Antagonistic Potentiality of Soil Chitinolytic *Aspergillus* Isolates and *Trichoderma harzianum* Against *Fusarium solani*

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ABSTRACT--- ***Chitinolytic enzyme producing by Trichoderma spp. have long been recognized as an agent for controlling plant diseases caused by various phytopathogenic fungi. Chitin is the main structural component of fungi. Chitinase is an enzyme responsible to metabolize the chitin. Primary screening of chitinase producing fungi is essential to obtain an efficient and novel biocontrol agent. In this study, A total of 20 species belonging to 4 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 depending 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. Four isolates showed high index of chitinolytic activity and three of them were belong to the genus Aspergillus. (one was Aspergillus fumigatus and two were Aspergillus awamori) and the last isolate was identified as Trichoderma harzianum. The 4 isolates were selected for biocontrol experiments against Fusarium oxysporium. Antagonism of T. harzianum and the three Aspergillus isolates were observed in vitro by using the dual culture techniques. All isolates showed high antagonism against Fusarium solani specially the isolates of Aspergillus awamori SUDA1,SUDA3 and Trichoderma harzianum SUDT which inhibit the growth 100% at day 14 and 12 respectively, while Aspergillus fumigates SUD5 reduced the growth to78.3% at day 14.***

Keywords--- *Trichoderma harzianum*, *Aspergillus* spp., chitinase enzyme, biocontrol

1. INTRODUCTION

Soil is highly complex system, with many components playing diverse functions mainly due to the activity of soil organisms [1]. Soil microflora plays a pivotal role in evaluation of soil conditions and in stimulating plant growth [2]. Microorganisms are beneficial in increasing the soil fertility and plant growth as they are involved in several biochemical transformation and mineralization activities. The most abundant and widely distributed micro-fungi in nature *are* filamentous mycoflora such as *Aspergillus* spp., *Penicillium and Trichoderma* which are economically, ecologically, and medically important and are 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 [3]. *Aspergillus and Penicillium* are ubiquitous fungi. *Trichoderma* is a prevalent filamentous imperfect fungi (Deutromycetes), found more or less in every soil. The distribution of genus *Trichoderma* is worldwide due to the high degree of ecological adaptability and survival shown by its strains under varied environmental conditions and substrates. Several *Trichoderma* species reduce the incidence of soil borne plant pathogenic fungi under natural conditions [4]; nevertheless the effectiveness of this depends mainly on the physical, chemical and biological conditions of the soil. 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 [5].

Efficient bio-control strains of the genera *Trichoderma* and *Aspergillus* are being developed as promising biological fungicides and their weaponry for this function also includes secondary metabolites with potential applications as novel antibiotics [6]. They are well known producer of chitinolytic enzymes and used commercially as a source of these proteins. Additional interest in these enzymes is stimulated by the fact that chitinolytic strains of *Trichoderma* are among the most effective agents of biological control of plant diseases [7] and [8]. Chitinases are chitin-degrading enzymes that

hydrolyze the β -1, 4- glycosidic bonds between the N-acetyl glucosamine residues of chitin and are widely distributed in nature [9].

The objectives of this study was isolation and screening of soil chitinolytic mycoflora. Selection the best chitinase producers for potential biocontrol of *Fusarium oxysporium.*

2. MATERIALS AND METHODS

Isolation of Soil mycoflora: Dilution plate technique described by [10] was 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}. One ml of soil sample suspension from each serial dilution was added on to different melted, cooled media namely Potato Dextrose Agar (PDA) which contained ; dextrose 20 g; agar 20 g; distilled water 1L [11], 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 [12] and Sabouraud's Dextrose Agar (SDA) composed of glucose 40g; peptone 10g; agar 15g; and distilled water 1L [13], 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 120ºC at 15 psi for 20 min. 1% Streptomycin was used as an antibiotic for the restrain of bacterial growth.

Screening of Fungal Isolates for its chitinolytic enzyme overproducing activity

The fungal isolates were screened for chitinolytic activity, by using the method of [14], using 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 then autoclaved at 121°C for 15 min. Selection of the isolates depend on the index of chitinolytic activity of the clear zone formed due to chitin hydrolysis. The fungal isolates producing clear zone over 1.0 cm alone were selected for further study. The index of chitinollytic activity was determined according to the following formula:-

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

Identification of Soil mycoflora: Identification of the soil isolates were made with help of the relevant literature [15]. Fungal morphology was studied macroscopically which included 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. All the four isolates were cultured on Potato Dextrose Agar (PDA), Czapek's Dox Agar(CZA) and Sabouraud's Dextrose Agar (SDA).

In vitro evaluation of fungal antagonism:

A dual culture method was carried out using the best four chitinolytic producers (two were *Aspergillus awamori*, one was *A. fumigates*, and the last one was *Trichoderma harizanum*.) 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 3 replicates. 5 mm. disc from 4 days old culture of *Fusarium solani* was placed on the center of the Petri dishes and then a loop full of suspension culture of each one of the four tested isolates was streaked at a distance of 3 mm. from inoculum. The control Petri dishes were inoculated with the *Fusarium solani* only and the experiment was conducted for 14 days. Colony diameter of the pathogenic isolate was measured on both control and dual culture plates and the percentage of growth inhibition was calculated starting from day 6 as describe by [16].

3. RESULTS

A total of 20 fungal genera and species were isolated from different soil samples obtained from five locations in Khartoum and River Nile States. The isolated mycoflora were screened for the production of chitinase enzyme depending on the index of chitinolytic activity which was carried out by measuring the diameter of the clear zone on agar chitin media. 10 isolates out of 20 showed positive results and four isolates recorded high index of chitinolytic activity, ranged from 1.5 -2.5 cm and were designates as SUDA1, SUDA3, SUDA5 and SUDT (Table 1). The best four isolates were selected for further investigation and were identified morphologically by using macroscopic and microscopic methods which revealed that all the isolates belonged to two genera, *Trichoderma* and *Aspergillus* spp.(Table 2).

Antifungal activity of the four isolates (*Aspergillus* SUDA1 and SUDA3, *A.* SUDA5 and *Trichoderma* SUDT) were tested against *Fusarium solani*, (Table 3a, 3b and plate1). The diameter of the pathogenic fungal colony was measured, starting from day six of inoculation till day 14 and the percentage of growth reduction was calculated. It was clear that the growth of *Fusarium solani* was inhibited completely (100%) by *Trichoderma* SUDT, *and isolates of Aspergillus* SUD1 and *A.*SUD3 at day 12 and 14 respectively, while the isolate of *Aspergillus* SUDA5 reduced the growth up to 78.3% at day 14. Plate 1(a) and 1(b) showed complete disappearance of *Fusarium solani* colony at the center of the petri dish and this was due to 100% of growth inhibition caused by *Aspergillus* isolates and *Trichoderma*.

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

Serial no.	Fungal Isolates	Source	Index of Chitinolytic Activity (in cm.)
1	SUD A1	Khartoum state - Soba	2.5
3	SUDA3	Khartoum state - Kafurey	2.2
5	SUDA5	Khartoum state- Alkadaro	1.5
8	SUDT	River Nile state- Hudiba	2.4

Table (2): Macro and microscopic features of different fungal genera and species isolated from various agricultural fields at different locations in Khartoum and River Nile States

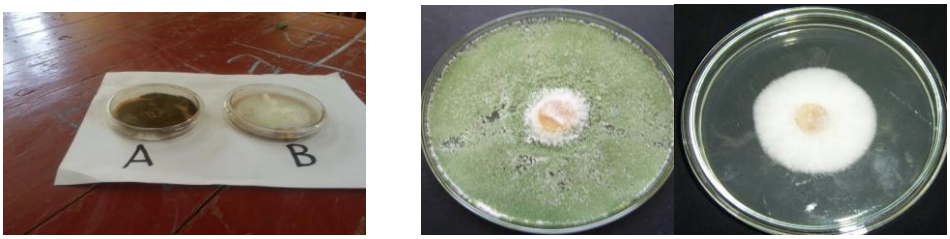
Fungal isolates	Name of species	Morphological features				Microscopic observations
		Diam (cm)	Texture	Upper surface	Lower surface	Conidiophores/ conidia/vesicles
SUDA1 and SUDA3	*Aspergillus awamori*	5	Radially furrowed,tough	brown to dark chocolate	reddish brown	Conidial heads globose loosely radiate. Conidiophores coloured in terminal areas.Phialides biseriate. Conidia globose. Vesicles globose, light brown in colour
SUDA5	*Aspergillus fumigatus*	6	Velvety to floccose	Dull blue green	colourless	Conidial heads columnar, compact, often densely crowded. Conidiophores short, smooth, light green. Phialides uniseriate. Conidia globose .
SUDT	*Trichoderma harzianum*	9.5	Velvety	Floccose, compact, whitish green-bright green.	Colorless to drab color	Conidia Moderate, compact, yellow- pale green . Smooth, subglobose. Conidiophores Much branched, form loose tufts which arise in ring like zone .

Table (3 a): Effect of different isolates of *Aspergillus awamori* and *A.fumigatus* on the growth of *Fusarium solani*

***Aspergillus awamori and A. fumigates* isolates**	**Day**	***Fusarium solani* colony diameter length in single culture.(control) cm.**	***Fusarium solani* colony diameter length in dual culture (cm.)**	**Reduction percentage of colony diameter (%)**
SUDA1	**4**	**7.6**	**2.7**	
	6	**8**	**2.3**	**14.8**
	8	**8.7**	**2**	**25.9**
	10	**9**	**1.2**	**55.6**
	12	**9**	**0.5**	**81.5**
	14	**9**	**Zero**	**100**
SUDA3	**4**	**7.6**	**2.7**	
	6	**8**	**1.5**	**44.4**
	8	**8.7**	**1.1**	**59.3**
	10	**9**	**0.8**	**70.4**
	12	**9**	**0.4**	**85.2**
	14	**9**	**Zero**	**100**
SUDA5	**4**	**7.6**	**2.3**	
	6	**8**	**2.1**	**9**
	8	**8.7**	**1.9**	**17.4**
	10	**9**	**1.5**	**34.8**
	12	**9**	**1.2**	**47.8**
	14	**9**	**0.5**	**78.3**

Table (3b): Effect of *Trichoderma harzianum* on the growth of *Fusarium oxysporium*

Trichoderma harzianum isolate	Day	*Fusarium solani* colony diameter length in single culture. (Control) cm	*Fusarium solani* colony diameter length in dual culture (cm.)	Reduction percentage of colony diameter (%)
SUDT	4	7.6	2.7	
	6	8	1.1	59.3
	8	8.7	0.8	70.4
	10	9	0.4	85.2
	12	9	Zero	100
	14	9	zero	100



1(a) 1(b)

Plate 1: Antagonistic effect of different species of *Aspergillus* and *Trichoderma harzinaum* on the growth of *Fusarium solani*

1 (a) A: dual culture of *Aspergillus awamori* and *Fusarium solani* B: control single culture of *Fusarium solani*.

1(b): A. dual culture of *Trichoderma harzianum* and *Fusarium solani* B. control single culture of *Fusarium solani*.

4. DISCUSSION

In this study survey and screening of chitinolytic soil fungi were carried out at Khartoum and River Nile states. Strong chitinolytic activity was detected by the appearance of a large clear zone around the fungal isolate on the agar chitin plates and this indicated their high ability to degrade and to utilize the crude chitin. Four isolates recorded high index of chitinolytic activity range from 1.5 -2.5 cm. were selected for further investigation, 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 [17]**.** Morphological characterizations of the selected isolates revealed that two isolates were *Aspergillus awamori*, one was *A. fumigatus* and the fourth isolate was *Trichoderma harzianum* as described by [18] and [19] for *Aspergillus* spp. and [19] for *Trichoderma* sp. respectively**.** Biocontrol results showed high efficacy of *Trichoderma harzianum* SUDT and *Aspergillus awamori* SUD1 and SUD3 which inhibited the growth of *Fusarium solani* completely (100%) at day 12 and 14 respectively, while the isolate of *Aspergillus fumigatus* SUDA5 reduced the growth up to 78.3% at day 14**.** Similar results were obtained by many researchers such as [20], who found positive potential efficacy of *Aspergillus awamori*, *A. fumigatus and Trichoderma harzianum* against many pathogenic fungi. Also in India Deepak [21] recorded positive antagonistic effect of several fungal genera and species such as *Trichoderma viride, Trichoderma harzianum, Aspergillus niger, Aspergillus fumigates and Aspergillus flavus* on the growth of two cumin fungal pathogens under *in vitro* and field conditions**.**

5. CONCLUSIONS

The high index of chitinolytic enzyme activity produced by isolates of *Trichoderma harzianum* and *Aspergillus* spp. prooved that these mycoflora were good and rich sources of chitinase enzyme. Biocontrol results showed the efficacy of these fungal isolates as biocontrol agents against pathogenic fungi.

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