Image analysis method to evaluate biological control of *Aspergillus flavus* growth by *Bacillus subtilis* and *Pseudomonas fluorescens*

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Abstract

This study was aimed to evaluate the ability of some biocontrol agents to suppress the growth of the *Aspergillus flavus* biologically. Five isolates of *Bacillus subtilis* and two of *Pseudomonas fluorescens* were examined to evaluate their ability to inhibit fungal growth *in vitro* on solid media. Growth inhibition estimated according to growth area measurement which made by analysis of images that picked up by digital camera and analyzed by UTHSCSA ImageTool, v. 2.0, software that developed by the University of Texas Health Science Center, San Antonio. Results of growth inhibition percentages showed superiority of *B. subtilis* isolate BSS4 (99.47%) and *P. fluorescens* isolates PFDL (92.29%) & PFMst (86.19%) respectively with no significant differences among them followed by *B. subtilis* isolates BSS2 (74.47%) & BSW (72.67%) which differed significantly from BSS4.

Introduction

Aspergillus flavus is a worldwide distributed fungus, its conidia could be found in air, soil and water with high ability to contaminate food, crops and feedstuff in the field and store due to their ability to grow at water activity (a_w 0.86-0.96) with a temperature ranged 12-48ºC and optimal growth at 37º C (Vujanovic *et al.*, 2001). Among other species of Aspergilli, *A. flavus* recorded to cause a broad spectrum of human diseases includes hypersensitivity reactions, human tissue infections which involved the skin, oral mucosa, or subcutaneous tissues in addition to producing Aflatoxins, specially AFB1 which is the most toxic and potent hepatocarcinogenic natural compound ever characterized (Hedayati *et. al.* , 2007).

Biological control gained a great attention during the last few years according to its environmentally friendly aspects compared with chemicals used for the same purpose (Kim, 2005; Gao *et. al.*, 2011). Bacteria were one of the potential agents that employed in this field as biocontrol agents which included the organism themselves or their metabolites. Generally, previous literature focused largely on *Bacillus subtilis* and *Pseudomonas fluorescens* as antifungal and mycotoxin degrading agents significantly affect *A. flavus* growth and aflatoxins production (Palumbo *et al.*, 2010; Mushtaq *et al.*, 2010 ; Goa *et al.*, 2011; Salem *et al.* 2012).

Growth inhibition in filamentous fungi usually estimated by the measurement of radial growth rates of mycelium in solid medium (Moyne *et al.*, 2001; Amzad *et al.*, 2008; Kong *et al.*, 2010; Kumar *et al.*, 2011). The huge technical development of digital photography interdependent with image processing and analysis computer software has enabled researchers to estimate biological parameters with high accuracy (Sainis *et al*, 1998). Growth area is one of the important and accurate parameters that employed to estimate growth inhibition (Kim, 2005; Renato *et al.*, 2006; Djonović *et al.*, 2006).

The objective of our study was to estimate the ability of *B. subtilis* and *P. fluorescens* to inhibit mycelial growth of *A. flavus in vitro* by using digital image analysis by measuring the mycelium growth area.

Materials and Methods

Microorganisms: *Aspergillus flavus* AFL14 was isolated from soil by dilution method and purified on MEA then preliminary identified morphologically according to Klich (2002) and the identification was confirmed molecularly according to Rodriguez *et al.* (2012) using Internal transcript spacer ITS1-ITS2:

(For: 5'TCCGTAGGTGAACCTGCGG3',

Rev:5' GCTGCGTTCTTCATCGATGC 3')

and ITS3-ITS4 region (For: 5'GCATCGATGAAGAACGCAGC3',

Rev: 5'TCCTCCGCTTATTGATATGC3') (Bellemain *et al.*, 2010).

B. subtilis isolates BSS1, BSS2, BSS3 and BSS4 obtained as a gift from Dr. Sami Al-Jumaily, Karbala Univ., College of Applied Medical Sciences; BSW was obtained as a gift from Dr. Wael Al-Waely, Basrah Univ., College of Agriculture Where *P. fluorescens* PFMst obtained from Dr. Mohammed Amir ; PFDL obtained from Dr. Dehya Al-Waely , Basrah Univ., College of Agriculture, Plant Protection Dept. ; All Bacterial isolates were confirmed biochemically (Henayl, 2000; Barrow and Filtham, 2003; Forbes *et al.*, 2007).

Antagonism treatments

B. subtilis isolates BSS1, BSS2, BSS3, BSS4 and BSW and *P. fluorescens* isolates PFMst and PFDL were activated on nutrient broth for 24h then 1ml (1 × 10^6 CFU) of each isolate mixed with 20 ml of PDA in 8.5 cm Petri plates, the cultures were incubated for 24 h at 37ºC after that a 0.5 mm plugs of *A. flavus* one week old culture grown on PDA used to inoculate Petri plates, the plates were incubated at 35ºC till the control treatment fill the plate. The experiment (Table 1) was carried in triplicate.

Table (1): antagonism treatments

Treatment	Description
T1	*B. subtilis* BSS1+ *A. flavus* AFL14
T2	*B. subtilis* BSS2+ *A. flavus* AFL14
T3	*B. subtilis* BSS3+ *A. flavus* AFL14
T4	*B. subtilis* BSS4+ *A. flavus* AFL14
T5	*B. subtilis* BSW+ *A. flavus* AFL14
T6	*P. fluorescens* PFMst + *A. flavus* AFL14
T7	*P. fluorescens* PFDL + *A. flavus* AFL14
Control	*A.flavus only*

Growth inhibition measurement

Plates were photographed using digital camera CASIO EXILIM EX-Z80 in JPEG format and the area of fungal growth on solid medium was measured using free software developed by the University of Texas Health Science Center, San Antonio called UTHSCSA ImageTool, v. 2.0. where the growth zone could be easily selected by selection tool (figure 2) then the area calculated automatically by the program through setting the measurement units according to known virtual length in the image like Petri dish diameter or any known length object in the image (Barguil *et al.*, 2005; Renato *et al.*, 2006; Campos *et al.*, 2008; Dušica *et al.*, 2012). The inhibition percentage was calculated according to the Abbott formula (Abbott, 1925) :

$$Inhibition = \frac{A1 - A2}{A1} \times 100$$

Where A1: Growth area in control treatment.

A2: Growth area in experimental treatments.

Statistical Design

The experiment was carried out using the completely randomized design and the data was analyzed using SPSS® ver. 16.0.

Results and discussion

Microorganisms: morphological characteristics results of *A. flavus* was matched the key traits of Klich (2002) and confirmed molecularly according to the sequence alignment of ITS1-ITS2 and ITS3-ITS4 regions with the standard strains in National Center of Biotechnology Information (NCBI).

Table (2): Morphological and molecular identification of *A.flavus* AFL14.

Isolate	Source	Morphological Identification	Molecular Identification			
			Final result	DNA region	Aligned reference strain	Range of alignment
AFL14	Soil	+	+	ITS1-ITS2	CBS 100558 ID:gb|KJ175473.1|	63-253
				ITS3-ITS4	M1204.653 ID:gb|KJ175474.1|	300-482

The biochemical test results of *B. subtilis* and *P. fluorescens* isolates presented in tables (3 and 4) respectively showed a consistent with the same test results in (Henayl, 2000; Barrow and Filtham, 2003; Forbes *et al*., 2007) which confirms the previous definition which was conducted in advance by the source donors.

Table (3): Biochemical confirmation tests of *B. subtilis*

Isolate	Gram stain	Shape	Spores	Oxidase	Catalase	Gelatine	IMVC				NaCl 6.5%	Amelase
							Indol	MR	VP	Citrate		
BSS1	+	+	+	+	+	+	-	+	+	+	+	+
BSS2	+	+	+	+	+	+	-	+	+	+	+	+
BSS3	+	+	+	+	+	+	-	+	+	+	+	+
BSS4	+	+	+	+	+	+	-	+	+	+	+	+
BSW	+	+	+	+	+	+	-	+	+	+	+	+

Table (4): Biochemical confirmation tests of *P. fluorescens*

Isolate	fluoresces under UV	Gram stain	shape	Oxidase	Catalase	Gelatine	IMVC			
							Indol	MR	VP	Citrate
PFMst	+	-	+	+	+	+	-	-	-	+
PFDL	+	-	+	+	+	+	-	-	-	+

Antagonism: Figures (1,2) illustrate the disparity in the ability of the *B. subtilis* and *P. fluorescens* isolates on the inhibition of the fungus growth represented by outweigh of *B. subtilis* BSS4 (99%) significantly of the rest of *B. subtilis isolates* followed by *P. fluorescens* isolates PFDL and PFMst (92.29% , 86.19%) respectively with no significant differences among them. The inhibition percentages of the rest of *B. subtilis* isolates BSS2, BSW, BSS1 and BSS3 were (74.47%, 72.65%, 67.16% and 59.91) with no significant differences among them. The results of *B. subtilis* ability to inhibit *A. flavus* growth matched what found by Klich *et al.* (1991), Kimura and Hirano (1988), Souto *et al.* (2004) and Ruiyu *et al.* (2012*)* who referred to the ability of *B. subtilis* to produce peptidolipid compounds, bacillomycin, protease, iturin A which had a high antifungal activity and other compounds that were reported to cause growth inhibition to *A.flavus* and other fungi. The disparity of antagonistic ability against *A. flavus* among isolates seems to be influenced by genetic factors that control the production of antifungal compounds as each gene could be induced by nutritional and environmental factors (Raaijmakers *et al*., 2002, Olteanu *et al*., 2011; Islam *et al*., 2012).

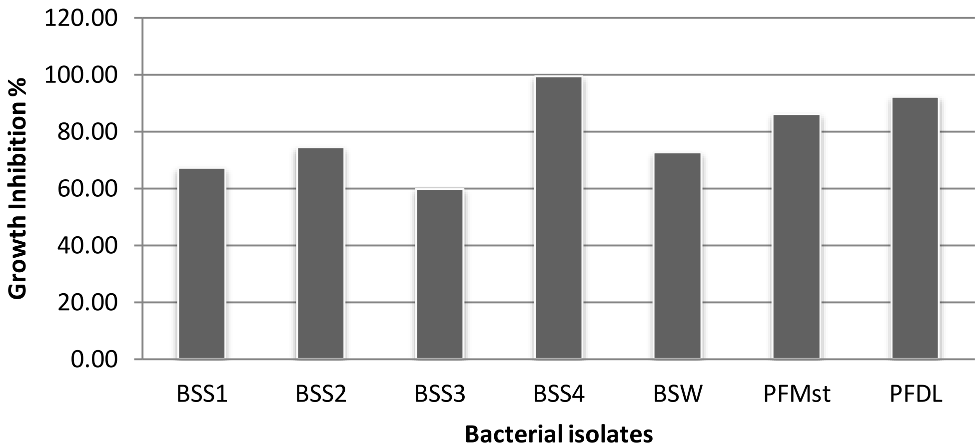

Figure (1): Growth inhibition of *A.flavus* AFL14 by *B.subtilis* and *P.fluorescens* LSD= 23.26 at P≤ 0.05.

A previous studies by Mushtaq *et al.* (2010) and Baig *et al.* (2012) exhibited growth inhibition activities against *A. flavus* by *P. fluorescens* that was compatible with the results of recent study. Generally, growth inhibition of *A. flavus*, showed in a recent study that it could be returned to several kinds of metabolites recorded to be produced by *P.fluorescens* had antifungal activity included 2, 4-diacetylphloroglucinol (DAPG), phenazine (Phz), pyrrolnitrin, oomycin A, viscosinamide, pyoluteorin and hydrogen cyanide (HCN), the metabolite production could exhibit variation from strain to strain (Raaijmakers *et al.*, 2002; Sageera *et al.*, 2012).

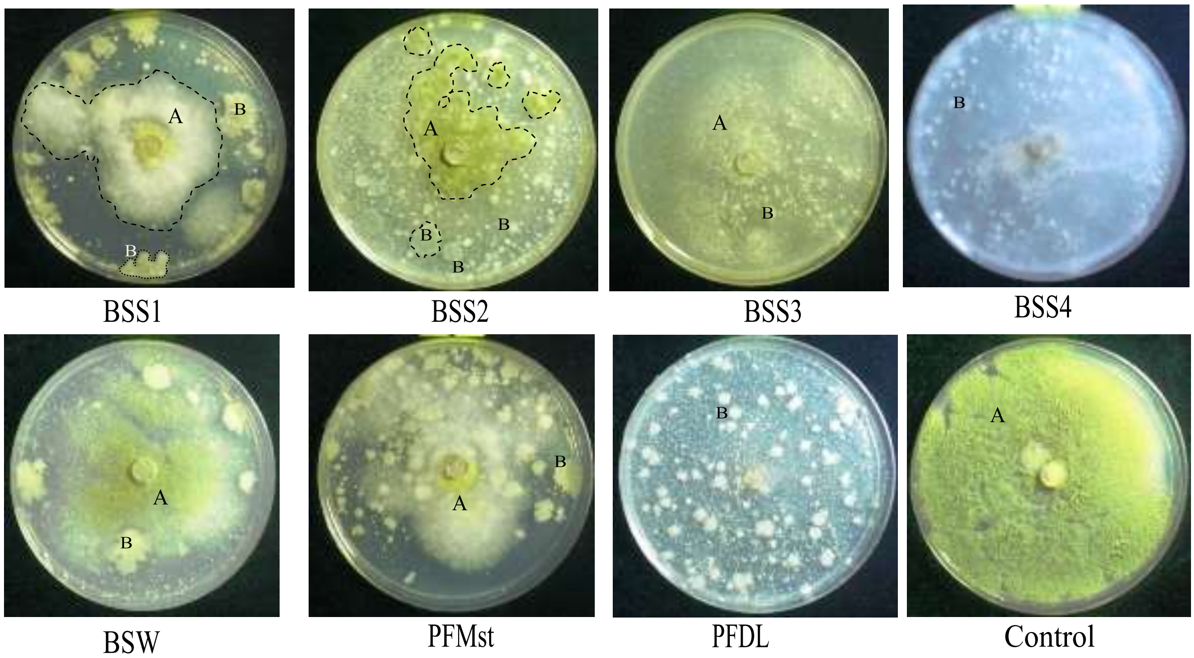

Figure (2): The antagonism results: *Bacillus subtilis* isolates (BSS1-BSS4 and BSW). *Pseudomonas fluorescens* isolates (PFMst, PFDL). A: *Aspergillus flavus* growth area; B: Bacterial growth area. The dotted lines show a sample of selected areas as it made by UTHSCSA ImageTool, v. 2.0 software.

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طريقة تحليل الصور الرقمية لتقييم المقاومة الحيوية للفطر *Aspergillus flavus* باستخدام عزلات من البكتريا *Bacillus subtilis* والبكتريا *Pseudomonas fluorescens*

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هدفت هذه الدراسة إلى تقييم قابلية بعض العوامل الأحيائية على كبح نمو الفطر *Aspergillu flavus* . تم اختبار قابلية خمسة عزلات من البكتريا *Bacillus subtilis* وعزلتين من البكتريا *Pseudomonas fluorescens* على تثبيط النمو الفطري على الوسط الصلب. قدرت معدلات تثبيط النمو اعتمادا على حساب مساحة النمو للغزل الفطري على الوسط الصلب باستخدام برنامج تحليل الصور الرقمية UTHSCSA ImageTool, v. 2.0 الذي طوره مركز علوم الصحة في جامعة تكساس . أظهرت النتائج تفوق عزلة البكتريا *B. subtili* BSS4 التي أعطت أعلى نسبة تثبيط بلغت (99.47%) و العزلة *P. fluorescens* PFDL (92.29%) والعزلة *P. fluorescence* PFMst (86.19%) على التوالي مع عدم وجود فروقات معنوية بينهم تلتها العزلات *B. subtilis* BSS2 (74.47%) و *B. subtilis* BSW (72.67%) والتي اختلفت معنويا عن العزلة *B. subtili* BSS4.