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Evaluation the pathogenicity and control of *Penicillium citrinum* isolates affecting the white fungus *Agaricus bisporus*

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Abstract

Five isolates of *Penicillium citrinum*were isolated from casing soils, copmpost and fruiting bodies of *Agaricusbisporus* from several culture stations in Iraq. According to the pathogenicity test, the highest percentage of inhibition was recorded for isolate *P.citrinum*(B1). The causal agent was molecularly diagnosed to the level of *P. citrinum*according to the nucleotide sequence analysis of the ITS genome region and it was recorded globally on the NCBI data base under the accission number ON738710.1.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 edable fungus *Agaricusbisporus* was studied with the presence of the pathogenic fungus. The results showed that the treatment of the combination of plant extracts + biocide + pathogenic fungus led to the highest rate of growth inhibition of the pathogenic fungus, increasing number of fruiting bodies up to 435 compared to the control treatment of pathogenic fungus 140. The total yield of the same treatment was 16.398 kg compared to 3.343 kg in the control treatment, and the biological efficiency was recorded by 37.2% compared to 10.2% in the control treatment. In general, this combination led to an increase in production and an improvement in the qualitative characteristics of the fruiting bodies, as the average diameter of the cap was 40.6 mm and the stalk length was 37.3 mm compared to 27.8 mm and 23.5 mm in the control treatment, respectively.

Keywords: *Agaricusbisporus, Penicillium citrinum* ,VEROX,

Introduction

Agaricus bisporus is one of the large edible mushrooms that are desired worldwide for their high nutritional value [1]. They are rich in many proteins and vitamins, and they also contain many minerals such as iron, phosphorous, calcium and others [2]. In addition to its medical importance as it acts as antitumor, Cancer, the effects of hypoglycemia, hypocholesterolemia and also represents a friendly state in the environment [3]. It is one of the most consumed species in the world [4]. China is the largest producer and consumer of food mushrooms in the world, while in Iraq, the first attempts to cultivate mushrooms began in 1976 in the Zafaraniya station, Later,

projects and researches related to food fungi were followed [5]. Edible mushrooms are exposed to many fungal, bacterial and viral diseases that affect their nutritional value and productivity [6]. Fungal diseases are one of the most serious disorders affecting mushroom crops, especially if hygiene standards during cultivation are not high [7]. Isolated several fungi that compete with the white edible fungus, including types of the fungus *penicillium spp*. that are found in casing soils or compost soils [8]. Infection with the pathogenic fungus was also recorded on the inoculum of the white edible *A.bisporus* on wheat grains [5] and it was found that it increases competition with the edible mushroom and caused an infestation causing green molds [9].

Several studiesIn different countries of the world have been conducted to control diseases that affect food mushrooms by several ways, of which biological methods, using several control factors, the most important of which are *Bacillus* and *Pseudomonas* bacterial strains [10].Also, several types of plant extracts were used to evaluate their efficiency in controlling fungal diseases that affect the white food mushroom such as alcoholic extracts of some desert plants to combat green mold disease in Iraq [11]. Due to the importance of diseases caused by competing organisms to edible mushrooms, especially the pathogenic fungus *penicillium spp*., and the damage and economic losses it causes in production, and its danger as it produces a group of highly toxic mycotoxins, therefor, *penicillium citrinum*. was isolated, diagnosed and studied for its effects on edible white mushroom and the possibility of controlling the pathogen using diffrent combination methods.

Materials and Methods

Isolation of *P. citrinum*

Samples of infected fruiting bodies of the white edible fungus, mulching soils, and compost were collected from several farms in different governorates of Iraq(Baghdad, Erbil, Diwaniyah, Kirkuk and Muthanna) and gave them the symbols (B1,E1,D1,K1 and M1) Straight. *P. citrinum*was isolated from mulching soils and compost by dilution method where it was reduced to 10^{-6} for all soil samples. 1 ml of the last dilution was taken and added to Petri dishes containing Potato dextrose agar medium with a slight stir to ensure homogeneous distribution and incubated at 25 ± 2 °C for 3 days[12].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 [13]. 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 hypochloriteat 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 P.D.A., and the same previous steps were performed [14].

Pathogenicity test in vitro

Pathogenicity was tested by dual culture method, as a petri dish was divided into two parts inoculated, one with the pathogenic fungus *p.citrinum* (treatment) and the other with the mushroom *A. bisporus* (control). The colony diameter was measured with a ruler and the percentage of inhibition was calculated using the folowing equation [15].

Inhibition rate %= Colony radius of the control-colont radius of a treatment/ Colony radius of the control×100

Molecular diagnosis

The molecular diagnosis of the most pathogenic fungal isolate *p.citrinium*(B1) was sent to the Korean Macrogen Company for the purpose of determining 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 that were 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 (Clove and conocarpus) was prepared with a weight of 20 g of dried plant powder and mixed with 100 ml of distilled water at 60 °C, 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 [11].

Preparation of the fungal inoculum

Rice was used as a carrier of the fungal inoculum for *P. citrinum*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-Wadaq 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 and a relative humidity of 85% [18].

The treatments

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

Extract + Conocarpus Extract.The inoculum of pathogenic *P. citrinum*was applied directly at a fertilizer rate of 100 g per replicate before covering the soil. After placing the covered soil, the plant extracts were sprayed 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 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 %= Quantity of fresh yield of the first harvest (Kg/ m^2)/ Culture medium dry weight (Kg/ m^2) × 100

Statistical analysis

The complete randomized design (CRD) was used for the experimental units distribution. Data analysis was carried out using the SAS computing program and 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 *P. citrinum* and pathogenicity test in vitro:

Five isolates of pathogenic *P. citrinum* were isolated from casing (mulching) soil and mushroom fruiting bodies. According to the pathogenicity test, the results (Figure 1) showed that the isolate B1 recorded the highest pathogenicity by resulting in the highest percentage of inhibition (58.33%), and thus it was selected for use in subsequent experiments.

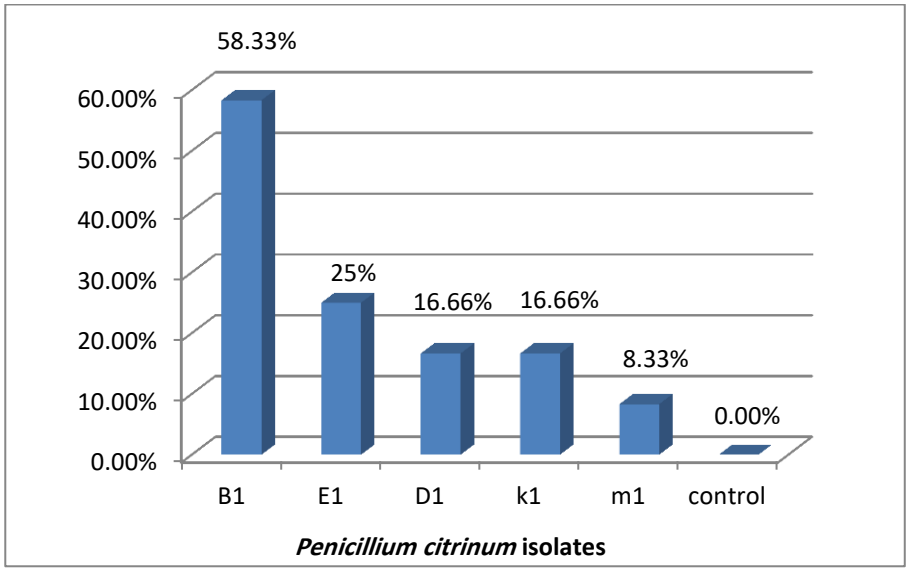

Figure (1): Pathogenicity test the percentage of *A. bisporus* growth inhibition by *P. citrinum* isolates

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 *P. citrinium* 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 the environmental conditions play a role in influencing the activity of fungal isolates [21].

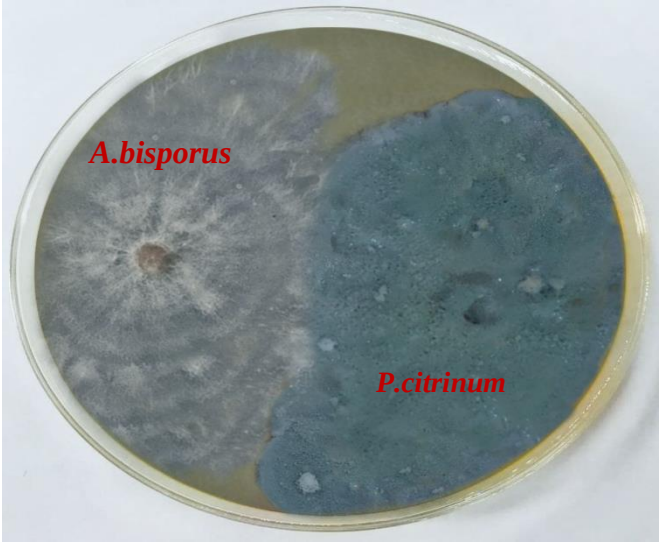

Figure (2): isolate *P. citrinum*B1 inhibitory the growth of fungal hyphae of *A.bisporus*

Molecular diagnosis

The results of the nucleotide sequence analysis of the pathogenic fungal isolate confirmed that it belongs to *P. citrinum.* The molecular nucleotide sequences achieved 100% match with the ITS genomic region when compared with the corresponding nucleotide sequences retrieved from the Genbank (Table 1). While the results of constructing the genetic tree (Figure 3) showed that this isolate appeared in the same clade, in which the Filipino isolates (MT597828.1) appeared. While, it was in separate clades far from the Chinese isolate (MT529843.1) due to the large genetic divergence between them.

Table (1): Compatibility (similarity) of the nucleotide base sequence of the fungus *P. citrinum* Y.N. 155 Haneen isolated from Baghdad province 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	*Pencilliumcitrinum*	Y.N.155 Haneen	iraq	ON738710.1	100%	11/6/2022
2	*Pencilliumcitrinum*	MEBP0017	Philippines	MT597828.1	100%	11/6/2020
3	*Pencilliumcitrinum*	DUCC5728	South Korea	MT582768.1	100%	9/6/2020
4	*Pencilliumcitrinum*	2010F2	China	MT558921.1	100%	27/5/2020
5	*Pencilliumcitrinum*	AVG35F2	Canada	MT543102.1	100%	29/5/2020
6	*Pencilliumcitrinum*	SF_699	China	MT529975.1	100%	23/5/2020
7	*Pencilliumcitrinum*	SF_590	China	MT529866.1	100%	23/5/2020
8	*Pencilliumcitrinum*	SF_567	China	MT529843.1	100%	23/5/2020
9	*Pencilliumcitrinum*	SF_540	China	MT529816.1	100%	23/5/2020
10	*Pencilliumcitrinum*	SF_210	China	MT529486.1	100%	23/5/2020

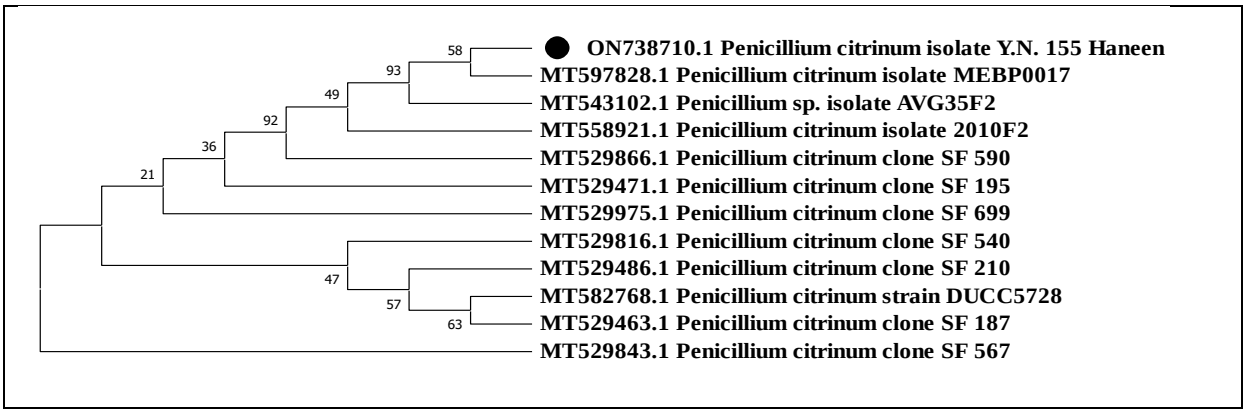

Figure (3): Genetic tree of the pathogenic fungus*P.citrinum*Y.N.155 Haneen (marked with a black dot) and global strain sequences for the same fungus obtained from the GenBank data site

The effect of plant extracts and the biocide verox on the productive traits (number of fruit bodies, yield and biological efficiency) of *A. bisporus* under conditions of infection with the pathogen *P.citrinum*

The results (Table 2) showed significant differences between the treatments, where the combination of pathogen + Biocide/compost + plant extracts, resulted in the highest number of fruiting bodies (435), while the lowest number of fruiting bodies was recorded in the treatment of pathogenic fungus only (140) fruiting bodies. As for the amount of yield and biological efficiency, the same combination recorded the highest amount of yield of 16.398 kg/m2 and biological efficiency of 37.2%, while the pathogenic fungus treatment alone recorded the lowest amount of yield of 3.343 kg/m2 and biological efficiency of 10.2%. 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 leads to stimulating the defensive resistance against pathogens. Or as a result of releasing compounds such as 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 *P. citrinum*

No.	Treatments	Total No. of fruiting body.m^2	Total yield Kg.m2	biological efficiency %
1	Control$^-$	580	23.376	40.8
2	*P. citrinum*	140	3.343	10.2
3	*P. citrinum*+ Clove extract	285	9.194	24.4
4	*P. citrinum*+ Kono Carpus extract	330	11.283	29.2
5	*P. citrinum*+ Biocide VEROX	340	11.056	30.08
6	*P. citrinum*+ extracts + Biocide VEROX	435	16.398	37.2
L.S.D 0.05		4.2556	0.0049	0.4728

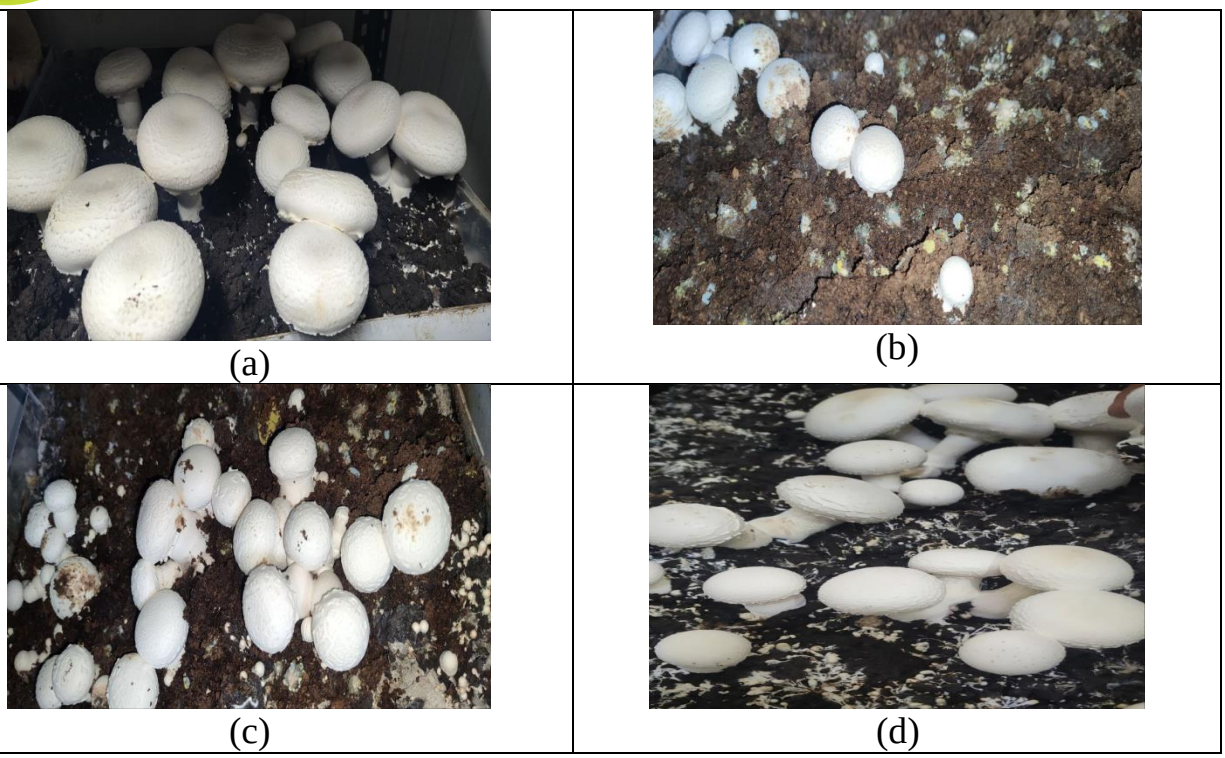

Figure (4): Productivity of the edible mushroom *A. bisporus* (a) control (b) with the presence pathogenic *P. citrinum* (c) with *P. citrinum*+ extracts + Biocide VEROX (d) with P. citrinum+ Biocide VEROX

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

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 40.6 mm and the highest average of fruiting body stalk length which was 37.3 mm, while the the infected untreated control recorded the lowest average of both indicators that 20.32 mm and 27.8 mm , respectively.

Table (3): Effect of plant extracts and the biocide VEROX on the phenotypic characteristics of *A. bisporus* in the presence of the pathogenic *P. citrinum*

No.	Treatments	Cap diameter mm	Stalk length mm
1	Control-	43.6	39.8
2	*P. citrinum*	20.32	18.3
3	*P. citrinum*+ Clove extract	32.35	27.8
4	*P. citrinum*+ Kono Carpus extrac	34.77	31.8
5	*P. citrinum*+ Biocide VEROX	36.42	32.1
6	*P. citrinum*+ extracts + Biocide VEROX	40.6	37.3
L.S.D 0.05		11.126	10.612

The variation of treatments in the 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 mushroom and affects the shape of the resulting fruiting bodies, which usually appears in the form of diformation in the fruiting body [17].

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

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