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CONTROL OF BACTERIAL WILT (POTATO BROWN ROT) DISEASE
I. ISOLATION AND IDENTIFICATION OF SOME BIOCONTROL MICROORGANISMS

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ABSTRACT: In the last years the biological control of plant pathogens has become a very powerful tool in order to decrease damage and losses caused by important diseases. In this work, rhizospheric soil samples were collected from healthy potato plants grown in infested potato fields to isolate the associated bioagents and examine their inhibitory effect against *Ralstonia solanacearum* the causal of potato brown rot disease, *in vitro* and *in vivo*. Among the isolated bacteria and actinomycetes, only six isolates of bacteria and actinomycetes showed antagonistic effect against *Ralstonia solanacearum*. These isolates were identified and tested under greenhouse conditions. Among the isolated bioagents *Pseudomonas* spp. and *Streptomyces* spp. was found to be very potential biocontrol agent against *Ralstonia solanacearum* under greenhouse conditions. Generally, the inoculum concentrations 10^8 cfu/ml of both *Pseudomonas* sp. and *Streptomyces* sp. was the most effective.

Key words: ***Ralstonia solanacearum*, biological control, rhizosphere, *Streptomyces* sp., *Pseudomonas* sp.**

INTRODUCTION

Potato bacterial wilt or tuber brown rot caused by *Ralstonia solanacearum* is a serious disease of potato and is a major limiting factor of potato production in Egypt. *Ralstonia solanacearum* causes devastating losses in many crops such as potatoes and tomatoes, throughout the world and affecting agricultural

productivity and economy worldwide. There is no effective chemical control against potato bacterial wilt (Well and Roldan, 1922 and Chantaraotan, 1982). In the last years the biological control of plant pathogens has become a very powerful tool in order to decrease damage and losses caused by important diseases Cabrefiga and Bonaterra (2007).

Biological control has an immense potential of bacterial wilt. The basic idea in biological control is that rhizospheric microorganisms colonize and antagonize *R. solanacearum* at the root infection site to reduce infection. Certain bacteria like *Pseudomonas fluorescens, Bacillus* spp. and actinomycetes have been found to delay the development and reduce incidence of bacterial wilt (Lopez and Biosca, 2004; and Ran *et al.*, 2005 a & b).

Bacteria antagonistic towards *R. solanacearum*, have been isolated from suppressive soils and rhizosphere host plants, such as avirulent strain or mutants of *R. solanacearum*, and *Pseudomonas fluorescens* (Karuna *et al.,* 1997), *P. cepacia*, and *Bacillus subtilis*. (Anuratha and Gnanamanickam 1990). Tolba, 1998 in Egypt isolated several effective biocontrol agents from the rhizosphere of healthy plants grown in infested areas in Egypt. These bioagents include *Pseudomonas fluorescens, Streptomyces griseus, Bacillus subtilis* and *Trichoderma harizanum*.

Tawfik *et al.* (2001) reported that, pre-plant treatment of seed tubers (*cv.* Sponta) with potato antagonistic rhizospheric isolates belonging to *Bacillus subtilis, P. fluorescens* and *P. putida* were evaluated for their effect on brown rot, soft rot and black leg development during their successive summer seasons.

In Petri plate assays, a number of *Streptomyces* species produce compounds that inhibit the growth of plant-pathogenic fungi (Trejo-Estrada *et al.*, 1998) and bacteria (El-Shanshoury *et al.* 1996). When these bioagents were used to treat soil or seeds, could protect plants from bacterial diseases (Liu *et al.*, 1997).

Thus, this research work aimed to isolate and identify the biocontrol agents from the rhizosphere of healthy potato plants. The antagonistic effect of the biocontrol agents was assayed under *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Isolation and Identification of Biocontrol Agents

Rhizosphere sampling

Rhizospheric soil samples were collected from healthy potato plants grown in infested potato fields at Ezbet Naser in El-Ismailia governorate. Suspension was made for each sample by washing soil particles adhesive rhizosphere of potato roots in flasks containing 99 ml of sterile tap water and shaking for 2 h at 1000 rpm. A series of dilutions, were prepared to isolate rhizospheric organisms. Potato dextrose agar (PDA); nutrient agar (NA) and starch nitrate (SN) media were used for the isolation of fungi, bacteria and actinomycetes, respectively. The three previously mentioned media were suggested by Lelliott and Stead (1987), Jacobs and Gerstein (1960) and Shirling and Gottlieb (1966) respectively. The plates were incubated at 30°C for two days to bacteria and for ten days to actinomycetes, the developed colonies were counted/g soil and picked up every two days. Isolates obtained from fungi, bacteria and actinomycetes were used to assay their antagonistic effect against *Ralstonia solanacearum*.

Assay of antagonism of bacterial isolates *in vitro*

For cultivation, preservation and maintenance of tested bacterial organisms, nutrient agar medium was used.

According to Loo *et al.* (1945), *R. solanacearum* was used for the preparation of seed layer by inoculating melted and cooled (43°C) agar medium with 2% (v/v) of *R. solanacearum*(tuber isolate T_7), mixed thoroughly and immediately used as the seed layer of plates.

Pathogen free agar medium was poured in 9cm Petri dishes at a depth of 2mm (base layer). After solidification, 5 mm of the previously prepared seeded agar was even distributed over the surface of the base layer and left for 15 min. to solidify.

Suspension bioagents isolated previously from rhizosphere were pipetted with four replicates symmetrically around the center of the dish. Plates were allowed to remain at 10 °C for one h and then incubated at 28 ±2 °C for 48 h, antagonistic activity was determined by measuring the diameter of inhibition zone to the nearest 1 mm.

Assay of antagonism of actinomycetes isolates *in vitro*

The antagonistic effect of the actinomycetes was determined by the technique described by Abd El-Hay (1958).

The plates were re-incubated at 30°C for 24 h. then examined for their antagonistic effect, the diameter of inhibition zones were measured and tabulated.

Assay of antagonism of fungal isolates *in vitro*

The antagonistic effects of the fungal isolates were determined by the technique described by Tolba, (1998). The diameters of inhibition zones were measured and tabulated.

Identification of antagonistic bacterial isolates by morphological and biochemical's characteristic

Morphological characteristics for the rhizospheric bacterial isolates were studied on nutrient agar (NA) medium and King's B (KB)medium discribed by King *et al.*, (1954). Shape of bacterial cells, sporulation and reaction to gram stain were recorded according to the method described by (Fahy and Persley, 1983). The reaction included tobacco hypersensitivity, arginin dihydrolase, potato soft rot, oxidase test (Kovacs), and levan production were applied according to Lelliot *et al.* (1966).

Identification of antagonistic streptomyces isolates by morphological and biochemical's characteristic

Strains of streptomyces were identified according to Hutter (1967). Determinations were made for the mass color of mature, sporulating aerial mycelium, substrate mycelium, sporophore morphology and diffusible pigments in starch nitrate (SN), oat agar (OA), yeast malt extract agar (YMA), glycerol asparagin (GA) media described by Shirling and Gottlieb (1966). The reaction of carbon source utilization test, (glucose, fructose, xylose, raffinose, rhamanose. and sucrose) was carried out according to Fahy and Pershey (1983).

Greenhouse Experiment

Preparation of pots and soil infestation

Clay pots, 20 cm in diameter, were sterilized by soaking in formalin solution 5% for 15 mins. and left in air for two weeks to get

rid the toxicity of formalin. Loam soil was autoclaved for 3 h at 120°C under 1.5 bar. Three kg sterilized soil were used in 20 cm-diameter pots. Inoculum was prepared by growing *R. solanacearum* isolate for 48 h. on nutrient broth medium. Sterilized soil was separately infested with *R. solanacearum* isolate Rs_{t9} previously isolated from tubers at the rate of 50 ml(10^6 cfu/ml) inoculum / kg soil. Infestation was done one week before sowing.

Treatment of tomato seedlings

Wounded roots of healthy tomato seedling (GS12 cv., two weeks old) were soaked in three concentrations (10^4, 10^6, and 10^8 cfu/ml) of rhizospheric antagonistic bioagents (previously prepared and incubated in liquid NA, medium for 48 h at 28°C) for ten min. The seedlings were planted in pots (20 cm in diameter) previously prepared with infested soil (150 ml/pot, 10^8 cfu/ml concentration of *Ralstonia solanacearum* suspension). All treated pots were kept under greenhouse conditions at 30±2 °C and 85 % relative humidity (RH.) and irrigated daily. Three replicates were used. Untreated tomato seedlings with bioagents tested were used as a control. Data were taken after 7 and 15 days from planting as disease severity, disease reduction, and disease index.

Disease assessment

The data were recorded as disease index, disease severity, and disease reduction.

Severity of tomato bacterial wilt was evaluated as percentage of wilted plants (W) showing wilt symptoms in relation to total plant numbers. Disease severity (disease index %) was calculated from disease rating for individual plants according to (Kempe and Sequeria 1983) using the scale based on the visual observation of the percentage of foliage wilt (0 =no symptoms , 1= up to 25% , 2=26-50%, 3=51-75%, 4=76-100% and 5= dead plants).

Disease index (DI) was calculated from the following equation

$$DI=\sum \frac{R.T\ X100}{5xN} X\ 100$$

Where, T= total number of plants in each disease category

R= disease severity scale (R=0, 1, 2, 3, 4, and 5).

N = total number of tested plants.

Also percentages of disease reduction (PDR) were calculated

from percentage of wilted plants using the following equation:-

$$PDR = \frac{(Wck - Wtr)}{Wck} \times 100$$

Where, Wck = percentage of wilted plants in check treatment.

Wtr = percentage of wilted plants in treated plants.

Most of the data were statistically analysed according to Snedecor and Cochran (1967) and the L.S.D. value was also calculated as described by Fisher (1948).

RESULTS AND DISCUSSION

Isolation from the Rhizosphere

Data obtained in Table (1) show the total count of isolated microorganisms fungi, bacteria and actinomycetes from potato rhizosphere on three specific media. Results revealed that the bacterial colonies were the most countable cells with the value of 6.49, 6.31 and 6.33 with 10^{-6} cfu/g on nutrient agar, PDA and starch Nitrate (SN) media, respectively. While, the number of fungal colonies as well as actinomycetes were less than the bacterial one, where the fungal colonies ranged from 4.78 – 4.90 with 10^{-6} cfu/g soil on the tested three media and actinomycetes colonies were in the range of 4.7-5.15 with 10^{-6} cfu/g soil.

Characteristics of Rhizospheric Bacteria

Only eight bacterial isolates from the rhizospheric experiment showed antagonistic effect against *Ralstonia solanacearum*. All the selected bacterial isolates were short rods, non spore formers gram negative bacteria, produce fluorescein or yellow pigment on King's B medium Oxidase reaction positive except Ps_2, Ps_{19} and H_2, isolates; and able to form levan colonies on sucrose except Ps_1, Ps_{16}, Ps_{19} and Ps_f isolates, fail to hydrolyses arginin except Ps_1, Ps_3, Ps_{14}, and Ps_f isolates; negative reaction against tobacco hypersensitivity test except Ps_2, Ps_{16}, and Ps_{19} isolates; and couldn't cause potato soft rot except Ps_f isolate. According to the data all the rhizospheric bacterial isolates

were closely related to fluorescent pseudomonads except H_2 isolate (Brendan *et al.*, 2001).

Table 1. Count of microorganisms isolated from rhizosphere of potato plants

Medium	Isolated microorganism 10^{-6}		
	Fungi	Bacteria	Actinomycetes
Potato Dextrose Agar	4.90	6.312	4.90
Nutrient Agar	4.78	6.49	4.7
Starch Nitrate (SN)	4.78	6.33	5.15

All seven fluorescent pseudomonads were identified by LOPAT scheme (Lelliott *et al.* 1966); which divided green fluorescent pseudomonads into five groups. The characteristics of the rhizospheric isolates were suggested to be as follows:

Ps_1:	*P. tolaasii*
Ps_2:	*P. syringae*
Ps_3, Ps_{14} and Ps_f:	*P. fluorescens*
Ps_{16}:	*P. cichorii*
Ps_{19}:	*P. syringae*

Characteristic of Rhizospheric Isolates of Streptomycetes

Data in Tables 2 and 3 indicate the growth characters, morphological and physiological of properties of six isolates of streptomycetes isolated from the rhizosphere of healthy potato plants. According to characters of the above mentioned in Tables 2 and 3 these isolates were assigned to the genus *Streptomyces* (Lechevalier *et al.* 1977). The tests used to characterize and identify Streptomyces isolates to the species level were described by Williams *et al.* (1972 a and b). According to the identification key of Hutter (1967) the obtained culturs for the *Streptomyces* spp is considered to be variable and was roughly given the names *St. albus, St. aureofaciens, St. malachitofuscus, St. antibioticus, St. alboniger* and *St. griseus*.

In vitro Antagonistic Activities of Rhizospheric Organisms on *Ralstonia Solanacearum* Growth

Data in Tables 4 and 5 represented the effect of some bacterial and streptomycetes

isolates on *Ralstonia solanacearum* growth on agar plates. It was clear that, all tested streptomycetes isolates were most effective than the bacterial isolates exhibiting inhibition zone values ranging from 42.0 mm to 75.0 mm and the highest values were recorded by *St. griseus*.

Table 2. Growth characters of streptomycetes colonies isolated from rhizospheare of healthy potato plant

No. of isolate	Spore	Sporophore morphology	Spore color Hutter(1967}	No. of section	Color of growth	Name of series
St_{18}	Present	Straight	Niveus(white)	II	Yellow brown	*St. alboniger*
St_{16}		To	Cinerus(gray)	III	Yellow brown	*St. antibioticus*
St_{19}		Flexible	Griseus(yellow)	V	Yellow brown	*St. griseus*
St_1		Spirals or	Niveus(white)	VI	Yellow brown	*St. albus*
St_3		hooks and	Cinerus(gray)	VII	Yellow brown	*St. aureofaciens*
St_7		open loop	Cinerus(gray)	VII	Yellow brown +green	*St. malachitofuscus*

Table 3. Morphological and physiological properties of antagonistic streptomycetes isolates and capability fermentation carbon sources

Characters	Isolates					
	St_1	St_3	St_7	St_{16}	St_{18}	St_{19}
Cell shape			Hyphae			
Sporulation	+	+	+	+	+	+
Gram stain	+	+	+	+	+	+
Carbon sources utilization:						
Glucose	+	+	+	+	+	+
Fructose	-	+	+	+	+	+
Xylose	-	-	+	+	+	+
Raffinose	+	-	-	-	-	-
Rhamanose	+	-	+	+	+	-
Sucrose	+	-	+	-	-	+

Table 4. Inhibition effect of different bacterial isolates against *Ralstonia solanacearum* growth measured as (mm)

Strains	Inhibition zone (mm)				
	S_2	T_6	T_7	W_{16}	mean
Bacteria:					
Ps1	36	32	34	28	33
Ps2	29	31	28	33	30
Ps3	21	24	26	23	23.5
Ps14	41	38	36	42	39
Ps16	36	39	38	39	38
Ps19	27	19	43	36	31
Psf	16	12	19	10	14
Eh2	18	30	29	29	27
L.S.D.		7.11			

Table 5. Inhibition effect of different Streptomycetes isolates against *Ralstonia solanacearum* growth measured as (mm)

Isolates	Inhibition zone (mm)				
	S_2	T_6	T_7	W_{16}	mean
Streptomyces albus	**52**	**57**	**49**	**60**	**55**
St. aureofaciens	**63**	**66**	**71**	**72**	**68**
St. malachitofuscus	**43**	**49**	**51**	**42**	**46**
St. antibiotcus	**63**	**62**	**60**	**61**	**62**
St. alboniger	**53**	**54**	**53**	**55**	**54**
St. griseus	**64**	**72**	**70**	**75**	**73**
L.S.D.		5.33			

However, the bacterial isolates also were able to inhibit the growth of *Ralstonia solanacearum*, but with lower effect than those caused by streptomycetes. In general, the bacterial isolate Ps14, and Ps16 were the most effective one against *Ralstonia solanacearum*.

Effect of rhizospheric bacteria against *R. solanacearum* on tomato seedlings

It's clear from the data in Table 6 that, the bacterial inoculum 10^8 cfu/ml was the most effective one with all tested bacterial isolates recorded disease severity value between 0-2. While inoculum concentrations 10^4, and 10^6 cfu/ml recorded disease severity values in the range of 1-3. On the other hand, disease reduction values ranged from 62.5 to 87.5, 40.0 to 93.33 and 70.0 to 100 using inoculum concentrations 10^4,10^6 and 10^8 cfu/ml, respectively. Regarding to disease index the data in the same table illustrate that, inoculum concentrations 10^8 cfu/ml recorded the lowest value varied from 0-24. While inoculum concentrations 10^4 and 10^6 disease index ranged from 4-36. Generally, the inoculum concentrations 10^8 cfu/ml was the most effective one.

Table 6. Effect of antagonistic rhizospheric bacteria on tomato plants as percentage of disease severity, disease reduction and disease index

Treatment	Disease severity concentrations			Disease index % concentrations			Disease reduction % concentrations		
	10^4	10^6	10^8	10^4	10^6	10^8	10^4	10^6	10^8
Ps_1	3	2	1	81.25	73.33	90	12	16	8
Ps_2	2	2	2	87.5	60	70	8	24	24
Ps_{14}	1	1	0	81.5	93.33	100	12	4	0
Ps_{16}	2	3	1	62.5	60	85	24	24	12
Ps_{19}	2	3	1	75	40	85	16	36	12
Eh_2	1	2	1	87.5	60	85	8	24	12
Healthy plants	0	0	0	100	100	100	0	0	0
Infested plants	4	5	5	0	0	0	64	60	80
L.S.D.	1.30	1.85	1.83	6.18	6.18	6.03	6.66	6.18	6.18

Bacterial isolates especially *Pseudomonas* sp. is known as antagonistic against plant pathogenic bacteria and has been found to be a very potential biocontrol agent against soil borne plant pathogenic bacteria under greenhouse and field conditions Cabrefiga, and Bonaterra (2007).

The findings of this study are in agreement with those obtained by Khalequzzaman *et al.* (2002). They reported that *P. fluorescens* was used in controlling wilt of tomato caused by *R. solanacearum* and increased the yield under greenhouse conditions.

This might be due to the production of an antibiotic substance on siderophores (Mulya *et al.*, 1996), or might be also due to inducing plant growth and disease suppression (Kumar *et al.*, 2001).

Effect of rhizospheric Streptomycetes against *R. solanacearum* on tomato seedlings:

It's clear from the data in Table 7 that, the streptomycetes inoculum at 10^8cfu/ml was the most effective one with all tested streptomycetes isolates recorded disease severity value between 0-2. While inoculum concentrations 10^4, and 10^6 cfu/ml recorded disease severity values in the range of 1-4. On the other hand, disease reduction (%) values ranged from 60 to 95, 43.75 to 87.5 and 85 to 100% using inoculum concentrations of 10^4, 10^6 and 10^8 cfu/ml, respectively. Regarding to disease index the data in the same table illustrate that inoculum concentrations of 10^8cfu/ml recorded the lowest value ranging from 0-12. While in inoculum concentration of 10^4 and 10^6 cfu/ml disease index ranging from 4-36. Generally, the inoculum concentration of 10^8 cfu/ml was the most effective dose.

The control of soil borne plant pathogens presents a challenge to potato growers. Although disease-resistant potato varieties are available, resistance is often incomplete. Chemical control, when available, might not be suitable due to the economic costs and environmental risks associated with application. Brown rot disease is important challenge to potato growers, as it cause significant yield and quality losses, and current control methods for this disease is inadequate (Hooker, 1981).

In recent years ,there has been spectacular development in using

Table 7. Effect of antagonistic rhizospheric *Streptomyces* spp. (St) on tomato plants as percentage of disease severity, disease reduction and disease index

Treatment	Disease severity concentrations			Disease index % concentrations			Disease reduction % concentrations		
	10^4	10^6	10^8	10^4	10^6	10^8	10^4	10^6	10^8
St_1	1	2	1	90	87.5	85	8	24	12
St_3	2	2	1	64	50	90	16	32	8
St_7	1	2	0	75	50	100	20	32	0
St_{16}	3	1	2	70	75	90	24	16	8
St_{18}	4	3	1	60	43.75	85	32	36	12
St_{19}	1	1	0	95	87.5	100	4	8	0
Healthy plants	0	0	0	100	100	100	0	0	0
Infested plants	4	4	5	0	0	0	80	64	80
L.S.D.	1.23	1.87	3.21	7.09	6.58	6.49	7.08	9.28	9.28

an alternative non toxic means of plant disease control. Biological control of root pathogens involved several strategies such as, (a) microbial suppression of infection ,the major of naturally occurring as well as introducing yeast or fungi seems to cause removal of infection or stimulating exogenous nutrients (Gava *et al.,* 2002). (b) microbial suppression of pathogen Sporulation, (Guo *et al.,* 2004). Suppression of the dissemination of the pathogen will reduce the progression of epidemics (Haas, and Défago 2005). This approach allows long interaction period between the antagonist and the pathogen and is successfully applied in the control of bacterial wilt disease (Cook, 1993). Streptomyces prolific antibiotic

production has made them the subject of numerous studies on the biological control of plant-pathogenic bacteria, fungi, and nematodes. The addition of antagonistic streptomycetes has been shown to reduce diseases caused by *R. solanacearum* (Tolba, 1998). The pathogen inhibitory activity of the indigenous soil microbial community, specifially the streptomycetes, offers a potential alternative for plant disease control. The result in this work revealed that streptomycetes isolates could reduce the bacterial wilt caused by *R. solanacearum* compared to the control .The antagonistic activities of the used isolates might be due to the production of antibiotic secondary metabolites which reduces the bacterial wilt disease. This result agree with the result mentioned by Rosales *et al.* (1995). Also, Dabour (2001) mentioned that antheracycline an antibiotic produced by *S.violacues* T118 was used for biological control of faba bean diseases.

Christopher and Bonnie (2005) reported that, Streptomycetes are a diverse family of gram-positive soil-dwelling bacteria that are of clinical relevance because they are a major biological reservoir of secondary metabolites, many of which are used as antibiotics (reviewed in Chater and Horinouchi 2003).

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مقاومة مرض الذبول البكتيري (العفن البني) في البطاطس
I. عزل وتعريف كائنات المكافحة الحيوية

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في السنوات الأخيرة أصبحت المقاومة الحيوية للمسببات المرضية للنباتات من الادوات القوية جدا في تقليل حجم الخسائر الناتجة عن الامراض.

وفي هذا البحث تم جمع عينات من منطقة الريزوسفير المحيطة بنباتات البطاطس السليمة المزروعة في حقول مصابة بالعفن البني وذلك لعزل كائنات المكافحة الحيوية واختبار مدي مكافحتها لمسبب مرض العفن البني بكتريا الرالستونيا سولاناسيرم تحت ظروف المعمل و الصوبة وقد تم الحصول علي ستة عزلات من البكتريا وستة عزلات من الاكتينومايسيتس تم تعريفهم وأختبار مدي قدرتهم علي تثبيط نمو المسبب المرضي في المعمل وكانت أفضل نتائج التضاد لعزلات جنس البكتريا بسيدوموناس و لعزلات الستربتومايسيس وتم اختبارهم في الصوبة علي شتلات الطماطم بتركيزات مختلفة وكانت افضل النتائج المتحصل عليها مع التركيز ١٠^٨ خلية/مل.