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Paenibacillus polymyxa and *Bacillus aryabhattai* as Biocontrol Agents against *Ralstonia solanacearum In Vitro* and *In Planta*

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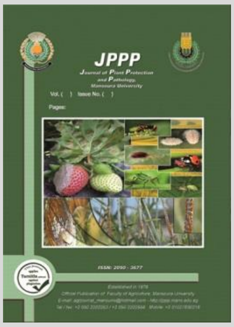

ABSTRACT

The present study aimed to biologically controlling the potato bacterial wilt disease which caused by *Ralstonia solanacearum* by antagonistic bacterial isolates which isolated from soil. Two bacterial isolates (coded, MAS400 and MAS100) obtained from soil of some field grown potato crops was tested to against *Ralstonia solanacearum* bacterium *in vitro* and *in planta*. The isolates showed various ability to inhibit *R. solanacerum* growth *in vitro*. The isolate MAS400 exhibited highly inhibitory activity (3.7 cm), while the isolate MAS100 showed moderate antagonistic activity (0.9 cm). Molecular identification and 16S rDNA sequencing for the antagonistic bacterial isolates showed that, the isolate MAS400 is *Bacillus aryabhattai* with percent identity 80.56% and the isolate MAS100 is *Paenibacillus polymyxa* with percent identity 97.63% compared to known bacterial sequences in the NCBI (National Center for Biotechnology Information) databases. The isolate MAS100 was deposited in Genebank NCBI with accession number MN971671. Assessment of antagonistic bacteria as biocontrol agents for suppression potato bacterial wilt disease showed that, symptoms were less severe and the appearance of wilt symptom on infected plants was delayed compared to the control. Trials with *Paenibacillus polymyxa* isolate on potato plants had a lower disease incidence (20 %) with maximum disease reduction (80 %), whereas *Bacillus aryabhattai* isolate had a higher disease incidence on potato plants (60 %), with lower disease reduction (40 %). Isolates *Paenibacillus polymyxa* showed the highest suppression on potato plants.

Keywords: Biological control**,** *Ralstonia solanacearum*, *Paenibacillus polymyxa*, *Bacillus aryabhattai* and bacterial wilt disease.

INTRODUCTION

Bacterial wilt in solanaceous plants which caused by *Ralstonia solanacearum*, (previously known as *Pseudomonas solanacearum*) is one of the important devastating soil inhabitant bacterium which distributed throughout different regions including the tropical, subtropical and some warmer regions of the world, and often results in colossal major loss in agricultural production Hayward (1991).

Different methods have been enhanced to control bacterial wilt disease, however it still lack an efficient and environmental friendly control measure for much of the host crops. Many international bacterial wilt symposia have been held to understand the bacterial wilt disease control differently in some locations in different countries across the world such as, Toulouse at 2016, Wuhan at 2011, York at 2006, White River at 2002, Guadeloupe at 1997 and Taiwan at 1992. The recent 6th IBWS organized in July 2016 in Toulouse, France successfully brought together a community of researchers worldwide including agronomists, farmers, and private companies involved in the study and control of bacterial wilt (Jiang *et al*., 2017).

The disease is widely distributed because it is mainly soil-borne disease, which make to it many hosts and therefore difficult to control with chemical compounds and cultural practices (Grimault *et al.*, 1993). It is appears to be a shift in the concept that biological control can play a serious role in controlling the bacterial wilt disease (Akiew *et al*., 1993). Biological control saves the environment from pollution with chemical compounds in addition to suppress the diseases. Many microorganisms have been experimented with variable success for biological control of bacterial wilt (Shekhawat *et al*., 1993). Efficiency of microorganisms are double role can increase the crop yield and also protect plant against the pathogens Higa (1999). An effective microorganisms against bacterial wilt have been suggested as *Pseudomonas* sp. (Castro *et al*., 1995). The most commonly microorganism agents applied are *Streptomyces* species (Lu *et al*., 2013 & Xiong *et al*., 2014), *Bacillus* species (Ran *et al*. 2005; Lei *et al*., 2010; Wei *et al*., 2011 & Wang *et al*., 2015), *Pseudomonas* species (Yang *et al*., 2012; Qiao *et al*., 2015 & Hu *et al*., 2016) addition to other microorganisms (Guo *et al*., 2004; Xue *et al*., 2009; Yang *et al*., 2012 & Huang *et al*., 2013).

Bio-control agents are promising methods to decrease bacterial wilt disease severity (jiang *et al*., 2017). Any microorganism which has efficacy to suppress *Ralstonia* spp. virulence or to decrease its population has the potential for biological control of bacterial wilt disease (jiang *et al*., 2017). Useful microbial combinations can make better use of available resources and produce antibiotic compounds help progress the consistency and effectiveness of bacterial biological control of bacterial wilt disease (Wei *et al*., 2015 and Yang *et al*., 2017).

The current study was aimed to, isolation and characterization of potential bacteri from plant rhizospheric soil as biocontrol agents and evaluate the antagonists effect

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on *R. solanacearum in vitro* and bacterial wilt disease on potato plants (*in planta*) by biocontrol measures, under Egyptian conditions.

MATERIALS AND METHODS

The Pathogenic organism

The pathogenic bacterium *Ralestonia solanicearum* race 3, biovar 2, used in this study was kindly provided by personal communication. The strain was originally isolated from potato tubers with typical symptoms of bacterial wilt and brown rot disease. The pathogenicity to the bacterium was tested on potato plants *Solanum tuberosum* cv. Spunta. The bacterium was showed typical wilt symptoms on potato plants.

Isolation of antagonistic bacteria

Ten grams of rhizospheric soil of some field grown potato crops were potted and mixed well in a 100 ml of sterilized distilled water in a 250 ml flask. About 100 µl of diluted soil suspension was streaked on potato dextrose agar (PDA) medium and then incubated at 28°C for 48 h. The single colonies appeared on PDA plates were individually tested against of the pathogenic bacterium *Ralestonia solanicearum* using the toothpick method (Kekessy and Piguest, 1970). The bacterial isolates which able to inhibit growth of *Ralestonia solanicearum* were transferred to slant tubes of PDA medium and incubated at 28°C for 48 h. then preserved at 5°C.

Inocula Preparing

Bacterial inocula of antigonestic bacterial isolates were prepared according to (Eastwell *et al*., 2006). The antigonestic bacterial isolates were grown in nutrient broth for 48 h at 28^{o} C. Cultures were chilled on ice for 30 min, concentrated by centrifugation and washed two times in sodium chloride solution (0.85% NaCl) to remove media and any extracellular components released by the bacteria. They were then diluted in saline solution and the concentration adjusted spectrophotometrically to OD $_{0.1}$ at 600 nm wavelength corresponding to about l0^{8} CFU / ml.

R. solanacearum was grown in king's medium at 28^{o} C. for 48 h and the bacterial growth was suspended in sterile distilled water. The population of bacteria were maintained to 10^{8} CFU/ml (0.1 OD at 600 nm) by using spectrophotometer.

Evaluation an antagonistic activity *in vitro*

Two isolates of bacteria were tested for their efficacy of inhibiting *R. solanacearum* growth by paper disc method (Dhingra and Sinclair, 1995). Ten µl suspension of *R. solanacearum* was spread by sterile swab onto Petri plates (9 cm) having Mueller Hinton agar medium (Standard formula grams / liter = Beef infusion from 300g, 17.50g casein acid hydrolysate, 1.50g starch and 17g agar final pH at 25^{o}C= 7.3 ± 0.1) to make a film of bacteria on the surface of the agar medium.

A sterilized filter paper (Whatman No. 42) measuring 5 mm in diameter were soaked in different antagonist broth for 3 minutes then dried and placed on plates with three replications. Filter paper discs dipped in sterile water were served as a control. The plates were incubated at 28±2°C for 48 h. Mean of inhibition zone diameters was measured by centimeters and inhibition percent was calculated by formula:

$$\textbf{\% inhibition} = \frac{\textbf{Mean of inhibition zone diameters}}{\textbf{Completely growth}} \textbf{X100}$$

Identification of antagonistic bacteria

The antagonistic isolates (coded MAS100 and MAS400) were investigated with microscopically tests, Gram staining, cultural features and KOH 3% adopted for the identification of unknown microbial organisms (Fahy and Hayward, 1983) & (Cappuccino and Sherman, 1996).

Molecular characterization of antagonistic isolates:

The antagonists bacterial isolates, MAS100 and MAS400 that showed the highest suppressive effect on *R. solanacearum in vitro*, were identified by the 16S rDNA.

DNA Extraction

Genomic DNA was extracted from isolates according to (Kawaguchi, *et al.*, 2005). All bacterial isolates were routinely cultured on nutrient agar medium. Single colonies grown on this medium were suspended in 20 µl sterile distilled water. The bacterial suspension was heated at 95 °C for 10 min and cooled for 5 min on ice. The suspension was centrifuged under cooling at 12000 rpm for 2 min and the resulting supernatants were used as DNA templates for PCR. All DNA samples were stored at -20°C.

PCR components

The amplification of 16S rDNA was carried out in a 50 µL final volume containing 10 µL of total DNA, using 0.5 mmol.L-1 of each primer from those listed in **Table (1)**. Ten µL of 2.5 mmol·L–1 of each dNTP, and 1 U of *Taq*DNA polymerase.

Table 1. Primers used to amplification of 16S rDNA of antagonistic bacterial isolates.

Primer Code	Sequence	Product Size	Reference
27F 1512R	5′-AGAGTTTGATCCTGGCTCAG-3′ 5′-ACGGCTACCTTGTTACGACT-3′	1500 bp	Weisburg *et al*., 1991

PCR condition (thermal profile)

The thermal reaction conditions in PCR were as follows: 94 °C for 5 min followed by 30 cycles of denaturation at 94°C for 30s, annealing at 56 °C for 30s, and primer extension at 72°C for 2 min; followed by a final extension at 72 °C for 5 min. The reaction products were separated by running 5 µL of the PCR reaction mixture (PCR cocktail) in 1.2% (w/v) agarose gel, and the bands were stained with ethidium bromide.

Purification of PCR Products

Amplified products for bacterial isolates were purified using EZ-10 spin column PCR. The purified product were transferred to 1.5 ml microfuge tube and three volumes was added of binding buffer 1, after that the mixture solution was transferred to the EZ-10 column and let it stand at room temperature for 2 minutes, after that centrifuged, 750 µl of wash solution was added to the column and centrifuge at 10.000 rpm for 2 minutes, repeated washing, 10.000 rpm was spine for an additional minute to remove any residual wash solution. The column was transferred into a clean 1.5 ml microfuge tube and add 50 µl of elution buffer, incubated at room temperature for 2 minutes and store purified DNA at -20 °C.

16S-sequencing analysis

A representative bacterial isolates were selected for sequencing analysis. A part of the rDNA region was amplified using the forward (16SF) or reverse (16SR) primer pairs. The sequencing of the product PCR was carried through in an automatic sequencer ABI PRISM 3730XL Analyzer using BigDyeTM Terminator Cycle Sequencing Kits following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using 16Sf-16SR primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Computational analysis (BLASTn) 16S.

The sequences were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) Sequences were aligned using Align Sequences Nucleotide BLAST.

Biocontrol activity *in planta*

The antagonistic isolates MAS100 and MAS400 were used to evaluate them to suppress bacterial wilt disease on potato plants in pots experiment. The experiment was carried out on potato cultivar Spunta by four treatments. The tubers were obtained from Potato Brown Rot project, Agricultural Research Centre, Cairo, Egypt. Tubers were certified to be free from *Ralstonia solanacearum*. One tuber per pot was used in all the treatments with five replications and maintained in a net house. Treatments T1 and T2 were inoculated with the antagonistic bacterial isolates (MAS 100 and MAS400), respectively. Treatment T3 was inoculated by pathogen only as a control. The treatment T4 was the negative control, without inoculation of pathogen or antagonistic bacteria. The experiment was conducted twice with completely randomized design.

The sandy clay soil was mixed (1:1) and sterilized using 5 % formalin solution. Soil were covered by plastic sheet for 17 days to destroy the microbial population in soil, then removed sheet with gap of 7 days and used for experiments. Three kg of soil mixture was filled in pot (25cm diameter).

Bacterial growth of the antagonistic isolates 48 h old were harvested by scraped from the Petri plates and mixed in 250 ml of sterile distilled water to reached bacterial concentration 10^8 CFU/ml (0.1 OD at 600 nm) by using spectrophotometer. The suspension was inoculated at root zone of each pot after planting directly.

A bacterial suspension of *Ralstonia solanacearum* was prepared in distilled sterilized water using 48 h old growth on king᾽s medium and concentration was maintained 10^8 CFU/ml.

The potato plants are infected by stem stab method as described by Winstead and Kelman (1952). When the plants are 15 - 20 cm tall, usually 3rd of 4th buds from the top is inoculated by injecting / placing a droplet of suspension (25-30 µl) on injury. Plants stabbed with sterilized distilled water as negative control. The inoculated plants were observed for wilt appearance three times, after 10 days, 15 days and 20 days of inoculation. Disease incidence percentage was calculated by formula:

$$\text{Disease incidence percentage} = \frac{\text{Counting of the wilted plants}}{\text{Total plants}} \times 100$$

Wilt reduction was calculated according to Aliye *et al.* (2008) as:

$$PR = [(P_C - P_T) / P_C] \times 100$$

Where PR is percent reduction, P_C and P_T are percentage values of control (pathogen only) and the treatment group, respectively.

Statistical analysis

All the data obtained in the present study were subjected to analysis of one-way ANOVA by costat version 6.311 and mean separation was performed using Fisher's Protected Least Significant Difference (LSD) method (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Evaluation an antagonistic activity *in vitro*

Twenty three different bacterial isolates were isolated from rhizospheric soil of potato crops. Nine bacterial isolates were able to inhibiting *R. solanacerum* growth. Among those isolates, two bacterial isolates (MAS100 and MAS400) showed high ability to inhibiting *R. solanacerum in vitro* were selected to accomplish this study. Isolate MAS400 exhibited highly inhibitory activity (3.7 cm.), while isolate MAS100 showed relatively low antagonistic activity (0.9 cm) (Table, 2 and Fig. 1). These results were agreed with (Aino, 2016; Singh *et al*., 2016; Sarkar and Chaudhuri, 2013; Nguyen and Ranamukhaarachch, 2010; Lwin and Ranamukhaarachch 2006 & Anuratha and Gnanamanickam, 1990).

Table 2. inhibitory effect of the antagonistic isolates on *Ralstonia solanacearum in vitro*.

Antagonistic isolate	Mean of inhibition zone (cm)*	% inhibition
MAS 100	0.9	10
MAS 400	3.7	41.11
control	0.00	0.00
LSD 5% =	0.163	

***=Data present means of the experiment within 3 replications each.**

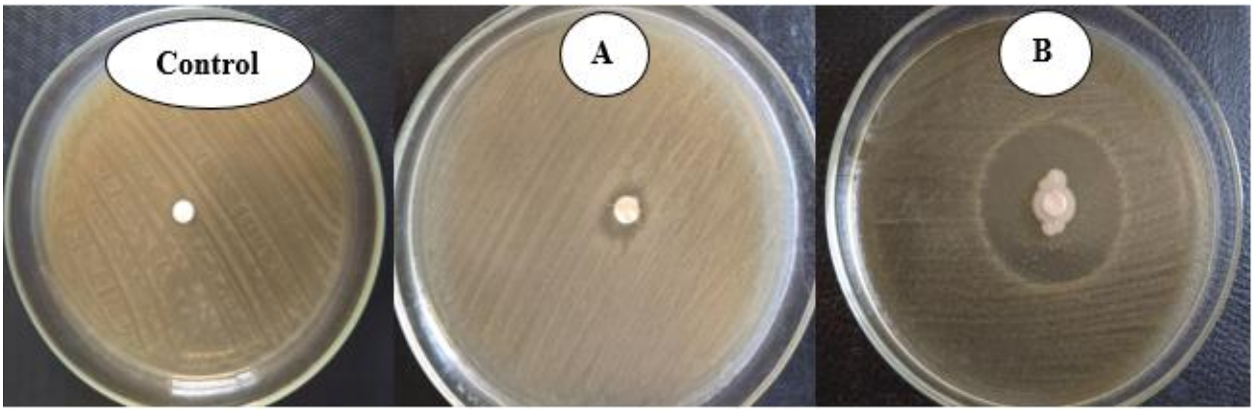

Fig.1. Inhibition zone resulted from the antagonistic effect of the antagonistic bacteria against *Ralstonia solanacearum*: (A) = MAS100, (B) = MAS400.

Identification of antagonistic bacteria

The antagonistic bacterial isolates were identified by some of conventional tests such as morphological, microscopical and biochemical tests. The bacterial isolates were positive with Gram stain, bacilli and able to grow at 28C°. The colonies were moist, round shape. With KOH 3% test the bacterial isolates showed negative reaction.

Molecular characterization

All bacteria contain 16S ribosomal RNA (rRNA) genes of approximately 1500bp in length. RNA genes contain regions of variable DNA sequence that are unique to the species carrying the gene. The sequences were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The sequences were aligned using Align Sequences Nucleotide BLAST. The study was focused on molecular characterization by used 16S rDNA sequences to identify these bacteria based on the nucleotide sequences of the 16S rDNA portions for bacterial strains which obtained from National Center for Biotechnology Information (NCBI) database. The sequences were compared to known bacterial sequences in the NCBI databases and were found to be the isolate MAS 100 is *Paenibacillus polymyxa* with identity percent 97.63% based on grouping of both strains showed in a phylogenetic tree of *Paenibacillus polymyxa*. The nucleotide sequence of MAS 100 isolate was deposited in Genebank NCBI with accession number MN971671 as *Paenibacillus polymyxa* (Fig.2) agreed with (Li *et al*., 2010 & Algam *et al*., 2010).

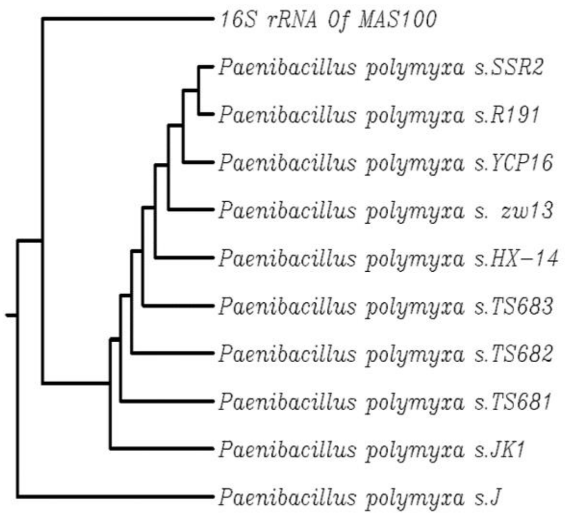

Fig.2. Phylogenetic tree illustrates the similarity of the isolate MAS 100 to *Paenibacillus polymyxa* strains sequenced deposited in GenBank database.

Paenibacillus polymyxa is non-pathogenic to plant and an endospore-forming bacterium and found in plant rhizosheric soil (Timmusk *et al.,* 2005 & Ravi *et al.,* 2007). *Paenibacillus polymyxa* is bacilli and positive with Gram stain (Zengguo *et al*., 2007). *P. polymyxa* can promote plant growth through three mechanisms. Mechanism number (1) is production of hormones such as gibberellins, cytokinins, auxins and ethylene (Timmusk *et al.,* 1999).These hormones enhance root expansion and plant growth. Mechanism number (2) is the production of antibiotics and the strengthening of immunity in the plant root. Mechanism number (3) is the microorganism's nitrogen fixation capacity, which can produce a form of nitrogen (NH_3 ammonia) that can be used by plants from the N_2 atmosphere.

The study indicated that the isolate MAS 400 identified as *Bacillus aryabhattai* with percent identity 80.56% using similarity with partial 16S rDNA sequencing obtained from NCBI database (Fig. 3).

Some of *Bacillus* species are common a biological control agents (Bacon and Hinton, 2002 & Choudhary and Johri, 2009). A lot of papers reported an efficacy of *Bacillus* species to inhibit many common plant diseases (Melo *et al*., 2009). The major biological control mechanisms of *Bacillus* species are considered to be the production of antibiotics (1st mechanism), such as lipopeptides (Ongena *et al*., 2005 & Ongena and Jacques, 2008) which induced great attention for inhibiting growth of plant pathogens and activating the innate immunity of plant system against various plant pathogens (Ongena *et al*., 2007; Romero *et al*., 2007 & Raaijmakers *et al*., 2010), the competition for ecological niches (2nd mechanism) (Compant *et al*,. 2005), or the inducement of systemic resistance (ISR) in host plants (3rd mechanism) (Van Loon and Bakker, 2006 & Saravanakumar *et al*., 2007).

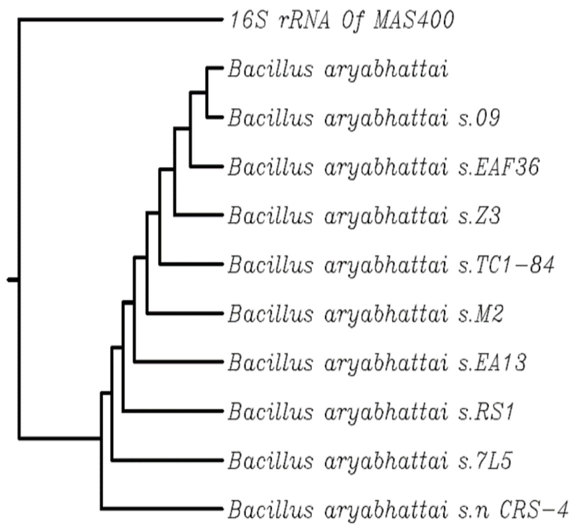

Fig.3. Phylogenetic tree illustrates the similarity of the isolate MAS 400 to *Bacillus aryabhattai* strains sequenced deposited in GenBank database.

Biocontrol activity *in planta*

On other hand, both the antagonistic bacterial isolates were caused a relatively reduction of wilt symptoms on potato plants compared to the control, this agreed with Hussain *et al.* (1993). The wilt symptoms was initiated only in control plants which infected by *R. solanacearum* after 8 days of inoculation, whereas in the other treatments MAS100 (*Paenibacillus polymyxa*) and MAS400 (*Bacillus aryabhattai*) symptoms was less severe and plants delayed appearance of wilt symptom after 19 days with MAS100 and 14 days with MAS400 of inoculation. Trials with MAS100 isolate on plants had a lower disease incidence (20 %) with maximum of disease reduction (80 %). Whereas, MAS400 (*Bacillus aryabhattai*) had a higher disease incidence on potato plants (60 %) with disease reduction (40 %). Negative control was not appeared wilt symptom.

Isolate MAS100 (*Paenibacillus polymyxa*) showed the highest disease suppression on potato plants. (Table 3 and Fig.4).

This result was agreed with Nguyen and Ranamukhaarachch (2010).

It is amazing that, *P. polymyxa* MAS100 strain when tested *in vitro* showed lowest inhibition to *R. solanacerum* while it was highly suppression for symptoms wilt *in planta* unlike *Bacillus aryabhattai* MAS400 strain.

Table 3. Effect of biocontrol agents on bacterial wilt disease incidence on potato plants, and percentage of wilt reduction.

Treatments	Bacteria	Disease incidence (%)	wilt reduction (%)
(T1) MAS100	*Paenibacillus polymyxa*	20	80
(T2) MAS400	*Bacillus aryabhattai*	60	40
(T3) Control	*Ralstonia solanacearum*	100	0.0
(T4) Negative control	without	0.0	100

Fig. 4. Efficacy of the biocontrol: (A) MAS 100= *Paenibacillus polymyxa,* (B) MAS400= *Bacillus aryabhattai*, (C) Control infected by *Ralstonia solanacearum* and (D) Healthy plant as negative control.

CONCLUSION

The study indicated that, *Paenibacillus polymyxa* MAS100 strain able to suppression of bacterial wilt disease of potato plants while *Bacillus aryabhattai* MAS400 strain was less able to that, other than it was large ability to inhibition *R. solanacearum* under *vitro* conditions.

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Paenibacillus polymyxa **و** ***Bacillus aryabhattai*** **كعوامل مكافحة حيوية تضاد بكتيريا** ***Ralstonia solanacearum*** **فى المعمل وعلى النبات**

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هدفت الدراسة إلى المكافحة البيولوجية للذبول البكتيري بواسطة العزلات البكتيرية المضادة المعزولة من التربة، تم الحصول على عزلتين بكتيريتين من منطقة الريزوسفير من الحقول المنزرعة و تم تقييم قدرتها على تثبيط البكتيريا *Ralstonia solanacearum* المسببة للذبول البكتيرى على نباتات البطاطس في المختبر، أظهرت العزلات البكتيرية قدرة عالية على تثبيط نمو البكتيريا المسببة للذبول وتم ترميز العزلتين المضادتين بالرموز MAS100 و MAS400. أظهرت العزلة MAS400 نشاطاً تثبيطياً كبيراً لحد ما (٣,٧ سم) ، بينما أظهرت العزلة MAS100 تثبيطاً نسبياً (٠,٩ سم)، تم التعريف على المستوى الجزيئي وعمل تتابع لنيوكلوتيدات Sequencing- DNA بإستخدام تقنية 16 S rDNA للعزلات البكتيرية المضادة ، حيث كانت العزلةMAS400 أقرب فى التعريف للسلاله البكتيرية *Bacillus aryabhattai* بنسبة ٨٠,٥٦٪ بينما كانت العزلة MAS100 أقرب فى التعريف للسلاله البكتيرية *Paenibacillus polymyxa* بنسبة ٩٧,٦٣٪ (مقارنة بالتتابعات المحفوظة فى قواعد بياناتNCBI) . تم ضم تتابع العزلة MAS100 الى التتابعات المحفوظة فى بنك الجينات بالرقم MN971671وتم تقييم قدرة العزلات البكتيرية المضادة كعوامل مكافحة بيولوجية لتثبيط مرض الذبول البكتيرى فى البطاطس. إنخفضت نسبة حدوث المرض على نباتات البطاطس مع المعاملة بالعزلة *Paenibacillus polymyxa* إلى (٢٠ ٪) بنسبة كفاءة (٨٠ ٪) في حين كانت نسبة حدوث المرض مع المعاملة بالعزلة *Bacillus aryabhattai* هى (٦٠ ٪) بنسبة كفاءة (٤٠ ٪). وخلصت الدراسة الى أن العزلة *Paenibacillus polymyxa* لها قدرة أعلى على تثبيط مرض الذبول البكتيرى فى البطاطس.