces of 0.5 cm of the fungus colonies growing on PDA medium [16].

Preparation spawn

A. bisporus inoculum was loaded on Wheat grains [17].

Incubation and growth

The compost was obtained commercially from Al-Wadaqq Company. The compost was placed in aluminum trays 40*40 cm with a height of 20 cm. The fungal inoculum of *A. bisporus* was added between compost layers. It was moisten by spraying with 2% water and incubated in a special growth room at 25 C° with a relative humidity of 85% [18].

The treatments

Five combinations of extracts of clove, conocarpus and biocide VEROX were used to study their effect on the fungal pathogen. As the treatments were Negative control, Positive (*A. flavus* Infected) control, *A. flavus* + Clove Extract, *A. flavus* + Kono Carpus extract, *A. flavus* + Biocide VEROX, and *A. flavus* + VEROX + Clove Extract + Conocarpus Extract.

The inoculum of pathogenic *A. flavus* was applied directly at a fertilizer rate of 100 g per replicate before placing the casing soil. After placing the casing soil, the plant extracts were sprayed at 100 ml/rep and the biocide at a concentration of 3 g/L at 50 mL/rep. The temperature was lowered to 16 °C and the humidity was raised to 90% to stimulate the white fungus to germinate [17].

Measurements

The measurements have been taken included the number of fruit bodies, the amount of yield per square meter, and morphological characteristics (cap diameter and stem length) periodically for four weeks. The biological efficiency ratio of the medium was evaluated [19].

Biological efficiency ratio % = $\frac{\text{Quantity of fresh yield of the first harvest (Kg/ m}^2\text{)}}{\text{Culture medium dry weight (Kg/ m}^2\text{)}} \times 100$

Statistical analysis

The complete randomized design (CRD) was used for the distribution of the experimental units. Data analysis was carried out using the SAS computing program (sas, 2012), and a means comparison was made among the treatments according to the least significant difference L.S.D. ($P \leq 0.05$) [20].

Results and Discussion

Isolation of pathogenic fungus *A. flavus* and pathogenicity test

Five isolates of pathogenic *A. flavus* were isolated from casing (mulching) soil and mushroom fruiting bodies. According to the pathogenicity test, the results showed that isolate B1 was recorded the highest pathogenicity by resulting in the highest percentage of inhibition (66.66%), compared with other isolates. Thus it was selected for use in subsequent experiments (Figure 1). Depending on the percentages of growth inhibition of *A. bisporus* by the pathogenic isolates (Figure 1), it was observed that some isolates inhibited the growth of the white edible mushroom at a higher rate than other isolates. as the Baghdad governorate isolate *A. flavus* B1 recorded the highest Pathogenicity against *A. bisporus* growth (Figure 2). This is attributed to the inhibitory activity of these isolates represented by the secretion of enzymes or mycotoxins, which affect the hyphae growth of *A. bisporus*. The reason may be that the nutrient medium is suitable for the growth of more virulent isolates than the other isolates, and environmental conditions play a role in influencing the activity of fungal isolates [21].

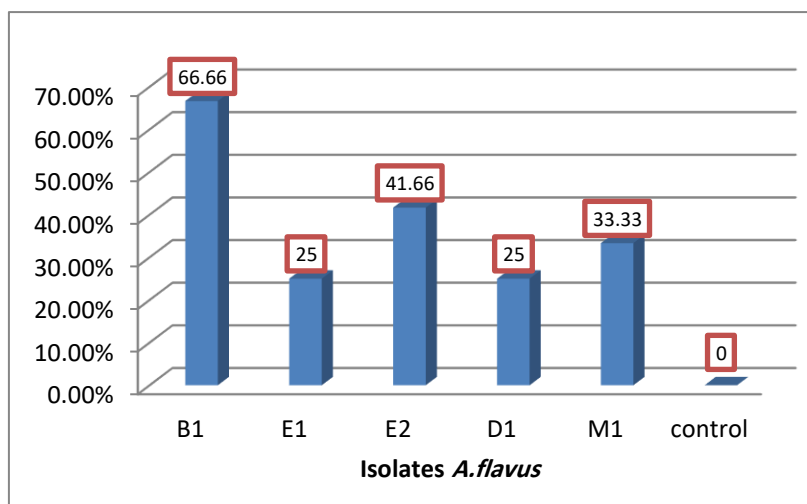


Figure (1): The percentage of *A. bisporus* growth % inhibition by *A. flavus* isolates



Figure (2): Isolate *A. flavus* B1 inhibitory the growth of fungal hyphae of *A. bisporus*

Molecular diagnosis

The results of the nucleotide sequence analysis of the pathogenic fungal isolate confirmed that it belongs to *A. flavus*. The molecular nucleotide sequences achieved 100% match with the ITS genomic region when compared with the corresponding nucleotide sequences retrieved from the genebank (Table 1). While the results of constructing the genetic tree (Fig. 3) showed that this isolate appeared in the same clade, in which the Egyptian isolates (ON745314.1 and ON714427.1) appeared. While, it was in separate clades far from the French (OW982417.1) and Belgian (OW982417.1) isolates (OW982327.1) due to the large genetic divergence between them.

Table (1): Compatibility (similarity) of the nucleotide base sequence of the fungus *Aspergillus flavus* Y.N. 150 Haneen isolated from Baghdad governorate with other fungal isolates of the same fungus registered in the NCBI

No.	Fungal name	Isolate name	Origin	GenBank Accession Number	similarity %	Registration date in NCBI
1	<i>A.flavus</i>	Y.N.150 Haneen	Iraq	ON738705.1	100	11/6/2022
2	<i>A.flavus</i>	————	Mauritius	OW986451.1	100	23/5/2022
3	<i>A.flavus</i>	————	Cuba	OW986401.1	100	23/5/2022
4	<i>A.flavus</i>	————	Belgium	OW982454.1	100	23/5/2022
5	<i>A.flavus</i>	————	France	OW982417.1	100	23/5/2022
6	<i>A.flavus</i>	————	Belgium	OW982332.1	100	23/5/2022
7	<i>A.flavus</i>	————	Belgium	OW982327.1	100	23/5/2022
8	<i>A.flavus</i>	AUMC 15404	Egypt	ON745314.1	100	13/6/2022
9	<i>A.flavus</i>	strain GKRS06	India	ON739012.1	100	12/6/2022
10	<i>A.flavus</i>	NORHAN	Egypt	ON714427.1	100	12/9/2021

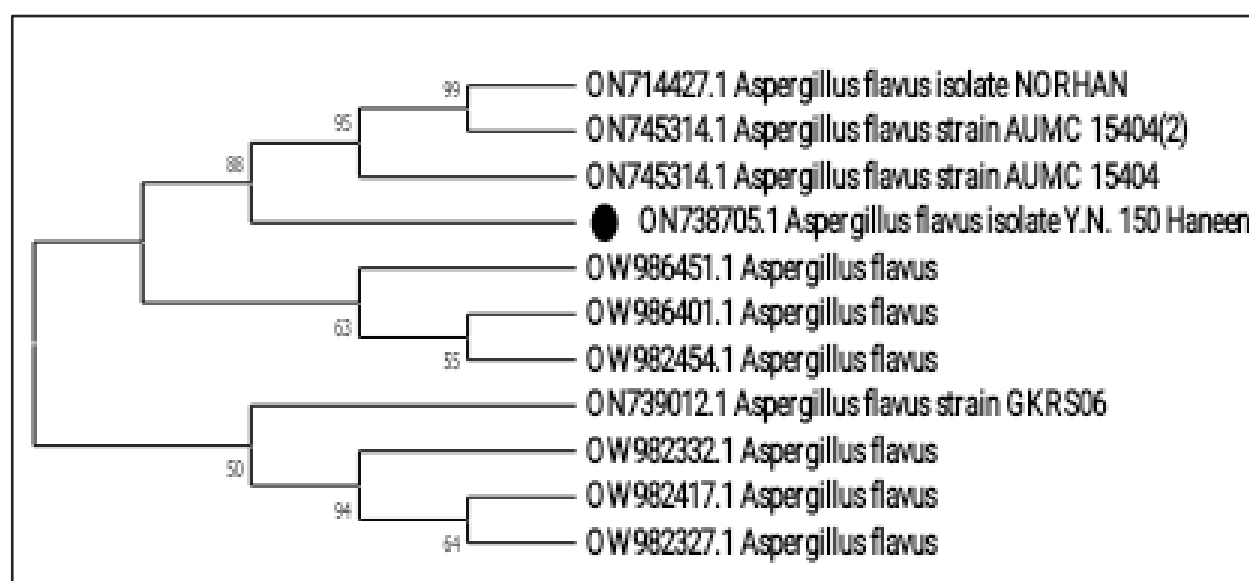


Figure (3): The Scale for the tree of the pathogenic fungus *A. flavus* Y.N.150 Haneen (marked with a black dot) and global strain sequences for the same fungus obtained from the GenBank data site

The effect of plant extracts and the biocide verox on the productive traits of *A. bisporus* under conditions of infection with the pathogen *A. flavus*:

The results (Table 2) showed significant differences between the treatments. The combination of pathogen + Biocide/compost + plant extracts, resulted in the highest

number of fruiting bodies (505), In contrast the lowest number of fruiting bodies was recorded in the treatment of pathogenic fungus only (195 fruiting bodies). As for the amount of yield and biological efficiency, the same combination recorded the highest yield of 19.894 kg/m² and biological efficiency of 33.7%, while the pathogenic fungus treatment alone recorded the lowest yield of 4.933 kg/m² and biological efficiency of 12.6%. This difference may be attributed to the nature of the active ingredients produced by plants as secondary metabolites such as terpenes, phenols, nitrogen and sulfur compounds that have a role in inhibiting pathogenic fungi [22]. Also, the obvious inhibition of pathogenic fungi by using the commercial probiotics combined with organic fertilizer, may be due to the biological activity of microorganisms in the compost. It has been found that the white food mushroom stimulates the defensive resistance against pathogens. Or as a result of releasing volatile compounds that prevent the growth of pathogenic fungi or disrupt their activity [23].

Table (2): Effect of plant extracts and biocide VEROX on the productive traits of *A. bisporus* in the presence of the pathogenic *A. flavus*

No.	Treatments	Total No. of fruiting body.m ²	Total yield Kg.m ²	biological efficiency %
1	Control	580	23.376	40.8
2	<i>A. flavus</i>	195	4.933	12.6
3	<i>A. flavus</i> + Clove extract	350	11.414	19.9
4	<i>A. flavus</i> + Kono Carpus extract	385	13.951	22.56
5	<i>A. flavus</i> + Biocide VEROX	310	11.105	21.4
6	<i>A. flavus</i> + extracts + Biocide VEROX	505	19.894	33.7
L.S.D 0.05		3.3282	0.0036	0.3305

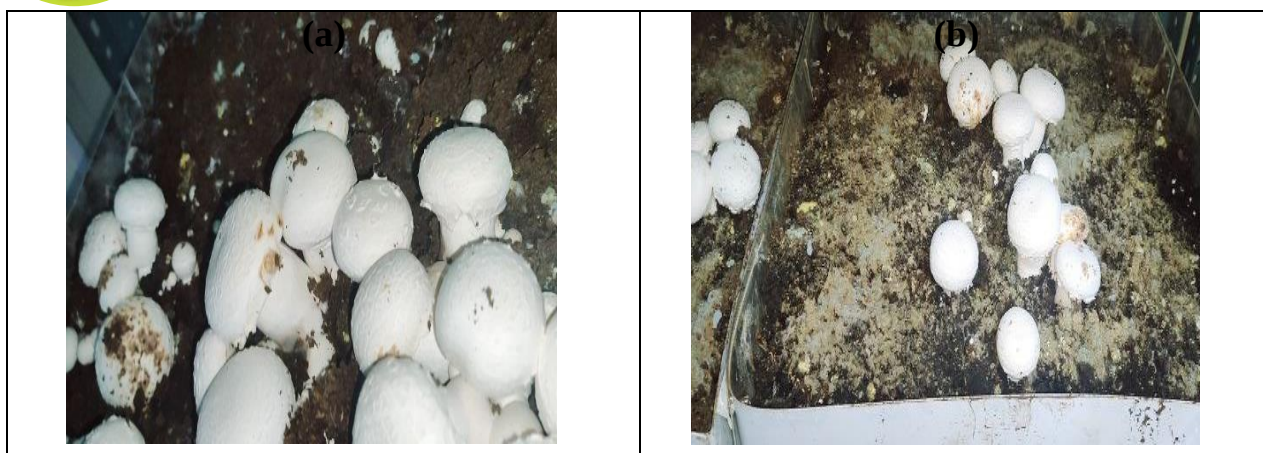


Figure (4) Productivity of the edible mushroom *A.bisporus* (a)with the treatment Pathogen + Biocidal + Extracts (b) with the presence of pathogenic *A. flavus*

Effect of plant extracts and the biocide VEROX on the phenotypic characteristics of *A. bisporus* under the conditions of infection with the pathogen *A. flavus*

The results of table (3) showed a differences between treatments, as the combination treatment of the pathogen + the mixture of the biocide + plant extracts recorded the highest rate of the fruit cap diameter of 43.1 mm and the highest average of fruiting body stalk length which was 39.1 mm, while the infected untreated control recorded the lowest average of both indicators that 28.5 mm and 23.5 mm, respectively.

Table3:Effect of plant extracts and the biocide VEROX on the phenotypic characteristics of *A. bisporus* in the presence of the pathogenic *A. flavus*

No .	Treatments	Cap diameter mm	Stalk length mm
1	Control	43.6	39.8
2	<i>A. flavus</i>	28.5	23.5
3	<i>A. flavus</i> + Clove extract	36.1	32.6
4	<i>A. flavus</i> + Kono Carpus extract	39.4	36.9
5	<i>A. flavus</i> + Biocide VEROX	37.9	33.8
6	<i>A. flavus</i> + extracts + Biocide VEROX	43.1	39.1
L.S.D 0.05		9.0795	5.1868

The variation of treatments in effect on the phenotypic characteristics of the fruiting bodies of food mushrooms may be attributed to the competition of pathogenic fungi of food fungi for nutrients and the absorption of important nutrients in the culture media.

Thus, such competition prevents the growth of edible mushrooms and affects the shape of the resulting fruiting bodies, which usually appears in the form of deformation in the fruiting body [17].

The study proved that plant extracts and biocide have an effective role in inhibiting the growth of the pathogen *A. flavus*. And reduced its effectiveness in infecting the white food fungus *A. bisporus*.

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