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BIOLOGICAL CONTROL OF SOME PLANT GROWTH PROMOTING RHIZOBACTERIA AGAINST *Ralstonia solanacearum* CAUSING POTATO BROWN ROT

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By

[**]O.M. El-Haj Saleh , N. F. Amin, H. E.Makboul and [*] M.N.A. Omar

Agriculture Microbiology Department, Faculty of Agriculture, Cairo University.
[*] Soils, Water and Environment Research. Institute, Department of Microbiology, Agriculture Research Center.
[**] Potato Brown Rot Project (PBRP), Giza, Egypt.

ABSTRACT

Laboratory and greenhouse experiments were conducted at the Potato Brown Rot Project (PBRP), Giza, Egypt to evaluate the ability of some strains of plant growth promoting rhizobacteria (PGPR) to alleviate the pathogenicity of *Ralstonia solanacearum* causing potato brown rot disease. The bacterial strain was isolated from naturally infected potato tubers and identified by PCR and Real-time PCR techniques as *Ralstonia solanacearum* race 3 biovar 2. Results of the antagonistic activity of the tested bio-agents against *Ralstonia solanacearum* showed that all of the tested (PGPR) strains variably exhibited antibacterial effects against *Ralstonia solanacearum*. Under greenhouse conditions PGPR significantly stimulated the growth of potato Draga CV. Shoot and root dry weights as well as root length and shoot height significantly increased when potato was inoculated with the biocontrol agents either as single inocula or in combination. The treatment which received the mixture of all tested bioagents (*Bacillus circulans, Bacillus polymyxa, Bacillus pasteurii , Bacillus megaterium and Pseudomonas fluorescence*) was the superior among the other treatments. A positive response was recorded on the NPK-contents of the plants as a result of inoculation with the PGPR strains.

***Key words**: antagonistic activity, biocontrol agents, PGPR,Potato (Draga CV.), Ralstonia solanacearum.*

1. INTRODUCTION

Ralstonia solanacearum is classified as one of the world's most important phytopathogenic bacteria due to its lethality, persistence, wide host range and broad geographic distribution. Although the pathogen causes major yield loss in the tropics and subtropics, it is currently a continuing threat in temperate climates (Denny, 2006). It causes a wilt disease in several important agricultural crops such as potato, tomato, tobacco, banana, pepper and eggplant. The disease is known as Southern wilt, bacterial wilt, and brown rot of potato (Denny, 2006). Originally, *Ralstonia solanacearum* is found in tropical, sub-tropical and warm temperate climates, but is not believed to survive in cold temperatures. *R. solanacearum* was classified by Denny (2006) into five races based loosely on host range and into five biovars based on differential ability to produce acid from a panel of carbohydrates. *Ralstonia solanacearum* race 3 biovar 2 is among the most serious diseases of potato worldwide, which is responsible for an estimated $950 million losses each year caused by potato brown rot **(**Elphinstone, 2005). The biological control of soil born pathogens depends mainly on the use of plant growth promoting rhizobacteria (PGPR) producing antibacterial substances, colonizing plant roots and reducing disease. There are many researches interested in PGPR and now there is an increasing number of PGPR being commercialized for different crops. It is also possible to inoculate seeds with bacteria that increase the availability of nutrients, including solubilizing phosphate, potassium, oxidizing sulphur, fixing nitrogen, chelating iron and copper **(**Benizri *et al*. 2001). In the

rhizosphere, bacteria have been shown to be effective as antagonists against pathogenic bacteria. *Bacillus* species producing antibiotics have been used as biocontrol agents against pathogenic bacteria (Yilmaz *et al*. 2005). Tubers inoculated with the bio-agents hosted significantly low numbers of *R. solanacearum* in the rhizoplane. The effect of *Pseudomonas* spp as a bioagent was also extended to the endorhizosphere. Moreover, inoculation with the bioagents increased the population of diazotrophs and P-solubilizers associated with different plant spheres (Tarek, 2010). The aim of this study was to evaluate the biological control of certain PGPR strains against the pathogenic bacterium *Ralstonia solanacearum* in potato plants.

2. MATERIALS AND METHODS

2.1. Isolation of *Ralstonia solanacearum* from potato tubers

Potato tubers having internal symptoms of the brown rot disease were used for the isolation of *R. solanacearum*. Samples were collected from the traditional potato districts in Ismailia Governorate from sonac farm Pivot 8 . Tubers were washed in running tap water, surface sterilized by flaming and the stolon ends were aseptically removed. The virulent *R. solanacearum* was selected and propagated on glucose nutrient agar (GNA) medium for 48 hours. The selected cultures were serologically examined using immunofluorescent antibody staining (IFAS) to confirm *R. solanacearum* identity. Positive isolates were tested for their pathogenic potential *via* inoculation into potato tubers (Janse, 1988 and Wenneker *et al*. 1999).

2.2. Pathogenicity of the isolates

The pathogenicity of *R. solanacearum* isolate recovered from different materials were confirmed by inoculating potato plants (3 leaves/seedling), grown in sterilized sandy soil in pots under greenhouse conditions, by the stem puncture technique (Janse, 1988). Injection was made at the leaf axis by a needle laden with the bacterial growth of the pathogen. Control treatments were prepared by applying few drops of sterile water instead of bacteria. Inoculated plants were covered with polyethylene bags for three days at 30°C, then bags were removed and pots were irrigated daily. The disease progress was determined according to the scale described by Kempe and Sequeira (1983). This scale was based on the visual observation of the percentage of foliage wilt (Zero = no symptoms; 1 = one or 2 leaves wilted (up to 25%), 2 = three leaves wilted (26-50%), 3 = four or more leaves wilted (51-75%), 4 = (76-100%) and 5=dead plant), then the disease index (DI)was calculated by the following formula:

$$\mathbf{DI=\sum(R.T)/ (5xN)_X\ 100}$$

where,**T=**the total number of plants in each category
R = disease severity scale (R=0, 1, 2, 3, 4, and 5).
N = the total number of tested plants.

2.3. Identification of the pathogens

The pathogenic isolate was identified according to Bergey's Manual of Determinative Bacteriology (Garrity , 2012). Tests were based on the biochemical (24 tests), physiological and morphological characteristics.

2.4. Immunofluorescence antibody stain (IFAS)

IFAS is a serological method for rapid detection and presumptive identification of bacteria. The polyclonal antibodies are produced in rabbits against living whole cells (Van der Merwe, 1989), heat killed (Coleno *et al*., 1976), formalized (Coleno *et al*., 1976) or glutaraldehyde-fixed (Robinson,1993). The anti-rabbit antiserum is conjugated with fluorescin isothyocyanate (FITC) and used along with IFAS testing.

2.5. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is one of the very sensitive methods used for the detection of *R. solanacearum*. It is based on the techniques described by Seal and Elphinstone (1993) using *R. solanacearum* specific oligonucleotide primer OLI-1 (5` GGG GGT AGC TTG CTA CCT GCC3`) and non-specific primer Y-2, (5` CCC ACT GCT GCC TCC CGT AGG AGT 3`).

2.6. Real-time, Fluoro genic PCR (Taq-Man) assay

The high specificity of this detection method and the availability of 2 specific probes (one for all *R. solanacearum* strains and one specifically for bv_2) makes it an excellent method for identification of *R. solanacearum* isolates.

2.7. Pot experiment

2.7.1. Potato tubers

Potato tubers (Draga CV.) were used in this experiment and obtained from Potato Brown Rot Project (PBRP), Dokki, Egypt. Sandy soil samples were collected from sonac farm Pivot 8 at Ismailia Governorate.

2.7.2. Bioagents strains

Some strains of PGPR were used in this study as biocontrol agents against *R. solanacearum*. Those are *Bacillus circulans*, *Bacillus polymyxa*,

Bacillus pasteurii, Bacillus megaterium and Pseudomonas fluorescence and they were obtained from Soils, Water and Environment Res., Ins.,epartment of Microbiology, ARC, Giza, Egypt.

2.7.3. Preparation of potato tubers for planting

Eyes of potato tubers and transactions were sterilized with sodium hypocloried (5.0%) for 5 minutes followed by washing with tap water four times. Individual eyes were left for 48 h to form calles. The eyes were inoculated with the biocontrol strains either singly or a mixture of all.

The treated eyes were left one day for developing the inoculated bacteria, then cultivated in the experimental pots.

2.7.4. Soil samples

Sandy soil was autoclaved for 3 h at 120°C, and transferred to the pots (40 cm in diameter). Sterilized soil was infested with the pathogenic isolate *R. solanacearum* one week before sowing.

2.7.5. Experimental design

Healthy surface sterilized potato tubers as whole tuber and/or seed pieces previously inoculated with either bio-agent bacteria (10^8 cfu/ml) were planted in 40 cm diameter pots containing infested soil with *R. solanacearum*. The disease was assessed after 21, 30,45 and 60 days of planting. The data were measured as shoot dry weight and height, root length and dry weight as well as NPK- contents of plant shoot.

2.8. Antagonistic assay

Disc diffusion method (Bajpai *et al.* 2009) was used and 0.5 ml from the suspension of R. solanacearum (107 cfu/m) was spread onto agar plates of King's B. (Proteose peptone 20.0 g, K2HPO41.5 g, MgSO41.5 g, Glycerol15ml, Distilled water1000 ml and Agar 20 g). Sterile filter paper discs (Whatman No. 1 1.5 mm diameter) were soaked with ca. 108 cfu/m of the bioagent suspensions, 4 discs per plate, with consideration of control plate where the filter paper discs were soaked in sterilized water. Plates were incubated upside down at 28°C for 48h. Antibacterial activity was evaluated by measuring the diameter of the clear inhibition zone against the tested pathogenic bacterium.

2.9. Statistical analysis

The analysis of variance of the presented data was carried out according to SPSS virgin 15. Means were compared using the LSD at 5% level of probability of experimentation (Agricultural research center, Central laboratory for design and statistical analysis research).

3. RESULTS

3.1. Isolation of the pathogenic bacterium

R. solanacearum was isolated from infected potato tubers grown in the field with previous history of the potato brown rot.

3.2. Characterization of the isolates

The colonies developed on the selective medium (SMSA) were milky, white, irregular and fluidal with red coloration in the center. The virulent forms were less fluidal or the fluidal colonies were completely pink to red as described by Elphinstone *et al.* (1996). The isolates are Gram- negative rod- shaped.

3.2.1. Immuno-fluorescent antibody staining

The isolates did not show any serological variation in IFAS test, either for cell morphology or the degree of fluorescence**.**

3.2.2. Polymerase chain reaction (PCR)

PCR was used in this work for verification identity of *R .solanacearum* using a forward oligonucleotide primer RS-1-F (5`-ACT AAC GAA GCA GAG ATG CTA TA - 3`) and Reverse Prime RS-1-R, (5-`CCC AGT CAC GGC AGA GAC T - 3`). The expected amplicon size from *Ralstonia solanacearum* template DNA is 718 bp. The isolates from symptoms in potato tubers in concern did not show any strain variation (Fig.1).

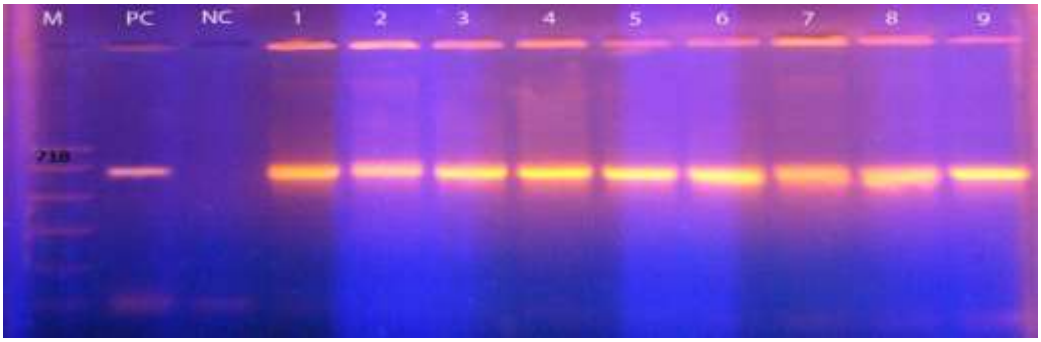

Fig. (1): *R .solanacearum* **bands in agrose gel electrophoresis isolates.**
where: M, marker, NC, negative control,PC, positive control, 1-9, potato tubers.

3.2.3. Real-time, Fluorogenic PCR (Taq-Man) assay

Taq-Man is a molecular detection method that combines polymerase chain reaction (PCR) with fluorescent detection of the amplicon (Weller *et al.* 2000) . In this assay, the isolates in concern did not show any pronounced variation in similarity (Fig.2).

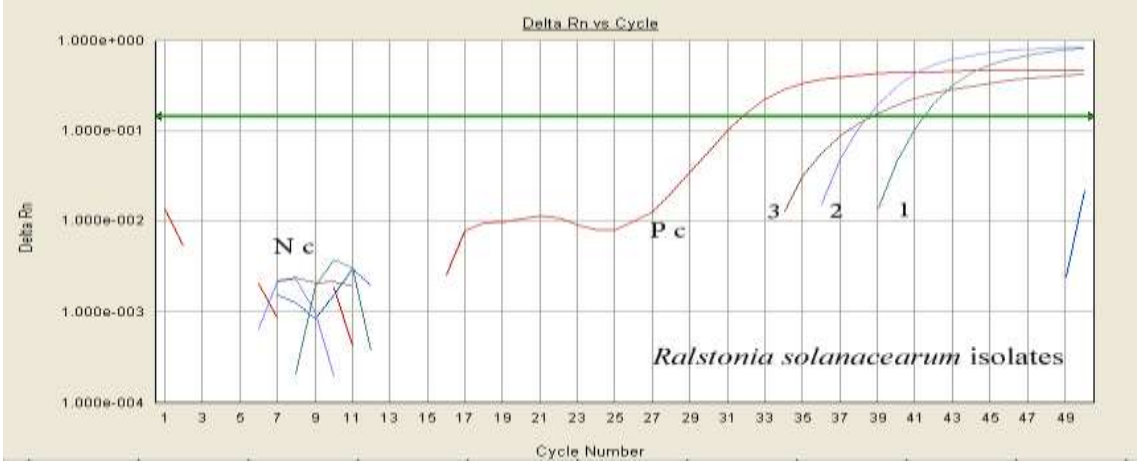

Fig.(2): Isolates of *R .solanacearum* in Real-time.
Where: NC, negative control, PC, positive control (*R .solanacearum*).

3.3. Pathogencity of the selected isolates

Pathogenicity of the selected isolates was determinated with a potato plant under greenhouse conditions. All isolates exhibited pathogenicity to potato plants. Variable degrees in wilting disease were noticed between the isolates as shown by the disease onset that ranged between 3 and 7 days after planting and the wilt severity as shown by collect wilting of the inoculated potato plant (Fig.3).

3.4. Antagonistic effect of some PGPR strains against *R .solanacearum*

Results in Table (1) show the direct confrontation between the tested strains of PGPR *(Bacillus circulans, Bacillus polymyxa, Bacillus pasteurii, Bacillus megaterium, Pseudomonas fluorescence* or mixture of all) and the pathogenic bacterium (*Ralstonia solanacearum*). All tested strains showed antagonistic effects against the pathogen. The highest inhibition zones were recorded with *Bacillus polymyxa, Bacillus megaterium* and the mixture of all (5 mm), followed by *Bacillus pasteurii* and *Bacillus circulans* (4 mm). *Pseudomonas fluorescence* exhibited the lowest effect with 3 mm inhibition zone.

As illustrated in (Fig.4), the growth of *Ralstonia solanacearum* in presence of the different bioagents completely disappeared as recorded using the Real-time technique.

3.5. Effect of bio-agents on the growth of potato plants infected with *Ralstonia solanacearum*

3.5.1. Root parameters

The root dry weights of potato variety Draga infected with *Ralstonia solanacearum* and treated with different bio-agents of PGPR are found in Table (2). All the bioagent treatments, either alone or in mixture, significantly improved the dry weight of roots compared with the untreated control infected with *Ralstonia solanacearum.* It is worthy to mention that the mixture of all the bioagents was the superior among the tested treatments.

At the early stage of growth (after 21 and 30 days of planting) there were no significant differences in the root length of potato plants observed between the treated or untreated control (Table.3). At the end of the experiment (60 days) the root length of potato plants infected with *Ralstonia solanacearum* and inoculated with the tested bio-agents significantly increased compared with the non inoculated infected control. The highest root length was recorded with the treatment inoculated with either *Bacillus circulans* or *Bacillus megaterium.* The root length value varied depending on the strain type of bio-agents used. Generally, all treatments inoculated with the bioagents recorded the same effect, except the treatment with *Pseudomonas fluorescence* which exhibited the least increase in root length.

Wilt diseased plant **Healthy plant (control)**

Fig.(3): Wilt diseased and a healthy plant (control).

Table (1): Antagonistic activity of the tested bioagents against *Ralstonia solanacearum**(R.S.)*.

Bacterial strains	Inhibition zone(mm)
Bacillus circulans	4
Bacillus polymyxa	5
Bacillus pasteurii	4
Bacillus megaterium	5
Pseudomonas fluorescence	3
mixture of all	5

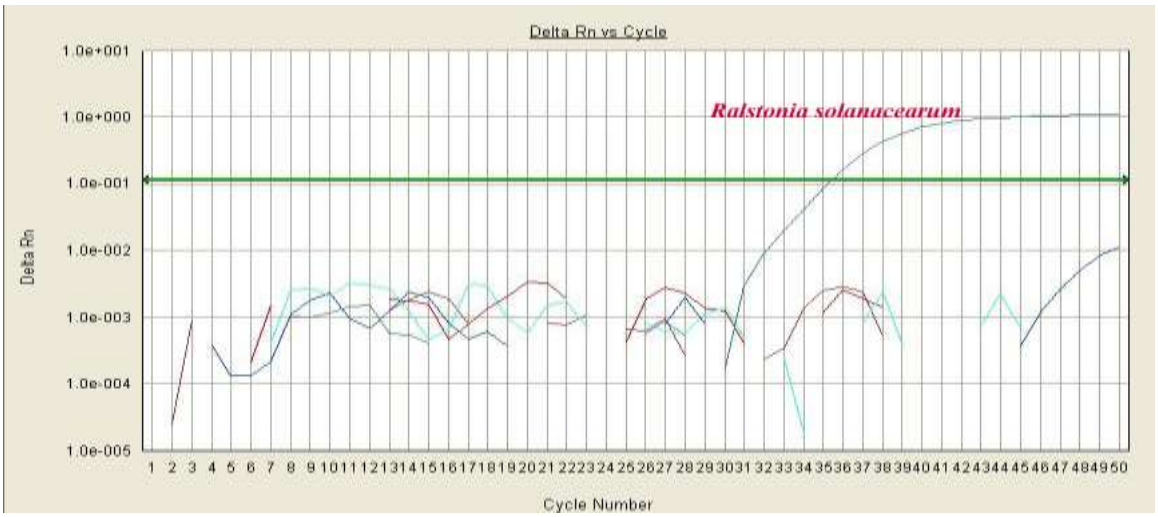

Fig.(4): Effect of Bioagents (PGPR) with *R .solanacearum* in Real-time.

Table (2): Influence of different strains of PGPR on root dry weight (g plant^{-1}) of Draga CV infected with *Ralstonia solanacearum*.

Treatments	Days after planting			
	21	30	45	60
Healthy plant(control)	**0.16**	**0.21**	**0.30**	**0.39**
R.solanacearum(control)	**0.11**	**0.10**	**0.08**	**0.06**
R.solanacearum+B. circulans	**0.12**	**0.14**	**0.17**	**0.28**
R.solanacearum+B. polymyxa	**0.12**	**0.15**	**0.20**	**0.28**
R.solanacearum+B. pasteurii	**0.12**	**0.16**	**0.20**	**0.27**
R.solanacearum+B. megaterium	**0.12**	**0.16**	**0.20**	**0.26**
R.solanacearum+P. fluorescence	**0.12**	**0.16**	**0.21**	**0.28**
R.solanacearum+ Mixture of all	**0.13**	**0.16**	**0.22**	**0.28**
L.S.D.5%	**0.03**			

Table(3): Influence of different strains of PGPR on root height (cm plant) of Draga CV infected with *Ralstonia solanacearum*.

Treatments	Days after planting			
	21	30	45	60
Healthy plant(control)	**5**	**8.5**	**15**	**18**
R.solanacearum(control)	**3**	**6**	**4.5**	**3**
R.solanacearum+B. circulans	**4**	**8**	**11**	**15**
R.solanacearum+B. polymyxa	**4**	**8**	**10**	**14**
R.solanacearum+B. pasteurii	**4**	**7**	**11**	**14**
R.solanacearum+B. megaterium	**4**	**8**	**11**	**15**
R.solanacearum+P. fluorescence	**4**	**6**	**9**	**12**
R.solanacearum+ Mixture of all	**4**	**8**	**10**	**14**
L.S.D.5%	**1**			

3.5.2. Shoot parameters

The shoot dry weight of potato plants Draga CV, exhibited highly significant decrease in the treatment infected with *Ralstonia solanacearum* when compared with the healthy plants which received no pathogenic bacteria (Table.4). The inoculation with the strains of PGPR, either in single inoculum or in combination, the shoot dry weight increased significantly compared with infected treatment with *Ralstonia solanacearum*. The rate of increase was positively correlated with the time of plant growth. The plants inoculated with *Bacillus polymyxa* recorded higher shoot dry weight level than that observed with the other treatments, even the mixture after 60 days of planting.

Table (4): Influence of different strains of PGPR on shoot dry weight (g plant^{-1}) of Draga CV potato infected with *Ralstonia solanacearum***.**

Treatments	Days after planting			
	21	30	45	60
Healthy plant(control)	**0.37**	**0.85**	**1.45**	**1.80**
R.solanacearum(control)	**0.32**	**0.56**	**0.33**	**0.25**
R.solanacearum+B. circulans	**0.35**	**0.74**	**1.20**	**1.33**
R.solanacearum+B. polymyxa	**0.35**	**0.75**	**1.25**	**1.40**
R.solanacearum+B. pasteurii	**0.35**	**0.70**	**1.22**	**1.34**
R.solanacearum+B. megaterium	**0.35**	**0.75**	**1.26**	**1.38**
R.solanacearum+P. fluorescence	**0.33**	**0.70**	**1.21**	**1.32**
R.solanacearum+ Mixture of all	**0.34**	**0.75**	**1.25**	**1.38**
L.S.D.5%	**0.02**			

Regarding the effect of different bioagent treatments on shoot height of potato plants Draga CV the inoculation with any of the bio-agents used, reduced the brown rot disease(Table.5),. Such disease reduction reflected an increase in shoot length of the potato plants compared with the infected control with *Ralstonia solanacearum* and untreated with bio-agents. The highest increase was recorded with the treatment inoculated with a mixture of all bioagents used.

Table(5): Influence of different strains of PGPR on shoot height (cm plant) of Draga CV potato infected with *Ralstonia solanacearum*

Treatments	Days after planting			
	21	**30**	**45**	**60**
Healthy plant(control)	**26**	**37**	**46**	**57**
R.solanacearum(control)	**23**	**32**	**24**	**14**
R.solanacearum+B. circulans	**24**	**35**	**45**	**51**
R.solanacearum+B. polymyxa	**24**	**34**	**45**	**52**
R.solanacearum+B. pasteurii	**25**	**33**	**44**	**51**
R.solanacearum+B. megaterium	**25**	**33**	**45**	**52**
R.solanacearum+P. fluorescence	**24**	**33**	**44**	**51**
R.solanacearum+ Mixture of all	**25**	**36**	**45**	**54**
L.S.D.5%	**1.87**			

NPK -contents of potato plants

3.6. NPK -contents of the potato plants

Table (6) represents the influence of the tested biocontrol agents on NPK-contents of the infected potato plants Draga CV. As expected, the infection with the pathogenic bacterium led to decreases in the contents of NPK in plant shoots. Contents evidently varied depending upon the type of bioagent used. Inoculation with *Bacillus pasteurii* recorded the highest nitrogen and phosphorus contents, while potassium was the superior when the plants were inoculated with *Bacillus circulans*, where the corresponding values were 2234,750 and 965ppm/plant, respectively. It is obvious that using such bacteria as a biocontrol improved the content of NPK in shoot plants more than that recorded in healthy plant.

4. DISCUSSION

Isolation of pathogenic bacteria from potato tubers showed characteristics relared to those described for race 3 biovar 2 of *R. solanacearum*. IFAS is one of the most sensitive serological tests for detecting pathogenic bacteria (De Boer *et al.* 1996). It is valid to confirm the presence of *R. solanacearum* but not able to detect the races or biovars. On the other hand, it is not completely reliable due to the possible cross reaction with other bacteria (Farag *et al.*, 2004; Matter, 2007). The isolates in concern did not show strain variation among them. Potato tuber isolates showed a single band at 718pb. Similar results

Table(6): Effect of different bioagents on NPK-contents (ppm) of potato plants Draga CV infected with *Ralstonia solanacearum*

Treatments	After 60 days planting		
	N	**P**	**K**
Healthy plant(control)	1532	678	676
R.solanacearum(control)	1112	611	611
R.solanacearum+B. circulans	1600	654	965
R.solanacearum+B. polymyxa	2175	632	765
R.solanacearum+B. pasteurii	2234	750	654
R.solanacearum+B. megaterium	1211	623	721
R.solanacearum+P. fluorescence	1476	655	612
R.solanacearum+ Mixture of all	1300	567	644
L.S.D.5%	1		

were obtained by Balabel (2006) and Farag (2007). The *R. solanacearum* primers and probe detected all biovars and races of virulent *R. solanacearum,* whereas the B2 primer and probe are specific for the detection of the race 3 biovar 2 strain. Positive results were obtained in both assays with all the three isolates, indicating that the isolates were all *R. solanacearum* biovar 2 race 3. The isolated strains of *R. solanacearum* exhibited pathogenicity and proved to have the ability to infect potato plants and cause brown rot disease. The antagonistic activity of *Bacillus circulans, Bacillus polymyxa, Bacillus pasteurii, Bacillus megaterium and Pseudomonas fluorescence* against the isolated strain of *R. solanacearum* was recorded in this study. All the tested bioagent strains exhibited antagonistic effect against the pathogenic *R. solanacearum* with different levels of growth inhibition. These results are in agreement with those obtained by Pederson and Reddy (1997) and Yilmaz *et al.* (2005). In another point of view, the antagonistic effects of these bioagents may be due to their potential activity to suppress bacterial wilt disease development and proved the role of such strains as plant growth-promoting rhizobacteria (PGPR) for potato (Aliye *et al.* 2008). Plant growth promoting rhizobacteria (PGPR) strains were reported to be a promising bio-control agents to control *R. Solanacearum*. It was found that they were able to reduce the disease in different levels and increased the yield of tomato plant (Guo *et al*. 2004). Under greenhouse conditions, the present results clearly confirm that the plants treated with PGPR strains significantly reduced the disease compared to the infected control, as well as supported greater plant of biomass (fresh and dry weights). Disease reduction by PGPR in colonization of plant roots may occur directly, through a competition for space and nutrients (Kloepper and Beauchamp, 1992; Liu *et al*., 1995) or may induce plant growth promotion by direct or indirect modes of action (Beauchamp, 1993; Lazarovits and Nowak, 1997). The present results agree with the reports that plants treated with *Bacillus* spp. and *Pseudomonas fluorescence* exhibited wilt disease reduction caused by *R. Solanacearum* and also improved tomato plant growth Aliye *et al.* (2008) and Guo *et al*. (2004).

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المقاومة الحيوية لبعض البكتيريا المنشطة للنمو ضد البكتيريا المسببة لمرض العفن البني في البطاطس

** أمامة محمد الحاج صالح - نادية فهمي أمين - حسين إمام مقبول - *محمد نبيل عمر

قسم الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة القاهرة.
*معهد بحوث الأراضي والمياه والبيئة- قسم الميكروبيولوجيا الزراعية- مركز البحوث الزراعية .
**مشروع حصر ومكافحة مرض العفن البني في البطاطس – الدقي – الجيزة- مصر.

ملخص

أجريت تجارب معملية وتحت ظروف الصوبة الزجاجية بمشروع حصر ومكافحة مرض العفن البني للبطاطس بالجيزة ، وذلك لتقييم دور بعض أنواع من البكتيريا المنشطة للنمو (PGPR) لمقاومة بكتيريا رالستونيا سولاناسيرم *Ralstonia Solanacearum* المسببة لمرض العفن البني في البطاطس. تم تعريف السلالات التي عزلت من درنات البطاطس المصابة بالمرض عن طريق استخدام تكنيك تفاعل البلمرة المتسلسل (PCR) وأيضا طريقة Real-time على أنها السلالة 3 الطرازالبيولوجي2. وقد أثبتت النتائج المتحصل عليها من اختبار التضاد الحيوي بين البكتيريا الممرضة (بكتيريا رالستونيا سولاناسيرم *Ralstonia Solanacearum*) والسلالات البكتيرية المختبرة للمقاومة الحيوية أن كل سلالات ال PGPR المختبرة كان لها تأثير مضاد على بكتيريا رالستونيا سولاناسيرم *Ralstonia Solanacearum* بدرجات متفاوتة. أما تحت ظروف الصوبة الزجاجية، فقد أدت بكتيريا ال PGPR إلى زيادة نمو صنف البطاطس(دراجا) زيادة معنوية والمتمثلة في زيادة الوزن الجاف للمجموع الجذري والخضري وأيضا زيادة أطوال السيقان والجذور سواء كانت المعاملات في صورة منفردة أو في صورة مختلطة. وأظهرت المعاملة المختلطة التي تحتوي على كل المعاملات الحيوية المستخدمة في الدراسة وهي (*Bacillus circulans, Bacillus polymyxa, Bacillus pasteurii, Bacillus megaterium and Pseudomonas fluorescence*). أعلى تأثير مقارنة بالمعاملات البكتيرية المنفردة. كما سجلت هذه المعاملة أعلى تأثيرعلى محتوى عناصر النيتروجين والفوسفور والبوتاسيوم في النبات.

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