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EVALUATION OF ANTAGONISTIC PROPERTIES OF RHIZO-BACTERIA *In vitro*

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ABSTRACT

Twelve bacterial isolates were recovered from the rhizosphere of cotton, flax, and tomato seedlings of the most predominat commerical cultivars in Egypt. Bacterial isolates determined for their activities against three phytopathogenic fungi: *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii*.When the six most antagonistic isolates were classified by standard tests, it was found that 4 strains (57 %) were belonging to *Bacillus* spp., 2 strains (40 %) were belonging to *pseudomonas* spp. *In vitro,Bacillus subtilus (2strains),Bacillus* sp. (2strains) , *Pseudomonas flurorescens* and *Pseudomonas* sp. were effective antagonists. Ammonia,chitinase, amylase and cellulase in bacterial culture by *Bacillus* sp., were produced and also, the production of siderosphore,ammonia, lipase and chitinase by *Pseudomonas* spp.may contribute to the antagonistic activities of the bacterial isolates.

Keywords:*Bacillus* spp.;Pseudomonads; Phytopathogenic fungi; Volatile materials; Hydrolytic enzymes.

INTRODUCTION

Antagonism between microorganisms is a common phenomenon in nature.Thus plant – pathogenic fungi can be affected by bacterial antagonists Cook and Baker,1983. Biological control of phytopathogenic fungi are better than chemical control Nautiyal,2001. Several genera and species of bacteria used as bioagent for many soil borne fungi Weller, 1988; Whipps, 2001.The biocontrol properties of *Pseudomonas,Bacillus* and Actinomycetes are due to their adaptive metabolism and their superior ability to extract some materials inhibiting the growth of several fungal pathogens Thomashow and Weller,1990. However, many *Bacillu*s strains are known to suppress fungal growth *in vitro* Katz and Demain, 1977 and *in vivo* Fravel, 1988. Different of enzymes and siderophores has been implicated as mechanisms used by biocontrol to limit the damage to plants by phytopathogens Glick and Bashan,1990; Bowen and Rovira,1994; Ashour *et .al*.2004..

The aim of our work, some genera of prokaryotes such as *Peudomonas* and *Bacillus*, well-adapted antagonistic species Pandey and Palni 1998 for controlling phytopathogenic fungi, *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii*, to evaluate the mechanisms of antagonism *in vitro*.

MATERIALS AND METHODS

Isolation,purifaction and maintenance of bacteria:
a) Isolation of Bacillus:-

Ten grams of soil samples collected from rhizosphere of plant seedlings were aseptically added to 90 ml of sterile tap water in pyrex flasks. The sample was shaken for 10 min. room temperature. The soil suspension was placed in thermostatically controlled water bath at 80℃ for 10 min , followed by cooling to 30℃.Aserial dilution was prepared one ml of each dilution was pipetted and plated on nutrient agar (NA) . The plates from each dilution were incubated in triplicate at 30℃. Surface colonies from each plate were grouped on the basis of colony morphological group were selected for further characterization. Cultures were purified by restreaking isolated colonies at least three times.

b) Isolation of *Pseudomonas*:-

Pseudomonas strains were isolated from rhizosphere of cotton , flax and tomato seedlings from soil Mansoura city in Dakahlia Governate during to 2014 / 2015 years the Egyptian soils. One gram of soil sample was inoculated in 100 ml of the kings medium B (KMB), and incubated at 20℃ for 24h. Bacterial growth was isolated and purified by streaking plates. All purified isolates were maintained on nutrient agar slants at 4℃.

Fungal isolates:-

The three isolates of *F. oxysporum* Schlech. , *R. solani* kühn and *S.rolfsii* Sacc. were isolated from roots of plant seedlings infected with damping –off disease. Isolation, purification and identification of these fungi were carried out at cotton pathology Lab., A. R. C. Egypt.

Antagonism:-

In vitro tests for antagonism of bacterial isolates towards damping – off fungi,*F. oxysporum*,*R. solani* and *S. rofsii* were secreened using plate assays. The assay plates were maintained at 28 – 30℃ and observations were made up to 7 days on the inhibition of the fungal growth Sivamani and Gnanamanickam 1988. Based on the *in vitro* antagonism, 6 bacterial strains showing maximum inhibition of pathogenic fungal growth were chosen.

Identification of the antagonistic bacteria:-

The six selected bacterial strains which showed antagonistic action to pathogenic fungi were transferred to a nutrient agar slant. Strains were identified by standard bacteriological tests based on Bergey's Manual of Determinative Bacteriology (2005).

Determination of antagonistic compounds :

Siderophore was determined on chrome-acurol S-agar (CAS),Schwyn and Neilands,1987.The bacterium was inoculated on CAS agar and incubated at 28℃ for 28h. When orange colour around the bacterial colony,siderophore was produced.To determine siderophore in the antagonism between *F.oxysporum*or *R. solani*or *S. rolfsii* and *Ps.fluorscenes,Pseudomonas* sp., the reduction in fungal growth by adding 100Um

$FeCl_3$ in KB broth.When ammonia was evaluated Dye 1962,the isolates were grown in peptone water in 30 ml tubes and incubated at 25℃ for 4 days. Afterwards, 1 ml of Nessler's reagent was added to each tube. Development of afaint yellow colour was indicative of weak reaction and deep yellow to brownish colour was indicative of strong reaction .The method of HCN Bakker and Schippers 1987. *Pseudomonas* bacteria and *Bacillus* spp. were inoculated individually on Petri dishes containing tryptone soya agar supplemented with 4.4 g glycine . Filter paper discs (9cm diameter , watman No . 1)soaked in 0.5 % picric acid in 2% sodium carbonate were placed in the lid of each Petri dish . The plates were sealed with parafilm and incubated at 25℃ for 4 days. Colour from yellow to brown and reddish brown was indicative of moderate and strong production of HCN by the bacterium, respectively. No change in colour indicated negative reaction.

Determination of enzymes by antagonistic bacteria:-

Determination of hydrolytic enzymes were detected on plate by streaking antagonistic bacteria individually on the medium containing enzyme substrate.Benson , 1990, Aneja ,1996, Basha and Ulaganathan , 2002.Ngarajkumer *et.al.*2004.

RESULTS AND DESCUSSION

Tweleve bacterial isolates were recovered from rhizosphere of seedlings, the most predominant commercial cultivars of cotton, flax and tomato. Of the 12 isolates, 4 were chosen from (Table1) and 2 were chosen from Table 2 for further study because they showed consistent *in vitro* antagonism against *F.oxysporum, R.solani* and *S.rolfsii* (Tables 1&2).

Table (1): Screening of diffirent endospores-forming bacteria against isolates of pathogenic fungi.

Endospores	Inhibation zone with fungal isolates		
Bacteria No.	***F.oxysporum***	***R.solani***	***S.rolfsii***
1	+	++	++
2	+	-	+
3	-	-	±
4	++	+	+
5	+	+	+
6	±	-	±
7	++	++	+

++ Inhibitation of fungal pathogen by overgrowth
+ Inhibitation of fungal pathogen on contact with the potential antagonist
- No inhibitation
± Inhibation of the potential antagonist by the pathogen

Table (2): Antagonism between some bacterial isolates (*Peudomonas* spp.)selected from the rhizosphere plants and pathogenic fungi

Isolate No.	Inhibation zone		
	F.oxysporum	***R.solani***	***S.rolfsii***
1	-	-	-
2	+	++	++
3	++	++	++
4	-	-	+
5	-	+	-

++ Inhibitation of fungal pathogen by overgrowth
+ Inhibitation of fungal pathogen on contact with the potential antagonist
- No inhibitation
± Inhibation of the potential antagonist by the pathogen

Based on the standard tests used to classify four isolated strains as endospore-forming (Table 3) , and two isolated strains (KMB) medium (Tables 4&5) , it was found that 4 strains (57%) were belonging to *Bacillus* spp. , 2 strains (40%) were belonging to *Pseudomonas* spp. The morphological and biochemical characteristics used for identification of these strains are as follows:

I. Bacillus: (Isolates No.1,4, 5 and 7):

All isolates were aerobic and facultative anaerobes, catalase producers, endospore forming rods (Table 3). They were classified according to their morphological, physiological and biochemical characteristics into *B.subtilis* (*B.s*-1 and *B.s*-4) and *Bacillus* spp. (*B.*sp-5 and *B.*sp-7).

II-*Pseudomonas*: (Isolates No. 2 and 3):

They were Gram – negative, rod-shaped,stricty aerobic bacteria, neither spores nor cysts were formed. They were able to use different carbon sources.

According to their various morphological and biochemical characters (Tables 5 & 6) they were classified into , *Pseudomonas fluorescenes* (*P.f*-2) and *Pseudomonas* sp. (*P.*sp-3) (Table 7)

Table (3): Morphological , physiological and biochemical characters of four endospore-forming antagonistic bacteria.

Character	Isolate No.			
	1	**4**	**5**	**7**
Cell diameter(µm)	5.0x1.2	2x0.7	2.7x7.5	3x0.7
Shape	CR	CR	CR	CR
Sporulation	EC	EC	EC	EC
Motility	+	+	+	+
Gram stain	+	+	+	+
Catalase production	+	+	+	+
Degration of :				
Galatinase	+	-	-	-
Casein	+	+	-	+
Starch	+	-	-	+
Aerobiosis	Aerobic or facultative anaerobic			
Anaerobic growth	+	-	-	-
V.P.assay	+	+	-	+
Indole formation	-	-	-	-
Tolerance of 7%NaCl	ND	+	+	+
Production of acid from :	-	+	+	+
Glucose	+	+	+	+
Mannose	+	+	+	+
Fructose	+	+	+	+
Arabinose	-	-	-	+
Xylose	-	+	+	+
Mannitol	-	-	-	+
Maximum temp.(°C)for growth	40	50	50	40

Spore: (E Ellipsoidal ;C Central ; T . terminal) CR; Chains Rods ND; Not determined

Table 4: Scientific name of endospore forming isolated strains.

No. of strain	Scientific name	Code name
1	*B.subtilis*	*B.s*-1
4	*B.subtilis*	*B.s*-4
5	*Bacillus* sp.	*B.*sp-5
7	*Bacillus* sp.	*B.*sp-7

Table (5): Morphological and biochemical characters of some bacterial isolates from (KMB) medium.

Character	No.of isolate	
	2	**3**
Cell shape	SR	SR
Spore formation	-	-
Fluorscent pigment	+	+
Motility	+	+
Gram stain	-	-
Oxidase reaction	+	+
Indole production	-	-
Nitrate prodiction	d	d
Starch hydrolysis	-	-
Fat hydrolysis	+	-
Gelatin liquefaction	+	+
Casein hydrolysis	+	-
Tween 80 hydrolysis	-	-
Growth in NaCl at 5%	d	d
Growth in NaCl at 7%	-	-
Voges-Proskauer (V.P)	-	-
Methyl Red (M.R)	-	-
Growth at 4℃	+	+
Growth at 41℃	-	-

SR =Short Rod. + = positive reactions - =negative reactions d= variable reaction

Table (6): Utilization of different carbon sources by bacterial isolates from (KMB) medium.

Carbon source	No. of isolate	
	2	3
Arabinose	+	+
Xylose	+	d
Fractose	+	+
Glucose	+	+
Galactose	+	+
Mannose	+	+
Rhamnose	-	-
Sucrose	+	+
Maltose	-	-
Lactose	-	-
Cellulose	-	-
Trehalose	+	+
Glycerol	+	+
Mannitol	+	+
Inositol	d	+
Dextrine	-	-
Starch	-	-

+ = positive reactions - = negative reactions d = variable reactions

Table (7): Scientific name of isolated strains on (KMB) medium.

No. of strain	Scientific name	Code name
2	*P.fluorescens*	*P.f*-2
3	*Pseudomonas* sp.	*P*.sp-3

Results on production of chemicals and enzymes, which may be involved in the antagonistic activities of *Bacillus* sp. and *Pseudomonas* sp., are presented in Table 8. Orange zones around the bacterial colony indicated the siderophores were produced . In $FeCl_3$-supplemented KB broth, *Pseudomonas* sp .showed siderophores are abcense in the presence of iron Meyer and Abdallah , 1978. The siderophore playes an important role in biocontrol of several fungal phytopathogens Scher and Baker 1982;KumerDileep , 1998**.**

Table (8): Detection of antagonistic properties of *Bacillus* sp. and *Pseudomonas* sp.

Antagonistic properties	Reaction with bacterial isolates	
	***Bacillus* sp.**	***Pseudomonas* sp.**
Production of :		
Siderophore	-	+
Ammonia	+	+
HCN	-	-
Production of enzymes :		
Chitinase	+	+
Lipase	-	+
Amylase	+	-
Cellulase	+	-
Pectinase	-	-
Protease	-	-

+ = Positive; - = negative

Ammonia and hydrogen cyanide are produced by rhizobacteria and play an important role in biocontrol Brimecombe *et. al.* , 2001. *Bacillus* sp. and *Pseudomonas* sp. do not produce HCN *in vitro.* Yellow colour of both isolates indicated the production of ammonia. Our results are agreement with Howell *et.al*., 1988 and Pavlica *et.al*.1978.

Petri dish-based assayes carried out for the production of hydrolytic enzymes indicated that *Pseudomonas* sp. produces chitinase, lipase while, *Bacillus* sp. produces chitinase, amylase and cellulase. Fridlender *et.al.,* 1993;Viswanatham and Samiyappan, 2001. In case of *Pseudomonas* spp. they have often not been described as important for biocontrol Bagnasco *et.al.,* 1998. For another point Agrawa and Kotasthane 2012 stated that chitinolytic strains of Trichoderma are among the most effective biocontrol agents for plant diseases .

Pandey *et.al*.,1998 , Shalaby , *et.al*. ,2013 found that *B.subtilis* and other bacteria were the most antagonistic isolates of the most pathogenic fungi .

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تقييم خصائص التضاد في بكتريا المجال الجذري معمليا

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تم في هذه الدراسة عزل 12 عزله بكتيريه من المجال الجذري من محيط بادرات بعض المحاصيل مثل القطن والكتان والطماطم حيث أنها من أكثر المحاصيل شيوعا في مصر . من بين هذه العزلات تم تحديد واختيار ستة سلالات أظهرت درجه عاليه من التضاد علي الإطباق في المعمل ضد فطريات *Fusarium oxysporum, Rhizoctonia solani , Sclerotium rolfsii.*استعملت مجموعه من الاختبارات القياسية لتصنيف هذه السلالات البكتيرية . ظهر أن أربعه سلالات منها تمثل(57%) كانتا تتبعان جنس *Bacillus* وسلالتان تمثلان(40%) كانت تتبع جنس *Pseudomonas*. وقد أظهرت احدي السلالات من جنس .sp *Bacillus* كـ فاءه عال ية في التضاد بإنتاجها الامونيا وكذلك إنزيمات الكيتينيز والاميليز والسليوليز في بيئه نموها – بينما أظهرت سلاله من .sp *Pseudomonas*كفاءة عاليه في انتاج السايدروفورز والامونيا وكذلك انزيمي الليبيز والكيتينيز ،ومن المعروف ان هذه المنتجات تزيد من النشاط التضادي لهذه البكتريا ضد الفطريات الممرضه للنباتات.