ANTIMICROBIAL ACTIVITY OF SUGARBEET PHYLLOSPHERIC MICROORGANISMS AGAINST *CERCOSPORA BETICOLA* SACC.

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(Manuscript received 8 October 2009)

Abstract

Screening some epiphytic microorganisms isolated from the phyllosphere of sugar beet plants for their potential antagonism against *Cercospora beticola* revealed a high potency of one isolate of each of *Pseudomonas fluorescens, P.aureginosa, Bacillus subtilis, Streptomyces albidoflavus, S.lavandulae* and *S. griseoviridis.* One isolate of *P.putida* showed moderate inhibitory effect. Among fungi, one isolate of *Trichoderma harzianum* exhibited moderate inhibitory activity. Some isolates were weak inhibitors. Isolates were evaluated for their ability to elaborate secondary metabolites, such as HCN, IAA, siderophores, and some hydrolytic enzymes such as chitinase, lipase, pectinase, protease and cellulose.

INTRODUCTION

Sugar beet (*Beta vulgaris* L.) is the second important sugar crop in Egypt after sugar cane in terms of acreage, total production and cash value. The cercospora leaf spot caused by *Cercospora beticola* is the most serious foliar disease, in Egypt, as in most of sugar beet growing area of the world. The traditional means to control this destructive disease is applying the harmful fungicides, however, using biological agents is encouraged at the time being. Many of previous investigators succeeded in reducing infection with leaf spot diseases of different crops by using some bioagents (Harman, 2006). Kiewnick and Jacobson (1998), reported the possibility of controlling cercospora leaf spot by Bacillus subtilis isolated from sugar beet phyllosphere.

The present investigation was carried out to isolate some antagonistic epiphytic microorganisms against *C.beticola* from the phyllosphere of sugar beet . Identification of these potent organisms and their antagonistic action were also evaluated.

MATERIAL AND METHODS

Isolation of epiphytic microorganisms from leaves of sugar beet plants

Leaf samples were collected from sugar beet-growing fields, at Shinno village, Kafr El-Sheikh governorate in 2006/2007 growing season. Samples were taken from healthy plants adjacent to cercospora-infected ones and immediately transferred to the Lab. in an ice chest for isolation. Leaf subsamples (10g each) were washed in tap

water and transferred to 100 ml of sterilized distilled water in 250 ml-flasks. Flasks were shaken at 120 rpm for one hour. Thereafter, serial 10-fold dilutions were prepared and 1 ml of each dilution was seeded into 250 ml from each of specific media., *Bacillus* spp. were recovered after pasteurization using nutrient agar. King`s medium B was used for isolating fluorescent pseudomonads, Martin`s medium for fungi and starch nitrate agar, starch caseinate agar and glycerol-yeast extract agar for isolating actinomycetes. Plates were incubated at 30C and four replicate plates of each of the respective media were used for each group. Pure cultures were grown on agar slants and kept at 10 °C till use.

Screening for potential antagonists

The recovered microorganisms were screened for their potency in inhibiting the growth of *C.beticola*. The pathogen was seeded into test agar media (17g agar/L). Spore suspension from *C.beticola* was prepared at the concentration of 10^6 in sterilized distilled water, from which 1ml was introduced into the prepared media at the rate of 1ml per 200 ml in 250 conical flasks of PDA, nutrient agar, King B & starch nitrate prepared for testing fungi, *Bacillus* spp., *Pseudomonas* spp. and actinomycetes, respectively. Plates were surface inoculated with the test organisms (0.5 cm diam. discs for fungi and actinomycetes), and heavily charged loop for bacteria, at the center of the agar plates, then incubated at 30 °C. Four replicate plates were used for each treatment and the antagonist-free media acted as control. Inhibition zones around the tested antagonists were measured after 3 days for bacteria and 5-8 days for fungi and actinomycetes. The diameter of inhibition zones was measured and means of replicates were calculated and indexed as low, moderate and high degree of antagonistic potential.

Identification of selected bioagents

Fungi were identified according to cultural characteristics and microscopic examination. *Pseudomonas* spp.were identified following the analytical purified identification system (API 20 E). *Bacillus* spp. were identified by a combination of API 20E and API CH strips as described by Borsodi, *et al.*(2007). Actinomycetes were identified according to their morphological characters (pigments, color of mycelium and spores), resistance to antibiotics, and biochemical tests (Williams *et al.*,1983).

Metabolic by-products

The selected isolates were screened for production of hydrogen cyanide (HCN), indole acetic acid (IAA), siderophores, and some extracellular hydrolytic enzymes as described below:

HCN production

Bacteria (48h-old culture) were streaked on slant of Luria Bertani agar medium (LB), triptone 10g, yeast extract 5g, NaCl 5g, agar 15g & distilled water 1 L, amended with glycine (4.4 gl/1). Sterilized filter paper were soaked in 0.5% (w/v) picric acid in 1% Na_2CO_3 and hanged with the plug inside the tubes. After incubation at 28±1°C, changing color of the hanging filter paper from yellow to brown, due the evolved HCN indicated a positive reaction.

Production of IAA

In vitro method was used for screening isolates for their ability to produce indole acetic acid (IAA) and the analogous products. Isolates were grown on Luria Bertani (LB) amended with 5 mM 1-tryptophan that was overlaid with an 82-mm-diameter nitrocellulose membrane disk. Agar plates were inoculated with bacterial cultures and incubated at 28 °C for 3 days. The membranes were overlaid with a Whatman No2 filter paper saturated with Salkowski reagent. Microorganisms producing IAA or analogous compounds exhibit a characteristic pink to red color after 0.5 to 3 h on the filter surface.

Production of Siderophores

Siderophore production was estimated on the universal medium, chromo-azurols (CAS) agar medium (sodium carbonate & ferric chloride). Bacterial inocula were spotted on the CAS agar medium and plates were incubated at 28±1°C for 48 h. Development of pink color around the bacterial colony indicates positive reaction.

In vitro assay of some extracellular hydrolytic enzymes

The activities of extracellular hydrolytic enzymes were detected on plate-based assays by streaking bacterial culture on the medium containing enzyme substrate and measuring the clearing zones of the degraded substrate around the bacterial colonies after incubation period of 5 days at 25± 1C.

Agar base (1.8 % w/v) was used for studying hydrolytic enzymes . Plates enriched with either of 5% skim milk, 0.2% tween 80 or 0.5% carboxymethyl cellulose, and citrus pectin were used for detecting the production of protease, lipase, cellulose and pectinase, respectively. Plates were examined for hydrolysis as judged by clearing area around the inocula. However, lipase activity was visualized as green color formed after adding 20% copper sulfate solution.

Chitinase activity was examined by adding colloidal chitin as substrate to the basal medium and incubation for 7 days at 25 C. Clear zone around the inocula indicates the positive reaction.

RESULTS

Isolation of epiphytic microorganisms from leaves of sugar beet plants

A number of six different fungal species were recovered from the phyllosphere of sugar beet leaves. *Aspergillus* spp. were isolated in the highest frequency (8 isolates) followed by *Penicillium* spp. (7 isolates). Other fungal species, namely, *Mucor* spp., *Alternaria* spp., *Fusarium* spp. were recovered at low frequencies (2, 4, and 3 isolates, respectively). *Trichoderma* sp., occasionally appeared at a very low frequency (1sp.). The fluorescent pseudomonads appeared on the isolation plates at very high frequencies (12 isolates). Six isolates of *Bacillus* spp., were recovered in the isolating plates. Regarding actinomycetes, a number of 14 different isolates were recovered.

Antagonism of the isolated microorganisms

Results (Table 1), indicate that one out of the 8 isolates of *Aspergillus* spp. (12.5 %) showed low antagonistic effect against *C.beticola*. The remaining recovered fungal isolates, except *Trichoderma* sp. have no antagonistic potential, while *Trichoderma* sp. efficiently inhibited the growth of the pathogen causing moderate degree of antagonism.

Results showed that 2 out of 6 *Bacillus* spp. isolates (33.3%) effectively inhibited the growth of the pathogen. *Bacillus* sp. (B_1) showed a high degree of antagonism, while, B_4 was less effective (low degree) against the pathogen. The fluorescent pseudomonads, however, actively antagonized the growth of *C.beticola*, where 4 isolates (33.3 %) could efficiently affect growth of the pathogen. The degree of the antagonism against the pathogen differ from low (Sp_4), moderate (Sp_1) to high (Sp_2 &Sp_6) as shown in Table 1.

Concerning the actinomycetes, 5 out of the14 candidates (35.7 %) could affect the growth of the pathogen. Three of these isolates efficiently affected the fungal growth causing a high degree of antagonism. These are isolates No. $Act_{1, 2}$ & $_5$), whereas, isolates No.$_8$ &$_{11}$ showed low effect against the pathogen (Table 1).

Table 1. Antimicrobial activity of isolated microorganisms in inhibiting the mycelial growth of *C. beticola*

Microorganism	Inhibition zone (mm)/ on			Degree of antagonism
	Potato Dextrose Agar	King`s medium B	Starch nitrate media	
Aspergillus sp.*	6	-	-	Low **
Trichoderma sp.	9	-	-	Moderate
Pseudomonas sp.(Ps_1)	7	9	-	Moderate
Pseudomonas sp. (Ps_2)	13	17	-	High
Pseudomonas sp. (Ps_4)	-	3	-	Low
Pseudomonas sp.(PS_6)	16	20	-	High
Bacillus sp.(B1)	17	13	-	High
Bacillus sp. (B4)	6	6	-	low
Actinomycete (Act_1)	20	-	21	High
Actinomycete (Act_2)	16	-	20	High
Actinomycete (Act_5)	15	-	15	High
Actinomycete (Act_8)	5	-	6	Low
Actinomycete (Act_{11})	6	-	6	Low

*Specific names are shown in Table (2)

** Low degree = diameter mean up to 6mm, moderate degree = diameter mean from 7 to 12mm, high degree = diameter mean above 12mm.

Identification of the recovered epiphytic organisms

The promising microbial isolates that showed potent effect against the target pathogen, in addition to some other candidates were subjected to identification according to the methods described under Material & Methods.

Bacillus spp., were identified using a combination of the API 20E and API 50CH strips of the analytical purified identification system. They were identified as *B. subtilis* (B_1) and *B.pumilus* (B_4) as shown in Table 2.

Fluorescent pseudomonads, were identified by using the system of API20E, in addition to some complementary test as described in Bergey`s Manual of Determinative Bacteriology (2006). Oxidase, nitrate reductase, growth at 41°C, fluorescent pigments on King`s medium B, blood agar haemolysis ...*etc* (Table 2).

For actinomycetes, the Probabilistic Identification Matrix developed by Williams *et al.* (1983) was followed. Isolates No.Act_1, Act_2, Act_5, Act_8 & Act_{11} were identified as *Streptomyces albidoflavus* (Act_1) ,*S.lavandulae* (Act_2), *S.griseoviridis* (Act_5), *S.purpureus* (Act_8) and *S. rimosus* (Act_{11}), respectively.

The fungal isolate of *Trichoderma* sp. was identified according to culture characters and microscopic examination as *T. harzianum*.

Major metabolic byproducts produced by the isolated microorganisms

Production of secondary metabolites by the epiphytic organisms under study that may regulate their antagonistic mechanisms are shown in Table 2. Indole acetic acid (IAA) was produced by some of the fluorescent pseudomonads (Table 2). These are *P.putida* (Ps_1 & Ps_{10}), *P.fluoroscens* (Ps_4 & Ps_7). Most of *Pseudomonas* spp. under study were found to have the potential to produce hydrogen cyanide HCN, however, at different degrees (+ to +++) according to the appearance of yellowish to brownish color on filter paper as described under Material & Methods (Table 2).

Regarding the production of siderophores, it was found that six out of the twelve screened pseudomonads were able to produce compounds which react with ferric chloride producing pink color around colonies as described previously. These are *P.putida* (Ps_1), *P.fluoroscens* (Ps_2), *P.* sp. (Ps_3), *P.fluoroscens* (Ps_4), *P. aureginosa* (Ps_6) & *P.* sp. (Ps_9) (Table 2). Some organisms produced the hydrolytic enzymes, as chitinase, lipase, pectinase, protease and cellulase on nutrient agar (Table 2). Only *Trichoderma* sp., was able to produce chitinase. Lipase was readily produced by six isolates of actinomycetes (*S.albidoflavus* Act_1, *S. Lavandulae* Act_2, *S. griseoviridis* Act_5, *S. purpureus* Act_8 and *S. rimosus* Act_{11} & Act_{12}). *Pseudomonas* spp. isolate Ps_2 and Ps_7 as well as *Bacillus* spp. isolates B_1 and B_4 actively produced lipase. With respect to pectinase and cellulase, they were only produced by *Bacillus* spp. (B_1, B_4) and *Trichoderma* sp. (Tr_1). Protease produced by *Pseudomonas* sp. (Ps_7), by 3 isolates of *Bacillus* spp. (B_1, B_2 & B_4) and 5 isolates of actinomycetes (Act_1, Act_2, Act_5, Act_9 & Act_{13})

Table 2. Characterization of the metabolic by-products and identification of microorganisms

Group	Test Organism	Inhibition zone (Antagonism)	IAA production	HCN Production	Siderophore Production	Enzyme activity				
						Chitinase	Lipase	Pectinase	Proteases	Cellulase
Pseudomonas Isolates (Fluorescent form)	*P. putida* (PS_1)	+	+	++	+	-	-	-	-	-
	P. fluorescens (PS_2)	+	-	+	+	-	+	-	-	-
	P. sp. (PS_3)	-	-	-	+	-	-	-	-	-
	P. fluorescens (PS_4)	+	+	++	+	-	-	-	-	-
	P. sp. (PS_5)	-	-	+	-	-	-	-	-	-
	P. aureginosa (PS_6)	+	-	++	+	-	-	-	-	-
	P. fluorescens (PS_7)	-	+	+++	-	-	+	-	+	-
	P. sp. (PS_8)	-	-	+	-	-	-	-	-	-
	P. sp. (PS_9)	-	-	-	-	-	-	-	-	-
	P. putida (PS_{10})	-	+	+++	+	-	-	-	-	-
	P. sp. (PS_{11})	-	-	-	-	-	-	-	-	-
	P. sp. (PS_{12})	-	-	-	-	-	-	-	-	-
Bacillus spp.	*B. subtilis* (B_1)	+	-	-	-	-	+	+	+	+
	B. sp. (B_2)	-	-	-	-	-	+	+	+	-
	B. sp. (B_3)	-	-	-	-	-	-	-	-	-
	B. Pumilus (B_4)	+	-	-	-	-	+	-	+	-
	B. sp. (B_5)	-	-	-	-	-	-	-	-	-
	B. sp. (B_6)	-	-	-	-	-	-	-	-	-
Actinomycetes	*S. albidoflavus* (Act_1)	+	-	-	-	-	+	-	+	-
	S. lavendulae (Act_2)	+	-	-	-	-	+	-	+	-
	Act_3	-	-	-	-	-	-	-	-	-
	Act_4	-	-	-	-	-	-	-	-	-
	S. griseoviridis (Act_5)	+	-	-	-	-	+	-	+	-
	Act_6	-	-	-	-	-	-	-	-	-
	Act_7	-	-	-	-	-	-	-	-	-
	S. purpureus (Act_8)	+	-	-	-	-	+	-	-	-
	Act_9	-	-	-	-	-	-	-	+	-
	Act_{10}	-	-	-	-	-	-	-	-	-
	S. rimosus (Act_{11})	+	-	-	-	-	+	-	-	-
	Act_{12}	-	-	-	-	-	+	-	-	-
	Act_{13}	-	-	-	-	-	-	-	+	-
	Act_{14}	-	-	-	-	-	-	-	-	-
Fungi	*Trichoderma harzianum* (Tr_1)	+	-	-	-	+	-	+	-	+
	Aspergillus niger.	+	-	-	-	-	-	-	-	-

DISCUSSION

A total of 57 different microbial isolate were recovered from the surface of sugar beet leaves collected from fields of Kafr El-Sheikh governorate. Screening for the antagonistic potential toward *Cercospora beticola* was carried out *in vitro.* Various isolated candidates were capable of antagonizing the target pathogen. This can be due mainly to the ability of these organisms to produce metabolic by-products in the growing media that affect the pathogen. Based on the evidences accumulated on the low sensitivity of fungi to many naturally produced antibiotics, with exception of gliotoxin, other by-products were considered in this work. The production of secondary metabolites as volatile substances, lytic enzymes, plant auxins, HCN and siderophores were looked upon with great concern. Two filamentous fungi (*Aspergillus niger* & *Trichoderma harzianum*), two *Bacillus* spp. (*B.subtilis & B.bumilus),* four *Pseudomonas* spp. (1 isolate of *P.putida,* 2 of *P.fluorescens &* 1 of *P.aureginosa*) and five actinomycetes (*Streptomyces albidoflavus, S. lavendulae, S .griseoviridis, S. purpureus & S. rimosus*) were found to inhibit the growth of *C.beticola.* Several previous investigations emphasized that *B.subtilis , Trichoderma* spp., actinomycetes and *Pseudomonas pp.* can antagonize and suppress many target pathogens in the Lab. (El-Assiuty *et al.,* 1986, Ahmadzadeh and Sharifi-Tehrani, 2009). The antagonistic effects of these microbes are attributed to the production of antibiotics in growing media. In addition to the action of hyperparasitism of *T.harzianum,* it has the potential to produce an extracellular antibiotic, namely, gliotoxin. Gliotoxin is produced by numerous fungi, including *Trichoderma* spp. (Howell and Stipanovic, 1983). This compound possesses a broad spectrum of activity against fungi and bacteria. It inhibits protein and nucleic acid synthesis as reported by Kerridge (1958). *T.harzianum* could suppress the radial growth of *Acremonium strictum* (syn. *Cephalosporium acremonium*) *in vitro* and control acremonium wilt of grain sorghum under greenhouse conditions (El-Assiuty *et al.*, 1986).

It is known that many actinomycetes have a broad spectrum of biological activities. In addition, the production of some volatile substances has been reported in *Streptomyces* spp. and other species (Dickschat *et al.,* 2005). Hence, the efficiency of actinomycetes recovered in the present study may be attributed to the production of a variety of secondary metabolites in the growing media.

Most microbial isolates having inhibitory effect against *C.beticola,* in addition to some other isolates, were identified and screened for their ability to produce some metabolites and lytic enzymes responsible for the antimicrobial activity in the Lab. Plant auxin, indole acetic acid (IAA) was produced only by four *Pseudomonas* spp.

These are Ps_1, Ps_2, Ps_4 & Ps_6. Many of the antagonistic agents belonging to fluorescent pseudomonads as well as some *Bacillus* spp. release metabolites that directly stimulate growth of many plants and improving its qualitative characters beside the major role of these microflora in biological control for many plant diseases. Recently, IAA has been shown to inhibit the growth of plant associated pathogens (Liu and Nester, 2006). Parmar and Dadarwal(2000) reported that *P. fluorescens* Pf-5, *P. fluorescens* 2-79, P. *fluorescens* isolated from rhizosphere and phyllosphere of various field crops, could stimulate plant growth and suppress plant pathogens.

Hydrogen cyanide (HCN) is produced by *P.fluorescens* (Voisard *et al.*, 1989). *P.putida* (Ps_1& Ps_{10}) and *P. aureginosa* (Ps_6) were also able to produce this metabolite, which may contribute to the potential of the bacterial strains to suppress the growth of the target pathogen. Qualitative detection of the presence of HCN in the culture media indicated that amounts produced differ greatly from one strain to another as judged by the intensity of the brown color. Strain No.7 &10 produced higher amounts of HCN compared to the others. However, these two isolates were not among those showing inhibitory potential indicating that HCN may not be the sole factor involved. Voisard *et al.* (1989) emphasized that HCN production by *Pseudomonas* strain CHAO contributes to the suppression of black root of tobacco. It is anticipated that HCN producers of *Pseudomonas* spp. under study could act as biocontrol agent towards cercospora leaf spot of sugar beet.

Production of siderophores was detected in six cultures of *Pseudomonas* spp. Siderophores are expressed as a high ability to chelate iron and create a biocontrol action through sequestering iron from pathogens, thus limiting their growth. Fluorescent pseudomonads are considered to be an excellent example for siderophore producers through the action of its fluorescent pigments, pyoverdines or pseudobactins under iron deficiency. Siderophores produced by strains of *Pseudomonas* spp., are considered as a constituent of biological products for plant disease control, and hence the bacterium could be safely employed for this purpose.

Results of hydrolytic enzymes indicated that some of the studied microorganisms were active producers of such extracellular enzymes. Production of hydrolytic enzymes by microorganisms is frequently involved in the attack of phytopathogenic fungi (Picard *et al.*, 2000). Chitinase was found to be excreted only by *T.harzianum.* It was suggested that efficiency of the bioagent in inhibiting growth of the pathogen and, in turn, controlling plant disease is attributed mainly to the excretion of chitinases that play an important role in biodegrading the cell wall of the pathogen. Some *Trichoderma* isolates were evaluated by Galletti *et al.* (2008) as possible biological control agents for the control of cercospora leaf spot.

An array of hydrolytic enzymes are known to be produced by some microorganisms causing perforations in the cell wall that may also lead to the release of chitinase by the antagonist that weakens the cell wall and increases the permeability of fungal cells, which assist in turn the penetration by the mycoparasite as suggested by Rousseau *et al.* (1996) and Lewis *et al.* (1991).

Among the studied microorganisms, *Bacillus sbtilis* ($B_{1)}$ produced lipase, protease, pectinase and cellulase. Likewise, *Trichoderma harzianum* produced chitinase, pectinase and cellulase. These results indicate that *B. subtilis* (B_1) and *T.harzianum* are promising organisms for further studies.

In conclusion, epiphytic phyllospheric microorganisms recovered from leaves of sugar beet can be promising agents for controlling cercospora leaf spot. Further study will be made to utilize these promising isolates either singly or in combination, on sugar beet plants for the control of cercospora leaf spot in the field.

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القدرة التضادية للكائنات الدقيقة من المحيط الورقى لبنجر السكر فى تثبيط سيركوسبورا بيتيكولا

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تناولت الدراسة عزل الكائنات الدقيقة من السطح الورقى لبنجر السكر بغرض دراسة قدرتها على تضاد الفطر سيركوسبورا بيتيكولا، المسبب لمرض تبقع الأوراق السركوسبورى لبنجر السكر. فقد وجد أن عدد أربعة من العزلات من أنواع بكتريا السودوموناس الفلوريسنتية وعزلتان من أنواع الباسيلوس وكذلك عدد خمسة عزلات من أنواع الأكتينوماسيتات وعزلتان من الفطريات الخيطية تملك القدرة على تثبيط نمو الفطر سيركوسبورا فى المعمل. واتضح من الدراسة أن البكتريا سودوموناس الفلوريسنتية ذات قدرة مميزة على إنتاج بعض المركبات الحيوية الثانوية ذات التأثير المثبط لنمو الكائن الممرض تحت الدراسة فى البيئات الصناعية بالمعمل. ويلي هذه المجموعة فى هذا التأثير الكائنات التابعة للأكتينوميسيتات. وقد وجد أن عزلتي الباسيلوس وكذلك عزلة التريكودرما المتحصل عليها كانت ذات كفاءة فى إنتاج الإنزيمات المحللة لجدر الخلايا. ومن ثم تم تعريف هذه الكائنات ذات القدرة التضادية معمليا. وقد أمكن تعريف الميكروبات المضادة على النحو التالى :

Pseudomonas spp., *P.putida, P.fluorescens* and *P.aureginosa.*

Bacillus spp., *B.subtilis* and *B.pumilus*

Streptomyces, S.albidoflavus, S.lavendulae, S.griseoviridis, S.purpureus and *S. rimosus*