Isolation and Screening of Different Chitinolytic Mycoflora Isolated from Sudanese Soil for Biological Control of *Fusarium oxysporium*

Elham S. Dawood[1*] and Alzaki A. Mohamed[2]

[1]Associate Professor at Department of life Science Correspondence
Department of life Science, Faculty of Education
Nile Valley university, Sudan

[2]Alzaki A. Mohamed researcher (MSC), PhD student at Department of Biotechnology
Omdurman Islamic University, Sudan

[*]*Corresponding author's email: eldawood [AT] nilevalley.edu.sd*

ABSTRACT--- ***A total of 40 species belonging to 8 genera of fungi were isolated from different agricultural fields in Khartoum and River Nile States during November 2014 to May 2015. The mycoflora were isolated by using soil dilution technique and soil plate technique on Potato Dextrose Agar and Czapek's Dox Agar medium supplemented with chitin and streptomycin. The fungal isolates were screened for the production of chitinase enzyme based on the index of chitinolytic activity. Morphological identification and characterization of the best chitinase producers were carried out with the help of authentic manuals of fungi. Six isolates which recorded high index of chitinolytic activity were selected for biocontrol experiments against Fusarium oxysporium. Identifiction of the mycoflora revealed that all the isolate were belong to the genus Aspergillus (three were Aspergillus niger and the rest three were Aspergillus terreus). All the isolates showed high antagonism against Fusarium oxysporium specially Aspergillus niger isolates SUDA7 and SUDA9 which inhibit the growth completely (100%) at day10 and 12 respectively, SUD8 reduced the growth 85% at day14 . Aspergillus terreus isolates reduced the growth to 63, 85.2 and 89.9% for SUD4, SUD2 and SUD6 in order at day 14.***

Keywords--- *Aspergillus niger, Aspergillus terreus,* chitinase enzyme, biocontrol

1. INTRODUCTION

Fungi are fundamental for soil ecosystem functioning especially in forest and agricultural soils, they play a key role in many essential processes such as organic matter decomposition and elemental release by mineralization [1]. Fungi are important component of the soil microbiota. Micro fungi play a focal role in nutrient cycling by regulating soil biological activity [1]. Soil fungi are important for industry of manufacturing such as antibiotics and enzyme…..etc., they produce many enzymes such as xylanase, cellulase, amylase and chitinase…..ect. The most abundant and widely distributed microfungi in nature are filamentous mycoflora such as *Aspergillus* spp., *Penicillium and Trichoderma* which are economically, ecologically, and medically important and large genera. They are important in view of health hazards. In addition, they are used in industrial and food fermentation processes, and they exist commonly in different types of soils, indoor and outdoor air, food and water [2]. *Aspergillus and Penicillium* are ubiquitous fungi. The genus *Aspergillus* encompasses organisms whose characteristics are of high pathological, agricultural, industrial, pharmaceutical, scientific and cultural importance and play an important role in the degradation of organic substrate, particularly plant material [3]. Chitinase is enzyme to digest chitin at the obligation area ß-(1,4) glycoside of N-acetylglucosamine (GlcNAc) results as oligomor of GlcNAc. Chitin is the essential structural component of fungal and insect pathogens of vascular plants. As it is absent from the vascular plants themselves, chitin could be used as a target molecule for fungicidal and insecticidal agents [4]. The cell wall of many phytopathogens contains chitin; hence, chitinases are exploited for their use as biocontrol agent [5].

Chitinase application increases the population of chitinolytic actinomycetes, bacteria and fungi The increase is shown to be correlated with the reduction in pathogenic fungi, nematodes and insects and are important for reduction of infection and, hence, crop damage [6]. Biological control of plant diseases particularly soil borne plant pathogens by micro-organisms has been considered as environmentally acceptable alternative to the existing chemical treatment methods [7]. The potential efficacy of *Aspergillus terreus, A. niger and A.fumigatus and Trichoderma harzianum* against the pathogenic fungi was carried by many researchers, in Sudia Arabia [8]. They showed the efficacy of *Aspergillus terreus against Fusarium oxysporium, Rhiztonia, Penicillum , Aspergillus niger and Chetomium sp*.

The objectives of this study was,isolation, screening and identification of chitinolytic soil fungi. Biocontrol of pathogenic *Fusarium oxysporium* by the best chitinolytic producers.

2. MATERIALS AND METHODS

Isolation of Soil mycoflora: Dilution plate technique described by [9] which used for the isolation of fungi from various soil samples collected in agricultural crop fields at different locations in Khartoum and River Nile States. Ten grams of soil samples were suspended in 90 ml of distilled water (in Erlenmeyer glass flask), then mix by using wrist action shaker for one hour at 120 rpm. The flasks were shaken thoroughly in order to get uniform distribution of the soil. The soil suspensions were diluted in 10 fold increment from 10^{-3} to 10^{-5}. The Volume of 1 ml of soil sample suspension from each serial dilution was pipetted on to different melted, cooled culture media namely Potato Dextrose Agar (PDA) which contained ; Dextrose 20 g; agar 20 g; distilled water 1L [10], Czapek's Dox Agar(CZA) contained sucrose 30g; $NaNO_3$ 2 g; K_2HPO_4 1g; $MgSO_4$+$7H_2O$ 0.5 g; KCl 0.5 g; $FeSO_4 7H_2O$ 0.01g; agar 15 g; and distilled water 1L [11] and Sabouraud's Dextrose agar (SDA) composed of glucose 40g; peptone 10g; agar 15g; and distilled water 1L [12], supplemented with 1% streptomycin (1gram of streptomycin was mixed thoroughly in 100 ml of sterilized distilled water). The pH of the culture media was maintained at pH 5.5 being optimal for the growth and sporulation in a majority of fungi. Each culture media was prepared in a liter of distilled water and autoclaved at 120°C at 15 psi for 20 min. 1% streptomycin was used as an antibiotic for the restrain of bacterial growth.

Screening of fungal species for its chitinolytic enzyme overproducing activity

The fungal isolates were screened for overproducing chitinolytic activity, by using the method of [13], Chitinase detection medium was used for measuring the chitinase activity. The final chitinase detection medium (all amounts are per liter) 4.5 g of colloidal chitin, 0.3 gm of $MgSO_4$. $7H_2O$, 3.0 g of NH_4SO4, 2.0 g $KH2PO_4$, 1 g of citric acid monohydrate, 15 g agar, 0.15 g bromocresol purple and 200 µl of tween-80), pH was adjusted to 4.7 and the medium was autoclaved at 121°C for 15 min. Selection of the isolates based on the index of chitinolytic activity of the clear zone formed due to chitin hydrolysis. The fungal isolates producing clear zone over 0.5 cm alone were selected for further study. The index of chitinolytic activity was determined according to the following formula:-

$$\text{Index of chitinolytic activity} = \frac{\text{Average diameter of the clear zone} - \text{Average diameter of the colony}}{\text{Average diameter of the colony}}$$

Identification of Soil mycoflora:

Identification of the soil isolates were made with help of the relevant literature [14]. Fungal morphology was studied macroscopically by observing colony features (colony diameter, colour texture, and colony colour) and microscopically by staining with lacto phenol cotton blue called as mounting fluid and observed under compound microscope for the observation of the conidia, conidiophores and arrangement of spores.

In vitro evaluation of fungal antagonism:

A dual culture method was carried out using the six best chitinolytic producers (3 were *Aspergillus niger* and 3 were *A. terreus*). Each chitinolytic isolate was streaked onto PDA agar plate supplemented with 1% chitin. A number of sterilized petri dishes were poured with PDA medium , each containing three replicates. 5 mm. disc from 4 days old culture of *Fusarium oxysporium* was placed on the center of the Petri dishes and then a loop full of suspension culture of each one of the 10 tested isolates was streaked at a distance of 3 mm. from inoculum. The control petri dishes were inoculated with the *Fusarium oxysporium* only and the experiment was conducted for 14 days. Colony diameter of the pathogenic isolate was measured on the dual culture plate and the percentage of growth inhibition was calculated starting from day 6 as describe by [15].

3. RESULTS

A total of 40 fungal genera and species were isolated from twenty soil samples obtained from different locations in Khartoum and River Nile States, Sudan. The soil mycoflora of fungal isolates were screened for the production of chitinase enzyme depending on the index of chitinolytic activity by measuring the diameter of the clear zone around the fungal colony on agar chitin media. 20 isolates out of 40 gave positive results and six isolates showed high index of chintolytic activity and were selected for further studies (Table 1). The best six isolates were designates as SUDA2, SUD4, SUD6, SUD7, SUD8 and SUD9. All these isolates showed high index of chitinolytic activity range from 1.8 -3.0 cm. Morphological identification was carried out by using macroscopic and microscopic methods which revealed that all isolates were belong to the genus *Aspergillus* sp (Table 2). Macroscopic features including the colony diameter, colony texture and colony colour (upper and lower surfaces). Microscopic features of mycelium including conidial heads, conidiophores, conidia and the vesicles for the identification of isolates of *Aspergillus* mycoflora. The systematic study for characterization of *Aspergillus* species was done by using the standard manuals and relevant literature of soil fungi.

All the six isolates were cultured on Potato Dextrose Agar (PDA), Czapek,s Dox Agar(CZA) and Sabouraud's Dextrose Agar (SDA) media. The six isolates of *Aspergillus* spp. were screened *in vitro* for their efficacy in the inhibition of growth of *Fusarium oxysporium.* The results were presented in tables (3a, 3b, and plate 1). Different range of growth inhibition was noticed among the six isolates. Initially all the antagonists and pathogen grew together without showing any zone of demarcation. The diameter of the of the pathogenic fungal colony was measured and the percentage of growth reduction was calculated from day six of inoculation till day 14. isolates SUDA7 and SUDA9 inhibit the growth of *Fusarium oxysporium* 100% at day 10 and 12 respectively while isolate SUDA8 inhibit the growth 85.2% at day 14 Table 3a). Isolates SUD4, SUD2 and SUD6 reduced the colony growth at the range of 63 - 88.9% at the day 14 in order (Table 3b). Plate 1(a) showed complete disappearance of *Fusarium oxysporium* colony at the center of the petri dish and this was due to 100% growth inhibition caused by *Aspergillus niger* isolates while at plate 1(b) the colony of *Fusarium oxysporium* appeared at the center with very small diameter and the it was surrounded with the culture of *Aspergillus terreus*.

Table (1): Screening of Fungal Isolates for the Production of Chitinolytic Enzymes

Serial No.	Fungal Isolates	Source	Index of Chitinolytic Activity (in cm)
2	SUD A2	Khartoum state - Soba	2.0
4	SUDA4	Khartoum state - Kafurey	1.8
6	SUDA6	Khartoum state- Alkadaro	2.3
7	SUDA7	Khartoum state-Haatab	3.0
9	SUDA8	River Nile state- Aldamr	2.0
10	SUDA9	River Nile state -Atbara	3.0

Table (2): Macro and microscopic features of different fungal species isolated from various agricultural fields at different locations

Fungal isolates Name of species		Morphological features				Microscopic observations
		Diam (cm)	Texture	Upper surface	Lower surface	Conidiophores/ conidia/vesicles
SUDA2, SUDA4 and SUDA6	*Aspergillus terreus*	5	Velvety to floccose	cinnamon, deep brown shades	Dull brown	Conidial heads long columnar, compact with uniform diameter. Conidiophores smooth and colorless. Exudates amber coloured. Conidia globose to sub globose, smooth.
SUDA7, SUDA8 and SUDA9	*Aspergillus niger*	6	Effuse, globose	Carbon black or deep brownish black	Colour less to pale yellow	Conidial heads large and black, at first globose then radiate or splitting in well- defined columns in age. Phialides biseriate, brownish .Vesicles globose. Conidia globose, spinulose, black in colour.

Table (3a): Effect of different isolates of *Aspergillus niger* on the growth of *Fusarium oxysporium*

Aspergillus niger isolates	Day	*Fusarium oxysporium* diameter length in single culture. (Control) cm	*Fusarium oxysporium* Diameter length in dual culture (cm.)	Reduction percentage of fungal diameter (%)
SUDA7	4	6	2.7	
	6	8.2	1.1	59.3
	8	9.2	0.6	77.8
	10	8.7	Zero	100
	12	9	zero	100
	14	9	Zero	100
SUDA8	4	6	2.7	
	6	8.2	1.7	37.1
	8	9.2	1.0	59.3
	10	8.7	0.9	66.7
	12	9	0.6	77.8
	14	9	0.4	85.2
SUDA9	4	6	2.3	
	6	8.2	2.1	9
	8	9.2	1.5	17.4
	10	8.7	0.5	34.8
	12	9	zero	100
	14	9	zero	100

Table (3b): Effect of different isolates of *Aspergillus terreus* on the growth of *Fusarium oxysporium*

Aspergillus terreus isolates	Day	*Fusarium oxysporium* diameter length in single culture.(Control) cm	*Fusarium oxysporium* Diameter length in dual culture (cm.)	Reduction percentage of fungal diameter (%)
SUDA2	4	7.6	2.7	
	6	8.0	1.5	44.4
	8	6	1.1	59.3
	10	7.2	0.8	70.4
	12	8.2	0.6	77.8
	14	9.2	0.4	85.2
SUDA4	4	7.6	2.7	
	6	8	2.3	14.8
	8	8.7	2.0	25.9
	10	9	1.7	37
	12	9	1.4	48.1
	14	9	1	63
SUDA6	4	4.5	2.7	
	6	4.7	2.1	29.6
	8	6.3	1.5	44.4
	10	8	0.8	70.4
	12	8.5	0.6	77.8
	14	9	0.3	88.9

A b

Plate 1: Antagonistic effect of different species of *Aspergillus* spp. on the growth of *Fusarium oxysporium*

A: dual culture B: control a: *Aspergillus niger* b: *A. terreus*

4. DISCUSSION

In this study isolation and screening of soil chitnolytic fungal isolates was carried out at different locations in Khartoum and River Nile states in Sudan. Selection of the best chitinase production was done by measuring the diameter of the clear zone appeared around the fungal colony on agar chitin media. All the selected isolates showed very clear zone around the growth. Appearance of this clear zone indicated their high ability to degrade and to utilize the crude chitin which reflected a strong chitinolytic activity. Regarding this, eight chitinolytic fungi (*Trichoderma viride*, *Chaetomium*, *Aspergillus fumigatus* ,*Cephalosporium* and three species of *Fusarium*) were isolated from agriculture soil in Al-Madinah Al-Munawarah, Saudi Arabia [16]. Chitinolytic *Apergillus terreus* was isolated from polluted soil at the market

of fishery, fish sale and preparation in Jeddah, Saudi Arabia [17]. On the other hand, two fungal species, *Alternaria alternate* and *Aspergillus flavus* were isolated from Egyptian black sand on plates containing crude shrimp shell chitin and were recorded as chitinolytic fungi [18]. Morphological identification was carried out by using macroscopic and microscopic methods which revealed that all the isolates belonged to the genus, *Aspergillus* and according to their characterization and morphological properties three were *Aspergillus niger*, and the rest three were *Aspergillus terreus* as described by Gaddeyya *et.al* [1]. Potential efficacy of of the tested isolates of *Aspergillus niger* and *Aspergillus terreus* against *Fusarium oxysporium* showed strong growth inhibition by all isolates except isolate SUDA4 which revealed moderate growth inhibition (63%). Similar results was obtained by many researchers, in Sudia Arabia [8] showed the efficacy of *Aspergillus terreus* against many pathogenic fungi such as, *Fusarium oxysporium, Rhiztonia, Penicillum , Aspergillus niger and Chetomium sp*. In Poland Maria, and Urszula [19] isolated *Aspergillus niger* LOCK 62 which produced an antifungal chitinase, inhibited the growth of many fungal phytopathogens such as *Fusarium culmorum*, *Fusarium solani* and *Rhizoctonia solani*.

5. CONCLUSIONS

It could be concluded that Sudanese soil is very fertile and is considered a good source of active mycoflora which can produce beneficial enzymes. The high index of chitinolytic enzymes produced by these tested fungal isolates is an indication of their efficacy as abiocontrol agents against pathogenic fungi.

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