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

Labeed A. Al-Saad [1], Adnan I. Al-Badran [1], Sami A. Al-Jumaily [2]

1. Dept. of Biology, College of sciences, University of Basrah.
2. College of Applied Medical Sciences,University of Karbala.

Email: la_alsaad@yahoo.com

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).

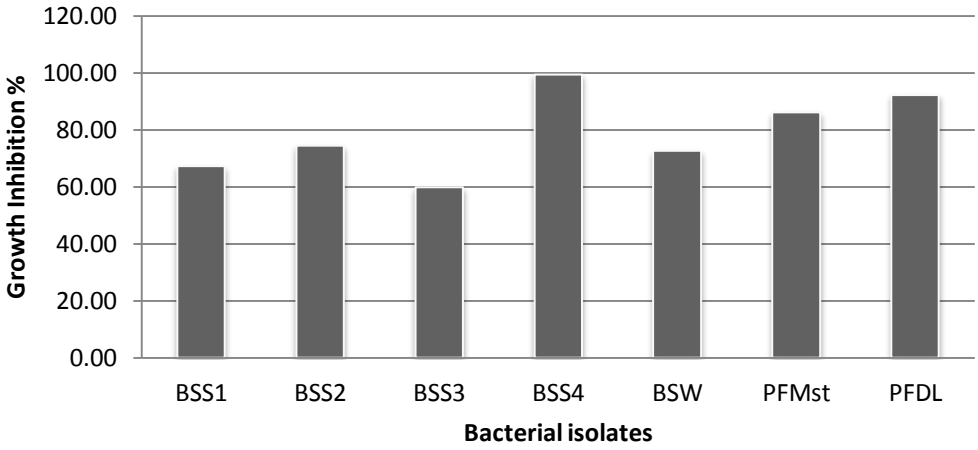

Figure (1): Growth inhibition of *A.flavus* AFL14 by *B.subtilis* and *P.fluorescens* LSD= 23.26 at P≤ 0.05.

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).

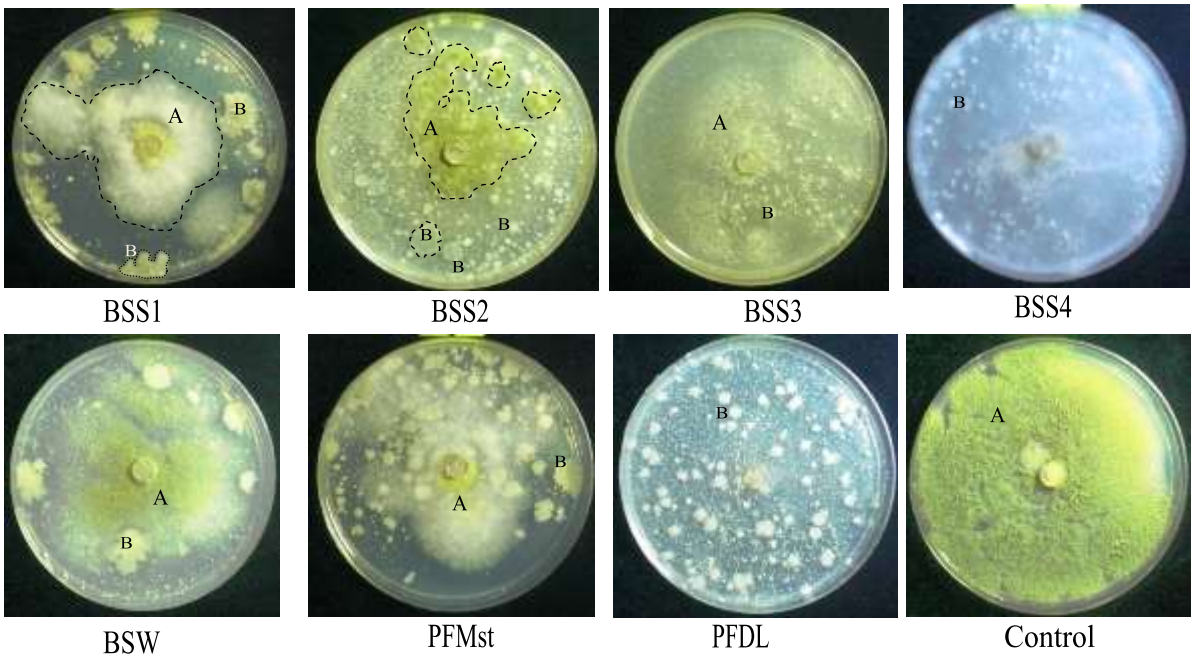

Figure (2): The antagonism results: *Bacillus subtilis* isolates (BSS1-BSS4 and BSW). *Pseudomonas fluorescens* isolates (PFMst, PFDL). A: *Aspergillus flavus* growth area; B: Bacterial growth area. The dotted lines show a sample of selected areas as it made by UTHSCSA ImageTool, v. 2.0 software.

References

Abbott WS (1925). A method of computing the effectiveness of an insecticide. Journal of Economic Entomology. 18: 265-267. In: Seye F, Faye O, Ndiaye M, Njie E, Afoutou JM (2009). Pathogenicity of the fungus, *Aspergillus clavatus*, isolated from the locust, *Oedaleus senegalensis*, against larvae of the mosquitoes *Aedes aegypti*, *Anopheles gambiae* and *Culex quinquefasciatus*. Journal of Insect Science. 9: 53.

Amzad HM, Ismail Z, Rahman A, Kang SC (2008). Chemical composition and anti-fungal properties of the essential oils and crude extracts of *Orthosiphon stamineus* Benth. Industrial Crops and Products. 2 7: 328–334.

Baig M, Fatima S, Kadam VB, Shaikh Y (2012). Utilization of antagonist against seed borne fungi. DAMA International. 1 (1): 42-46.

Barguil, BM, Resende MLV, Resende RS, Beserra JEA, Salgado SML (2005). Effect of extracts from citric biomass, rusted coffee leaves and coffee berry husks on *Phoma costarricencis* of coffee plants. Fitopatologia Brasileira. 30:535-537.

Barrow GI, Feltham RKA (2003). Cowan and Steel's Manual for the identification of medical bacteria. Cambridge University Press, The Edinburgh Building, Cambridge CB2 2RU, UK. pp.331

Bellemain E, Carlsen T, Brochmann C, Coissac E, Taberlet P, Kauserud H (2010). ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. BMC Microbiology: 10-189.

Campos MA, Silva MS, Magalhães CP, Ribeiro SG, Sarto RPD, Vieira EA, de Sá MFG (2008). Expression in *Escherichia coli*, purification,

refolding and antifungal activity of an osmotin from *Solanum nigrum*. Microbial Cell Factories. 7:7 doi:10.1186/1475-2859-7-7.

Djonović S., Pozo MJ, Kenerley CM (2006). Tv-bgn3, a β-1,6-Glucanase from the biocontrol fungus *Trichoderma virens* is involved in mycoparasitism and control of *Pythium ultimum*. Appl. Environ. Microbiol. doi: 10.1128/AEM.01607-06.

Dušica Ć., Devrnja N, Milojević J, Zdravković-Korać S, Tubić L, Djuričković MS, Vinterhalter B (2012). Pollen morphology and variability of *Tulipa hungarica* Borb. African Journal of Biotechnology. 11(3): 616-620.

Forbes BA, Sahm DF, Weissfeld AS (2007). Bailey and Acott's Diagnostic Microbiology. Mosby, Inc., an affiliate of Elsevier Inc.China. pp.1031.

Gao X, Ma Q, Zhao L, Lei Y, Shan Y, Ji C (2011). Isolation of *Bacillus subtilis*: screening for aflatoxins B1, M1, and G1 detoxification. Eur Food Res Technol. 232:957–962.

Hedayati MT, Pasqualotto AC, Warn PA, Bowyer P, Denning DW (2007). *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. Microbiology. 153:1677-1692.

Henayl WR (2000). Bergey's Manual of Determinative Bacteriology. Lippincott Williams & Wilkins. USA. pp.599.

Islam M, Rezuanul D, Jeong YT, Lee YS, Song CH (2012). Isolation and identification of antifungal compounds from *Bacillus subtilis* C9 inhibiting the growth of plant pathogenic fungi. Mycobiology 40(1): 59-66.

Kim J (2005). Antifungal activity of lactic acid bacteria isolated from kimchi against *Aspergillus fumigatus*. Mycobiology. 33(4): 210-214.

Kimura N, Hirano S (1988). Inhibitory strains of *Bacillus subtilis* for growth and aflatoxin production of aflatoxienic fungi. Agric. Biol. Chem. 52:1173-l179. Cited in Zuo R, Chang J, Yin Q, Wang P, Cheng W, Wang X, Liu J, Zheng Q (2012). Inhibiting *Aspergillus flavus* growth and degrading aflatoxin B1 by combined beneficial microbes. African Journal of Biotechnology. 11(65): 12903-12909.

Klich MA (2002). Identification of common *Aspergillus* species. Centraalbureau voor Schimmelculture, UTRECHT, The Netherlands. pp. 116.

Klich MA, Lax AR, Bland JM (1991). Inhibition of some mycotoxigenic fungi by iturin A a peptidolipid produced by *Bacilluss subtilis*. Mycopathologia. 116: 77-80.

Kong Q, Shan S, Liu Q, Wang X, Yu F (2010). Biocontrol of *Aspergillus flavus* on peanut kernels by use of a strain of marine *Bacillus megaterium*. International Journal of Food Microbiology. 139: 31-35.

Kumar P, Dhiman S, Bhatt RP, Singh L (2011). In-vitro Antifungal activity of *Sapium sebiferum* L. against *Aspergillus niger* and Aflatoxigenic *Aspergillus flavus*. Journal of Applied Pharmaceutical Science. 1(9): 108-110.

Moyne A, Shelby R, Clevel TE, Tuzun S (2001). Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*. Journal of Applied Microbiology. 90:622- 629.

Mushtaq S, Ali A, Khokhar I, Mukhtar I (2010). Antagonisitic potential of soil bacteria against food borne fungi. World Applied Sciences Journal. 11(8): 966-969.

Olteanu V, Sicuia O, Ciuca M, Carstea DM, Voaides C, Campeanu G, Cornea CP (2011). Production of biosurfactants and antifungal

compounds by new strains of *Bacillus spp*. isolated from different sources. Romanian Biotechnological Letters. 16(1): 84-91.

Palumbo JD, O'Keeffe TL, Kattan A, Abbas HK, Johnson BJ (2010). Inhibition of *Aspergillus flavus* in soil by antagonistic *pseudomonas* strains reduces the potential for airborne spore dispersal. Phytopathology. 100(6): 532-538.

Raaijmakers JM, Vlami M, de Souza JE (2002). Antibiotic production by bacterial biocontrol agents. Antonie van Leeuwenhoek. 81: 537-547.

Renato MC, de Souza PJR, da Eira1 AF (2006). Digital monitoring of mycelium growth kinetics and vigor of Shiitake (*Lentinula edodes* (Berk.) Pegler on agar medium. Brazilian Journal of Microbiology. 37:90-95.

Rodríguez A, Rodríguez M, Luque MI, Justesen AF, Córdoba JJ (2012). A comparative study of DNA extraction methods to be used in real-time PCR based quantification of ochratoxin A-producing molds in food products. Food Control. 25: 666-672.

Ruiyu Z, Chang J, Yin Q, Wang P, Cheng W, Wang X, Liu J, Zheng Q (2012). Inhibiting *Aspergillus flavus* growth and degrading aflatoxin B1 by combined beneficial microbes. African Journal of Biotechnology. 11(65):12903-12909.

Sageera S, Imtiyaz M, Omi L, Arif A (2012). Biological Control of *Fusarium oxysporum* and *Aspergillus sp*. by *Pseudomonas fluorescens* isolated from wheat rhizosphere soil of kashmir. IOSR Journal of Pharmacy and Biological Sciences. 1(4): 24-32.

Sainis JK, Rastogi R, Chadda VK (1998). Application of image processing in biology and agriculture. Nuclear India. 32 (5-6):12-13.

Salem E, Djébali N, Tabbene O, Hadjbrahim A, Mnasri B, Mhamdi R, Shaaban M, Limam F (2012). Evaluation of antifungal activity

from *Bacillus* strains against *Rhizoctonia solani*. African Journal of Biotechnology. 11(18): 4196-4201.

Souto G I, Correa OS, Montecchia MS, Kerber NL, Pucheu NL, Bachur M, García AF (2004). Genetic and functional characterization of a *Bacillus sp*. strain excreting surfactin and antifungal metabolites partially identified as iturin-like compounds. J. Appl. Microbiol. 97:1247-1256.

Vujanovic V, Smoragiewicz W, Krzysztyniak K (2001). Airborne fungal ecological niche determination as one of the possibilities for indirect mycotoxin risk assessment in indoor air. Environ Toxicol. 16:1-8.

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

لبيد عبدالله السعد [1]، عدنان عيسى البدران [1] ، سامي عبدالرضا الجميلي [2]

1. قسم علوم الحياة ، كلية العلوم، جامعة البصرة.
2. كلية العلوم الطبية التطبيقية، جامعة كربلاء.

Email: la_alsaad@yahoo.com

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.