Current Science International Volume : 07 | Issue : 02| April- June | 2018
ISSN: 2077-4435 Pages**:** 242-256

Use of rhizobacteria as biocontrol agents against *Ralstonia solanacearum*: Principles, mechanisms of action and characterize its bioactive compounds

Enas A. Hassan[1], Abeer E.A. El Hadidy [2],Naglaa M. Balabel [3], Nerhan A. Eid[2], El shahat M. Ramadan[1]

[1]*Agric. Microbial. Dept., Fac. Agric., Ain Shams Univ., Cairo, Egypt*
[2]*Plant Prot. Dept., Desert Res. Center, Cairo, Egypt*
[3]*Bact. Dis. Res. Dept., Plant Pathol. Res. Instit., Agric. Res. Center, Giza, Egypt*

Received: 21 Feb. 2018 / Accepted: 30 April 2018 / Publication date: 24 May 2018

ABSTRACT

Five rhizobacterial isolates were had potent effect against *Ralstonia solanacearum* race 3 which causing bacterial wilt *in vivo* expressed as biocontrol efficiency. In this study the five isolates were identified as: *Bacillus pseudomycoides* M3, *Brevibacillus brevis* M4, *Stenotrophomonas maltophilia* M5, *Stenotrophomonas maltophilia* BG4 and *Streptomyces toxytricini* C5 based on 16S rRNA gene sequences. Further studies with antagonistic bacterial strains were characterized for production of antibiotics, metabolites and volatiles. The assessment of cyanide hydrogen and siderophore production for selected antagonists revealed that *Bacillus pseudomycoides* M3 and *Stenotrophomonas maltophilia* M5 isolates gave the highest values of HCN whereas maximum siderophore production was recorded by *Brevibacillus brevis* M4 and *Stenotrophomonas maltophilia* M5. The recovered of antimicrobial compounds from supernatants of these strains were conducted using chlorophorm, petroleum ether and ethylacetat individually to find that chlorophorm phase at 100 ppm for *Bacillus pseudomycoides* M3, *Streptomyces toxitricini* C5 and *Stenotrophomonas maltophilia* M5 gave high broad spectrum of antagonistic effect against selected *R. solanacearum* strains whereas petroleum ether phase at 50 ppm inhibited the most selected strains of *R. solanacearum in vitro*. The efficient organic phase of each strain was separated to many fractionated bands by Thin Layer Chromatography (TLC).

Key words: Bacterial wilt, *Ralstonia solanacearum*, biological control, rhizobacteria, antimicrobial compounds

Introduction

Ralstonia solanacearum is an important soil borne bacterial plant pathogen with a worldwide distribution and a wide host range. Some of economical important plant hosts include tomato, potato, eggplant, pepper, tobacco, banana, chili and peanut. However, different strategies for the management of bacterial wilt have been used in the world, no effective control methods exist for bacterial wilt disease (Champoiseau *et al.,* 2010). Increasing the use of chemical pesticides for plant diseases control causes several negative effects on human and environment health. Furthermore, increasing public awareness about the side effects of them led to a research to find alternatives for these products. One of the alternative methods is biocontrol utilizing plant associated antagonistic microorganisms (Safdarpour and Khodakaramian 2018).

Some bacteria are referred to as plant growth promoting rhizobacteria (PGPR) and their effectiveness in controlling a number of plant diseases caused by soil-borne pathogens have been widely documented (Glick *et al.,* 2007 and Abdelwareth *et al.,* 2012). PGPR are endophytic bacteria may have an ecologically beneficial position as they could grow and compete on the root surface, perhaps develop within the root where they are relatively safe form competition and environmental stresses (Whipps, 2014). These PGPR which mostly belong to genera (*Bacillus* sp. and *Pseudomonas* sp.) are antagonists of recognized root pathogens.

The mechanisms of PGPR include: the ability to produce phytohormones, N_2 fixation, antagonism against phytopathogens and solubilization of insoluble phosphates. It was also suggested that PGPR can also prevent the deleterious effects of stresses from the environment (Lugtenberg and Kamilova, 2009). The widely recognized mechanisms of biocontrol mediated by PGPB are competition

Corresponding Author: Abeer E.A. El Hadidy, Plant Prot. Dept., Desert Res. Center, Cairo, Egypt
E-mail: elhadidyabeer@gmail.com

for an ecological niche or a substrate, production of inhibitory allelochemicals, and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens (Usharani *et al.,* 2013).

Offensive PGPR colonization and defensive retention of rhizosphere niches are enabled by production of bacterial allelochemicals, including iron-chelating siderophores, antibiotics, biocidal volatiles, lytic enzymes, cyanide and detoxification enzymes (Mehjabeen *et al.,* 2017).Whenever possible, modes of action involved in each type of interaction are assessed with particular emphasis on antibiosis, competition, parasitism, and induced resistance. None of the mechanisms are necessarily mutually exclusive and frequently several modes of action are exhibited by a single biocontrol agent. Indeed, for some biocontrol agents, different mechanisms or combinations of mechanisms may be involved in the suppression of different plant diseases (Singh *et al.,* 2017).

Antibiotics are heterogeneous group and are low molecular weight of organic compound that are harmful to the growth and metabolic activity of microorganisms. Most of the antibiotics are peptides in nature and effective or active against bacterial growth. Antagonistics produced by bacteria include volatile compounds (hydrogen cyanide, aldehydes, alcohols, ketones, and sulfides) and nonvolatile antibiotics: polyketides (diacetylphloroglucinol; DAPG and mupirocin), heterocyclic nitrogenous compounds (phenazine derivatives: pyocyanin, phenazine-1-carboxylic acid; phenazine-1- carboxylate (PCA), and phenazine-1-carboxamide (PCN) and hydroxyphenazines) (Singh *et al.,* 2017).

The objective of the present study aims to identify, assay of biochemical activities of the most active bacterial isolates used for bio control of *R. solanacearum*.

Material and Methods

1. Bacterial pathogen:

Bacterial pathogens used in this investigation are eleven *R. solanacearum* (race3 biovar2) isolates which selected according to their morphological characteristics on SMSA medium and their severe reaction by pathogenicity test in previous study (Hassan *et al.,* 2017).

2. Antagonistic bacteria:

Five antagonistic isolates were used in this investigation, which were isolated from some plants and were screened according to their antagonistic activity *in vitro* against *R. solanacearum* in previous study (Hassan *et al.,* 2017). The most efficient antagonistic isolates (M3, M4, M5, BG4&C5) were identified by 16S rRNA sequence .Isolation of cellular DNA was performed as described by Ausubell *et al.* (1987) and amplification of 16S rDNA according to (Lane,1991) using the universal 16S primers (F1 5' AGAGTTT(G/C)ATCCTGGCTCAG 3' R1 5' ACGG/C) TACCTTGTTACGACTT 3').

3. Evaluation of antagonistic potential of bacterial isolates on bacterial wilt under greenhouse:

The experiment was carried out under greenhouse conditions for verification bio-control potentials of five rhizobacterial isolates. The 4 weeks old tomato seedlings (*Solanum lycopersicum* cv. Super Strain B) were transplanted in pots (18 cm in diameter) filled with 800 gm sterilized potting mixture (1 soil: 1 peat moss), 5 ml of antagonistic bacterial broth containing 10^8 cfu/ ml were added individually into soil directly for transplanting tomato seedling. Then, soil drenching method was applied to infest the soil by bacterial wilt pathogen and inoculated the root system of each seedling after wounding with a scalpel and dipped of inoculum broth (10^8 cfu/ ml), planted pots were maintained under greenhouse conditions with 5 replicates for each treatment, at temperature ranging between 25 to 30°C and relative humidity between 70 to 90%. Control treatments without either the pathogen or the tested antagonist bacterial isolates were considered as negative controls. Completely randomized block design with five replicates for each treatment was followed. The treated plants were monitored for disease development up to 14 days period after inoculation. Disease development was recorded as disease incidence and biocontrol efficiency according to Song *et al.* (2004) as follows. % Disease incidence = (No. of infected plants/ total plants) ×100. Biocontrol efficiency= {(Disease incidence of control - Disease incidence of antagonist-treated group) / Disease incidence of control} × 100.

4. Assay of antagonistic activities:

4.1. Hydrogen cyanide (HCN) production:

Production of HCN was detected according to the method of Wei *et al*. (1991).
Each potent bacterial isolate was streaked on the Trypticase soy agar (TSA) medium in triplicate plates. The filter padding in each plate was soaked with 2 ml of sterile picric acid solution (2.5g/l picric acid, 12.5 g/l Na_2CO_3) under aseptic condition and the lids were closed. After incubation for a week at 30ºC, the color change of the filter paper pad was noted and the HCN production potential of the antagonists was assessed as shown below:-
1. No colour change (-) : no HCN production
2.Brown colouration (+) : weak HCN production
3.Brownish to orange (++) : moderate HCN production
4.Complete orange (+++) : strong HCN production

4.2. Siderophore production:

Siderophore production was assayed by Chrome Azurol S (CAS) method described by Alexander and Zuberer (1991). 1 ml of modified CAS assay solution was mixed with 1 ml culture filtrate of studied isolates. After allowing the mixture to equilibrate for 3-4 h, absorbance was measured at 630 nm using Jasco V-630 Spectrophotometer (UV 120-20).

4.3. Production of extracellular bioactive metabolites:

4.3.1. Extraction of antibacterial substances:

Conical flasks (100 ml volume) containing 50 ml of King´s broth medium or glycerol nitrate broth medium were inoculated with1 ml standard inoculum of bacteria or actinomycetes cultures. After inoculation, the flasks were incubated at 30°C on a rotary shaker of 200 rpm. At the end of the incubation period, the cultures were centrifuged at 10000 rpm for 20 minutes at 4ºC.The supernatant was adjusted to pH 8 and extracted with an equal volume of ethyl acetate and chloroform or petroleum ether. The organic phase was separated from the aqueous phase and evaporated at 40°C using rotary evaporator under evacuation 130 mille bar to evaporate the solvent and water from the extract till little volume, then transferred to small glass beaker (weighed before receiving the extract) and allow to dry using oven with fan at 30°C. The obtained residue was weighed then stored at -4ºC till use.

4.3.2. Assay of antibacterial activity of crude extract in vitro:

The antibacterial activity of crude extract recovered from each solvent was determined against selected *R. solanacearum* strains by paper disk test as the following; the partially purified extract obtained by evaporation of solvent used was dissolved in ethyl alcohol (70%) making successive serial concentrations (50,100,200 ppm) for each crude extract. *R. solanacearum* tested isolates were grown over night in nutrient broth at 30°C individually,1ml of bacterial suspension containing approximately 10^8 cfu/ ml was added to 100 ml melted nutrient agar in Petri dish and allowed to set. Autoclaved 5mm diameter filter paper disk were dipped in the crude extract solution, then three disks were placed on each plate. Ethyl alchol replacing the bacterial extraction was pipetted in control dishes. The diameter of the zone of inhibition was recorded after 48 h. Each treatment had two replicates. The data were represented by relative power of antibiosis by this equation:

RPA = Diameter of inhibition zone / Diameter of filter paper disk.

4.3.3 Assay of antibacterial activity of purified fractions of crude extract in vitro:

The most potent active crude extract was fractionated by thin layer chromatography (TLC) to assess qualitatively the components of crude extract on $60F_{254}$ silica gel plates (Merk, Germany), using solvent system which was chloroform: methanol(9:1) then the plate were air- dried, and detected at 535

nm using GAMMAG TLC scanner system .The migration velocity of the mixture was characterized by numerical value RF which expresses the retention shape between the distance travelled by the compound from the origin (Δ x)and the solvent front from origin (Δ y) (Sherif *et al*., 2008).The sample was applied on the readymade plate on the form of band , run by the previous solvent system and the separated bands were localized using UV lamp .The bands were then scrapped off the plate , each band was transferred in 1 ml of ethanol (70%) the antibiotic effect of fractions was investigated by filter paper desk in petri dishes containing King´s medium cell suspension of *R. solanacearum* as described above to rebioassay of each band against *R. solanacearum* tested isolates individually(Yahya *et al*., 1995).

4.3.4 Characterization of the partially purified antibacterial compounds:

The most active bands which obtained by TLC, were identified and characterized by using High Pressure Liquid Chromatography (HPLC) and Mass Spectroscopy (M/S) profile at Central laboratories of Desert Research Center, and Faculty of Pharmacy, respectively Data were employed in partially complete characterization of the chemical structure and molecular formula of active compounds.

5- Statistical analysis:

Data were statistically analyzed according to Duncan, (1955). LSD test at 5 % level of significance was used for comparison between the means of different treatments.

Results and Discussion

A. Evaluation of antagonistic potential of bacterial isolates on bacterial wilt under greenhouse:

In this study, the five rhizobacterial isolates which selected from previous study were assessed for their effectiveness in controlling tomato bacterial wilt disease resulting from eleven strains of *R.solanacearum* individually in pot experiment under greenhouse conditions. Table (1) illustrate that the bacterial antagonistic are significantly different in their ability to suppress bacterial wilt expressed by percentage of disease incidence comparing to control treatment. Accordingly, the antagonistic isolates reduced disease incidence from 10 to 40% compared to the control treatment. Isolate *B.pseudomycoides*, was the most effective in reducing the percentage of disease incidence, as it gave 10% incidence with all tested isolates of *R.solanacearum* followed by *S. toxytricimi*.

Table 1: Suppression of bacterial wilt development caused by strains of *R. solanacearum* Race 3 biovar 2 in treated tomato plant with antagonistic bacteria under greenhouse conditions

Bacterial isolates	Disease incidence (%)										
	Ralstonia solanacearum strains										
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11
Untreated	100a	90a	100a	90a	100a	100a	100a	100a	100a	100a	100a
Bacillus pseudomycoides M3	10d	10d	10c	10c	10c	10d	10e	10d	10d	10e	10e
Brevibacillus brevis M4	30b	10d	10c	10c	10c	30b	30c	30b	30c	30c	40b
Stenotrophomonas maltophilia M5	20c	10d	10c	10c	10c	30b	40b	30b	40b	40b	30c
Stenotrophomonas maltophilia BG4	20c	40b	30b	40b	40b	20c	20d	20c	10d	20d	20d
Streptomyces toxytricini C5	10 d	20c	10c	10c	10c	10d	10e	10d	10d	20d	20d

*Means having similar letters in the same column are not statistically differed at P≥0.05.

Fig. (1) Presented the performance of antagonistic bacteria isolates against eleven strains of *R.solanacearum* race 3 biovar 2 (pathogenic bacteria) as a percentage of biocontrol efficiency of isolates. The isolates *B.pseudomycoides*, *B.brevies* and *S. toxytricimi* recorded the highest disease control on the most of pathogenic bacteria. Champoiseau *et al.* (2010) showed that, among biological control agents, a number of soil bacteria and plant growth promoting rhizobacteria (PGPR) are currently being investigated for their role in the control of *R. solanacearum* race3 biovar2.The results of this

study were agreement with Yun *et al.,* (2014) who reported that Bacillus isolates had a strong capacity to reduce the wilt disease intensity. Six wild isolates as well as the model strain were particularly effective in protecting against *Ralstonia solanacearum* all achieving more than 50 % biocontrol efficacy.

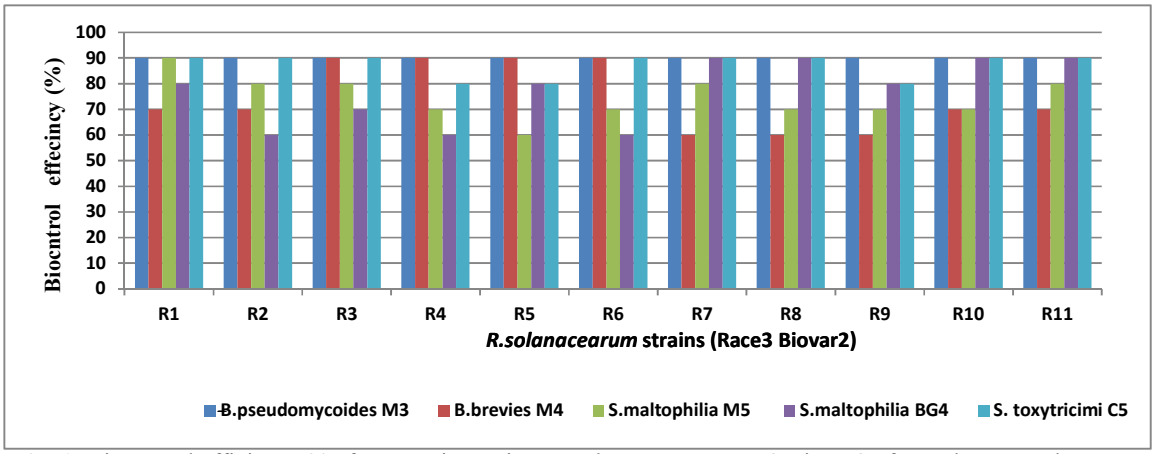
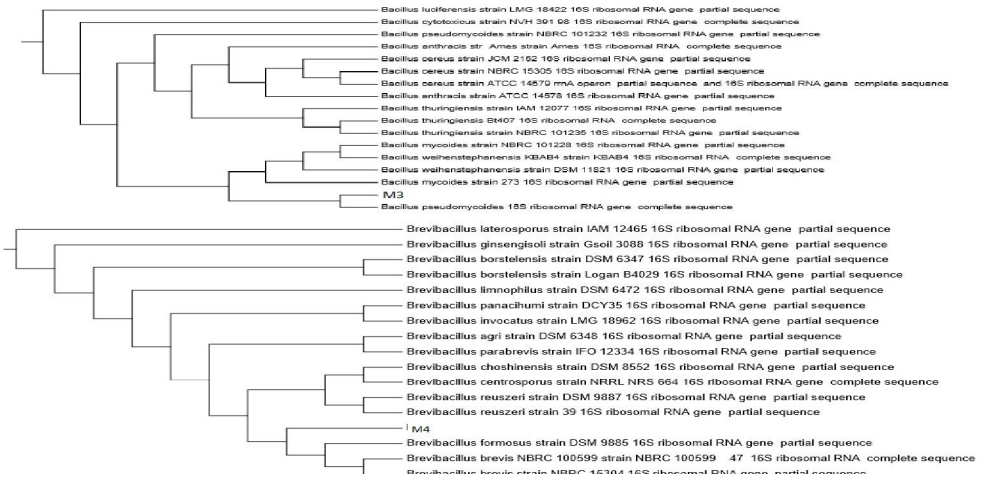


Fig. 1: Biocontrol efficiency % of antagonist against *R.solanacearum* Race3 Biovar2 of tested tomato plant

B. Identification of antagonistic bacteria by molecular analysis

The antagonistic bacteria were identified by amplifying and sequencing the 16Sr DNA techniques by sigma scientific services. The results showed that 16S rDNA of M3,M4,M5 ,BG4,C5 isolates had 98%, 98% , 67 %,97%,96% identities with Bacillus *pseudomycoides* 101228, *Brevibacillus brevis* NBRC 15304, *Stenotrophomonas maltophilia* ATCC 19861 , *Stenotrophomonas maltophilia* IAM 12423, Streptomyces toxytricini NRRL B-5426 respectively. Construction of a phylogenetic tree based on comparative analysis of the 16S rRNA genes was performed with the use of various algorithms implemented in CLC free workbench, version 4.5.1. The phylogenetic analysis based on 16S rRNA gene sequences indicated that strain M3.M4, M5, BG4 and C5 formed a phyletic lineage, within *Bacillus pseudomycoides*, *Brevibacillus brevis*, *Stenotrophomonas* malt philia, *Stenotrophomonas maltophilia*, *Streptomyces toxytricini* respectively (Fig.2).



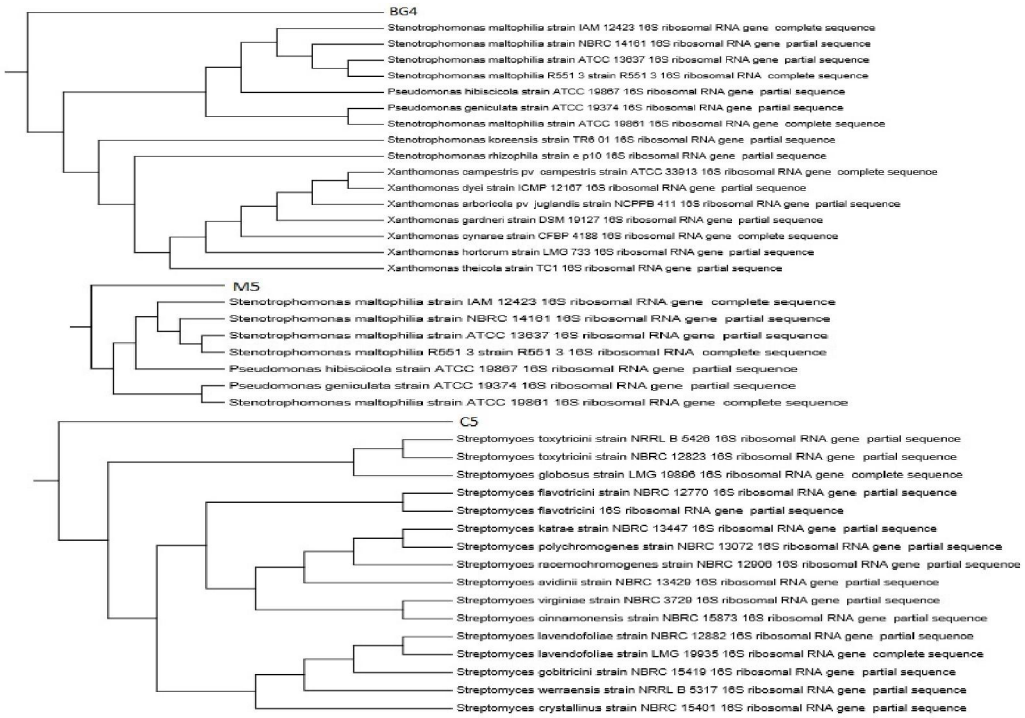


Fig. 2: Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between isolated strains M3, M4, M5, BG4, C5 and related taxa. Bootstrap percentages (based on 1000 replicates) are shown if greater than 50 %.

C. Assay of antagonistic activities:

1. Hydrogen cyanide (HCN) production:

Saber *et al*. (2015) reported that hydrogen cyanide (HCN) is a broad spectrum antimicrobial compound involved in biological control of root disease by many plant associated bacteria. In this study, *in vitro*, HCN production by the antagonistic strains was tested by the picric acid assay. Table (2) shows the ability of studied isolates; *Bacillus pseudomycoides* M3, *Brevibacillus brevis* M4, *Stenotrophomonas maltophilia* M5, *Stenotrophomonas maltophilia* BG4, and *Streptomyces toxytricini* C5 to produce HCN, which identified as high HCN producers which turned the colour of the filter paper to orange completely.

2. Siderophore production:

Siderophores are low molecular weight ferric iron chelating compounds that are secreted extracellularly under iron limiting conditions and whose main function is to supply iron to the iron starved cells. Some PGPR strains produce siderophores that bind Fe3+, making it less available to certain members of native microflora**.** The strains of rhizobacteria that produce siderophore under Fe limiting conditions in the rhizosphere chelate Fe^{3+}, the form that is insoluble in water, hence not available to bacteria (Singh *et al.,* 2017). To examine the ability of these isolates to produce siderophores, they were subjected to CAS assay. Table (2) shows that *Brevibacillus brevis* M4 showed substantial amounts of siderophore production. Its corresponding value is 1.6 µMDFOM. followed by the *Stenotrophomonas maltophilia* M5 which showed 1.3 µMDFOM. The lowest amount was recorded by *Streptomyces toxytricini* C5 isolate being 0.78 µM DFOM. Most evidences to support the siderophore theory of biological control by rhizobacteria come from the work with pyoverdin, a class of siderophores that comprise the fluorescent pigment of fluorescent pseudomonads (Mishra and Arora 2017).

Table 2: Assessment of sidrophore (μM DFOM) and hydrogen cyanid production of tested antagonistic isolates *in vitro*.

Antagonist rhizobacteria	Sidrophore (μMDFOM)	Hydrogen Cyanide
Bacillus pseudomycoides M3	1.2	++++
Brevibacillus brevis M4	1.6	+++
Stenotrophomonas maltophilia M5	1.3	++++
Stenotrophomonas maltophilia BG4	0.98	+++
Streptomyces toxytricini C5	0.78	+++

3. Production of extracellular bioactive metabolites:

3.1 Assay of antibacterial activity of crude extract in vitro:

Antimicrobial compounds of five selected strains were recovered from their filtrate individually by three different solvents extraction (Ethyl acetate, petroleum ether and ,chlorophorm), at three concentrations (50,100,200 ppm) of their crude extract and were evaluated against the selected strains of *R. solanacearum* in vitro to select the most efficient solvent and the suitable concentration which inhibit the bacterial pathogen. Table (3) showed that the antibacterial metabolites of *Bacillus pseudomycoides* M3 recovered by chlorophorm gave the strongest antagonistic activity toward of the most bacterial pathogens followed by ethyl acetate solvent. The chlorophorm extraction has a broad spectrum on the selected bacterial pathogen at all used concentrations but 100 ppm of chlorophorm phase has the highest score on tested bacteria. Data in Table (4) revealed that the antimicrobial compounds of *Stenotrophomonas maltophilia* BG4 were strongly recovered by chlorophorm and petroleum ether because of its broad spectrum effect on the tested bacteria expressed as relative power of antibiosis at 50, 100 and 200 ppm concentrations respectively. The highest figures of relative power of antibiosis (RPA) recovered against pathogenic bacteria were 6.8, 6.7 and 6.5 in case of *R. solanacearum* R6, R5 and R10, respectively. Streptomyces toxitricini C5 gave the highest antagonistic effect by chlorophorm phase followed by the recovered compounds by chlorophorm and ethyl acetate, the wide effect of chlorophorm phase was recorded at 100 ppm for both solvents (Table 5).The highest values of (RPA) were recorded at chlorophorm and petroleum ether phase of *Stenotrophomonas maltophilia* M5 at 100 and 200 ppm respectively (Table 6).

Table 3: Growth inhibition of several *Ralstonia solanacearum* strains affected by different organic fractions of culture filtrates of *Bacillus pseudomycoides* M3 *in vitro* expressed by Relative power of antibiosis (RPA).

Strain	Organic solvent	Crude Con. (ppm)	*Ralstonia solanacearum* strains										
			R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11
Bacillus pseudomycoides M3	Petroleum ether	50	4.4 c	4.4 c	4.2 d	3.4 e	4.2 b	4.4 c	3.4 e	2.4 c	5.3 a	3.4 c	4.4 b
		100	4.4 c	4.4 c	4.6 b	3.4 e	4.2 b	4.6 c	4.2 d	2.4 c	4.2 b	3.4 c	4.3 b
		200	5.3 b	4.4 c	4.4 c	4.4 d	4.2 b	6.5 b	4.2 d	4.4 b	5.3 a	6.5 a	3.4 c
	Ethyle acetate	50	5.3 b	4.6 b	4.6 b	4.6 d	5.3 a	6.5 b	5.3 c	4.6 b	5.3 a	3.4 c	3.4 c
		100	4.6 c	4.6 b	4.6 b	6.5 b	3.4 c	6.5 b	8.5 a	4.2 b	1.6 c	1.6 d	5.3 a
		200	2.4 f	4.6 b	4.6 b	5.3 c	3.4 c	7.4 a	5.3 c	4.2 b	1.6 c	1.6 d	4.8 ab
	chloroform	50	4.2 d	4.6 b	4.2 d	6.5 b	3.4 c	4.6 c	3.5 e	4.4 b	4.6 b	5.5 b	5.5 a
		100	6.8 a	4.6 b	6.5 a	5.2 c	5.2 a	6.7 b	6.3 b	5.5 a	4.4 b	5.5 b	4.8 ab
		200	4.6 c	5.3 a	4.6 b	6.8 a	3.5 c	4.2 c	4.2 d	4.4 b	4.7 b	6.3 a	5.6 a

*Means having similar letters in the same column are not statistically differed at P≥0.05.

Table 4: Growth inhibition of several *Ralstonia solanacearum* strains affected by different organic fractions of culture filtrates of *Stenotrophomonas maltophilia* BG4 isolate *in vitro* expressed by Relative power of antibiosis (RPA).

Strain	Organic solvent	Crude Con.(ppm)	*Ralstonia solanacearum* strains										
			R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11
Stenotrophomonas maltophilia BG4	Petroleum ether	50	5.2 a	5.3 b	6.3 a	5.2 b	6.7 a	6.8 a	6.3 a	5.5 a	4.7 a	6.5 a	4.4 c
		100	3.4 c	3.4 d	6.5 a	5.5 b	4.2 b	4.4 c	3.5 d	4.8 b	2.4 c	4.3 c	5.2 b
		200	5.5 a	5.5 b	5.1 b	6.2 a	3.4 c	5.4 b	5.2 b	4.4 bc	4.4 b	6.3 a	6.1 a
	Ethyle acetate	50	3.2 c	4.6 c	4.6 c	6.2 a	3.5 c	4.6 c	3.7 d	4.2 c	4.5 b	5.5 b	5.2 b
		100	4.4 b	4.6 c	4.6 c	6.2 a	3.5 c	3.6 d	3.6 d	4.4 bc	4.4 b	5.5 b	5.5 b
		200	4.6 b	5.3 b	5.6 b	5.8 ab	3.7 c	4.4 c	4.2 c	3.3 c	4.8 a	5.2 b	5.5 b
	chloroform	50	4.6 b	6.2 a	5.1 b	6.2 a	4.6 b	4.4 c	4.2 c	4.4 bc	4.4 b	6.1 a	4.5 c
		100	3.7 c	3.5 d	6.2 a	5.5 b	4.2 b	4.4 b	3.6 d	4.4 bc	2.5 c	4.6 c	4.2 c
		200	3.5 c	3.2 d	5.1 b	5.5 b	4.2 b	3.5 d	3.2 e	4.5 bc	2.1 d	4.6 c	3.2 d

*Means having similar letters in the same column are not statistically differed at P≥0.05.

Table 5: Growth inhibition of several *Ralstonia solanacearum* strains affected by different organic fractions of culture filtrates of *Streptomyces toxytricini* C5 *in vitro* expressed by Relative power of antibiosis (RPA).

Strain	Organic solvent	Crude Con.(ppm)	*Ralestonia solanacearum* strains										
			R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11
Streptomyces toxytricini C5	Petroleum ether	50	3.4 d	4.2 b	3.2 c	3.4 d	4 .0 a	4.4 c	3.4 c	2.4 c	4.3 a	3.4 c	4.4 b
		100	4.1 c	4.4 b	4.5 b	3.4 d	3.1 b	4.6 c	4.2 b	2.3 c	4.2 ab	3.4 c	4.3 b
		200	5.3 b	3.4 c	4.4 bc	4.3 c	4.2 a	5.5 b	4.2 b	4.1 b	3.3 b	6.5 a	3.4 c
	Ethyle acetate	50	5.1 b	4.6 b	4.3 bc	4.6 d	4.3 a	6.5 a	4.3 b	4.3 b	4.3 a	3.4 c	3.1 c
		100	4.6 c	4.6 b	4.3 bc	6.5 a	4.4 a	6.5 a	5.5 a	4.2 b	1.6 c	2.6 d	5.3 a
		200	2.4 e	3.6 c	4.6 b	5.3 b	3.4 b	6.4 a	5.3 a	4.2 b	1.6 c	1.6 e	3.8 c
	Chloroform	50	4.2 cd	3.6 c	4.1 bc	5.5 b	2.4 c	4.6 b	2.5 d	4.4 b	4.6 a	5.5 b	5.5 a
		100	6.8 a	3.6 c	6.5 a	5.2 b	4.2 a	6.7 a	5.3 a	5.5 a	4.4 a	5.5 b	4.8 b
		200	4.6 c	5.1 a	4.5 b	6.8 a	3.4 b	4.2 c	4.2 b	4.4 b	4.7 a	6.3 a	5.6 a

*Means having similar letters in the same column are not statistically differed at P≥0.05.

Table 6: Growth inhibition of several *Ralstonia solanacearum* strains affected by different organic fractions of culture filtrates of *Stenotrophomonas maltophilia* strain M5 isolate *in vitro* expressed by Relative power of antibiosis (RPA).

Strain	Organic solvent	Crude Con. (ppm)	*Ralstonia solanacearum* strains										
			R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11
Stenotrophomonas maltophilia M5	Petroleum ether	50	3.8 d	4.2 b	4.2 b	3.4 d	4 .0 b	4.3 d	3.4 e	2.1 c	5.3 a	3.4 c	4.4 b
		100	4.1 cd	4.4 b	4.6 b	3.4 d	4.1 b	4.3 d	4.2 d	2.4 b	4.2 b	3.4 c	4.3 b
		200	5.3 b	4.4 b	4.4 b	4.3 c	4.2 b	6.5 b	4.2 d	4.1 b	4.3 b	6.5 a	3.4 c
	Ethyle acetate	50	5.1 b	4.6 ab	4.6 b	4.6 c	5.3 a	6.3 b	5.3 c	4.6 b	5.3 a	3.4 c	3.4 c
		100	4.6 c	4.6 ab	4.6 b	6.5 a	3.4 c	5.5 c	7.5 a	4.2 b	1.6 c	1.6 d	5.3 a
		200	2.4 e	4.6 ab	4.6 b	5.3 b	3.4 c	7.4 a	5.3 c	4.4 b	1.6 c	1.6 d	4.8 ab
	chloroform	50	4.2 cd	4.6 ab	4.2 b	5.5 b	2.4 d	4.7 d	3.4 e	4.4 b	4.6 b	5.5 b	5.5 a
		100	6.8 a	4.6 ab	6.5 a	5.2 b	5.2 a	6.7 b	6.3 b	5.5 a	4.4 b	5.5 b	4.8 ab
		200	4.6 c	5.3 a	4.6 b	6.8 a	3.5 c	4.2 d	4.2 d	4.4 b	4.7 ab	6.3 a	5.6 a

*Means having similar letters in the same column are not statistically differed at P≥0.05.

Table (7) indicates that the petroleum ether phase of *Brevibacillus brevis* M4 gave broad spectrum on bacterial pathogens .The strong effect on most of selected strains was shown at 50 ppm presented by RPA. Generally speaking, the antimicrobial compounds recovered from the selected cultures of rhizobacteria highly varied from one strain to another and from the strain of *R.solanacearum* to another. On the other hand *R.solanacearum* strains R4, R5, R6 and R7 were highly affected by antimicrobial fractions where the RPA ranged from 6.5 to 8.5.

3.2 Assay of antibacterial activity of purified fractions in vitro.

Thin layer chromatography (TLC) plates of silica gel on aluminum support 60 F_{254} was performed to assess the qualitatively the supernatent which was extracted by the most previously efficient solvent .The antibiotic activity of all fractions of crude extract of *B.brevis* M4, *Stenotrophomonas maltophilia* BG4 and *Streptomyces toxitricini* C5 strains was performed on tested strains of *R. solanacearum* (eleven strains) and the data recorded as diameter of inhibition zone in Table (8). *B.brevis* M4 (Ethyl acetate phase) showed six fractions (six bands) with Rf values 0.4, 0.5 or

0.6. All bands showed high antibiotic activities against tested pathogenic bacteria but the highest effect was observed by F6 fraction presented by diameter of inhibition zone ranged from 2.6 to 3.6 cm. But TLC bioantagraphy of *Stenotrophomonas maltophilia* BG4 strain (Ethyl acetat phase) showed four bands from crude extract, with various Rf value .F4 fraction exhibited the highest antagonistic effect on tested pathogenic bacteria. Five bands with Rf value 0.4 , 0.5 or 0.6 were fractionated from component of crude extract of *Streptomyces toxitricini* C5 strain by TLC plate (chlorophorm phase). The highest effect on tested pathogenic bacteria was obtained by F4 fraction with Rf value equal 0.6.

The antibiotic effect actually increased for the fractionated bands in comparison to crude extracts. This effect could be due to the purification process which concentrates the active molecule(s). Moreover, some metabolite showed high solubility in certain solvent. These findings are comparable with results obtained by Oliveira *et al*. (2011) who observed that the same F3 fraction had high antibiotic activity against *X. axonopodis* pv.*citri* (*Xcc*), decreasing by 93.5% the number of lesions of citrus canker in orange leaves. Ji *et al*. (2008) reported that five fractions, obtained by supernatant extraction with ethyl acetate and fractionated by TLC, also showed inhibitory zones against *X. axonopodis* pv.*citri (Xcc).* The compounds with antibiotic activity obtained from *L.antibioticus* were produced during the cultivation of antagonist cells and exhibited solubility in ethyl acetate.

The results of the present study suggest that the highest fraction obtained by TLC from crude extracts, produced by *B.brevis* M4,*Stenotrophomonas maltophilia* BG4 and *Streptomyces toxitricini* C5 may be a new alternative for the control of *R. solanacearum* . However, further studies will be performed to purify, determine and effectively identify the molecules involved in antibiosis.

Table 7: Growth inhibition of several *Ralstonia solanacearum* strains affected by different organic fractions of culture filtrates of *Brevibacillus brevis* M4 *in vitro* expressed by Relative power of antibiosis (RPA).

Strain	Organic solvent	Crude Con.(ppm)	*Ralstonia solanacearum* strains										
			R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11
Brevibacillus brevis M4	Petroleum ether	50	5.2 b	5.3 b	6.3 a	5.2 b	6.7 a	7.4 a	6.3 a	5.5 a	4.7 a	6.5 a	4.4 c
		100	3.4 c	3.4 d	6.5 a	5.5 b	4.2 b	4.4 c	3.5 d	4.8 b	2.4 b	4.6 c	5.2 b
		200	5.5 a	5.5 b	5.1 b	6.2 b	3.4 c	6.8 b	5.2 b	4.4 b	4.4 a	6.3 a	6.3 a
	Ethyle acetate	50	4.2 b	4.6 c	4.6 c	6.2 a	3.5 c	4.6 c	3.7 d	4.2 b	4.5 a	5.5 b	5.2 b
		100	4.4 b	4.6 c	4.6 c	6.2 a	3.5 c	4.6 c	3.6 d	4.4 b	4.4 a	5.5 b	5.5 b
		200	4.6 b	5.3 b	5.6 b	6.8 a	3.7 c	4.4 c	4.2 c	4.4 b	4.8 a	6.2 a	5.5 b
	Chloroform	50	4.6 b	6.2 a	5.7 b	6.2 b	4.6 b	4.4 c	4.2 c	4.4 b	4.4 a	6.1 a	5.5 b
		100	3.7 c	3.5 d	6.2 a	5.5 b	4.2 b	4.4 c	3.6 d	4.4 b	2.5 b	4.6 c	5.2 b
		200	3.6 c	3.2 d	6.1 a	6.5 a	4.2 b	4.5 c	3.2 d	4.5 b	2.4 b	4.6 c	3.2 d

*Means having similar letters in the same column are not statistically differed at P≥0.05.

Table 8: Activity of different fractions of crude extract for the most efficient antagonistic isolates against several strains of *R. solanecearum* on King's medium at 27˚C for 48 hours *in vitro*.

Fractions (M3, BG4 and C5)		*Ralestonia solanacearum* strains Diameter of inhibition zone(cm)										
		R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11
B.brevis M4	F1 (Rf = 0.6)	2.2 b	2.2 bc	1.4 b	2.3 b	2.7 bc	1.4 b	1.4 b	2.4 b	2.6 b	2.4 b	2.3 b
	F2(0.4)	2.3 b	1.5 cd	1.4 b	2.2 b	3.2 ab	1.3 b	1.3 b	2.3 b	2.4 b	2.4 b	1.3 c
	F3(0.4)	2.3 b	1.6 cd	1.3 b	2.3 b	3.2 ab	1.3 b	1.3 b	2.3 b	2.8 b	2.2 b	1.6 c
	F4(0.5)	2.4 b	1.3 d	1.4 b	2.9 ab	2.3 c	1.6 b	1.6 b	2.3 b	2.3 b	2.1 b	1.5 c
	F5(0.6)	1.4 c	2.5 b	1.5 b	2.9 ab	1.2 d	1.5 b	1.5 b	2.4 b	2.2 b	2.4 b	1.2 c
	F6(0.5)	3.2 a	3.4 a	3.4 a	3.4 a	3.4 a	2.6 a	2.6 a	3.4 a	3.4 a	3.6 a	3.4 a
Stenotrophomonas maltophilia BG4	F1)0.4)	2.2 b	2.2 b	2.4 b	2.3 b	1 b	2. 4 b	1.9 b	2.5 b	2.5 b	1.4 c	1.7 b
	F2(0.5)	2.6 b	1.5 b	2.3 b	2.1 b	1.4 b	2.3 b	1.5 b	2.3 b	2.3 b	1.6 bc	3.1 a
	F3(0.4)	2.4 b	1.3 b	2.5 b	2 b	1.1 b	2.3 b	1.1 b	2.3 b	1.5 c	2.1 b	2.1 b
	F4(0.6)	3.4 a	3.3 a	3.4 a	3.2 a	3.5a	1.6 a	2.5 a	3.6 a	3.7 a	2.7 a	3.4 a
Streptomyces toxitricini C5	F1(0.6)	1.5 b	1.8 b	1.6 b	2.5 b	2.4 b	1.5 c	2.5 b	2.5 b	1.6 b	1.6 b	0.9 c
	F2(0.4)	2.3 b	2.4 b	2.3 ab	2.2 b	2.3 b	1.7 c	2.3 b	2.3 b	2.7 b	2.7 b	2.4 b
	F3(0.5)	2.6 ab	2.6 b	2.5 ab	2.4 b	2.2 b	2.4 b	2.4 b	2.4 b	2.4 b	2.4 b	1.4 c
	F4(0.6)	3.6 a	4.3 a	3.6 a	3.4 a	4.2 a	5.3 a	3.4 a	3.4 a	4.7 a	3.7 a	3.4 a
	F5(0.6)	1.7 b	3.3 ab	3.2 a	2.6 b	1.4 b	2.5 b	3.1 a	3.1 a	2.1 b	2.1 b	2.2 b

*Means having similar letters in the same column are not statistically differed at P≥0.05.

3.3 Characterization of the partially purified antibacterial compounds:

There are many compounds released by rhizobacteria used in this study, especially from the three most effective bacterial strains, *B.brevis* M4, *Stenotrophomonas maltophilia* BG4 and *Streptomyces toxitricini* C5, profiles. The most promising antibacterial metabolites in microbial extracts were identified by using High-Pressure Liquid Chromatography and mass spectrometry. The data are presented in Figs. (3-5) and Tables (9-11) that, showed some fractions for each bacterial crude extract; retention time and, mass/z for each fraction lead to identify the present compounds. HPLC profile of *B.brevis* M4 is presented by Fig. (3) and Table (9) that, the first identified compound with molecular weight 168 deduced from m/z 207 [M+1] might be Vanillic acid which has the molecular formula ($C_8H_8O_0$). The second antibacterial compound corresponding to molecular weight 224 deduced as m/z 248 [M+1] might be N-acetyl-D-glucosamine which has the molecular formula ($C_8H_{15}NO_6$). The third antibacterial compound corresponding to molecular weight 265 which own m/z 303 [M+1] suggested to be 2-Methylbutyric acid glucoside which has the molecular formula ($C_5H_{10}O_2$). The fourth antibacterial compound identity to molecular weight 272 and possess m/z 278[M+1] might be Bacilysin which has the molecular formula (CF_3 - CO -NH: CH- CH_3). The fifth antibacterial compound corresponding to molecular weight 265 which own m/z 265 [M+1] suggested to be Tyrocidines which has the molecular formula ($C_{66}H_{87}N_{13}O_{13}$). The sixth antibacterial compound identity to molecular weight 310 and possess m/z 348 [M+1] might be Feruloyl aspartate which has the molecular formula ($C_{31}H_{44}N_7O_{19}P_3S$). The seventh antibacterial compound corresponding to molecular weight 566 deduced as m/z 568 [M+1] might be Uridine 5'-diphospho-glucose which have the molecular formula ($C_{15}H_{22}N_2O_{17}P_2Na_2$).Extracted compounds of *Stenotrophomonas maltophilia* BG4 were shown in HPLC profile. Data presented in Fig. (3) and Table (9) indicated that the first identified compound with molecular weight 192 deduced from m/z 231 [M+1] might be citric acid which has the molecular formula ($C_6H_8O_7$). The second antibacterial compound corresponding to molecular weight 168 deduced as m/z 207 [M+1] might be vanillic acid which have the molecular formula ($C_8H_8O_0$). The third antibacterial compound corresponding to molecular weight 224 deduced as m/z 248 [M+1] might be N-acetyl-D-glucosamine which has the molecular formula ($C_8H_{15}NO_6$). The fourth antibacterial compound corresponding to molecular weight 328 deduced as m/z 328 [M+1] might be 4-Hydroxyphenylpropionic glucoside which has the molecular formula ($HOC_6H_4CH_2CH_2CO_2H$). The fifth antibacterial compound corresponding to molecular weight 627 deduced as m/z 278 [M+1] might be 6-phosphogluconic acid which has the molecular formula ($C_{15}H_{26}N_2$). The sixth antibacterial compound corresponding to molecular weight 326 deduced as m/z 326 [M+1] might be –Hydroxy feruloyl malate which have the molecular formula ($C_4H_4O_7$). The seventh antibacterial compound corresponding to molecular weight 369 deduced as m/z 369 [M+1] might be Feruloyl quinate which have the molecular formula ($C_{17}H_{19}O_9$). Various antimicrobial substances from *Streptomyces* sp. and actinomycetes bacteria have been isolated and characterized (Hrvoje *et al.,* 2006). *Streptomyces toxitricini* C5 extract shows some compounds as present in HPLC profile (Figs. 4 and Table 10). The first identified compound with molecular weight 192 deduced from m/z 231 [M+1] might be Citric acid which has the molecular formula ($C_6H_8O_7$). The second antibacterial compound corresponding to molecular weight 224 deduced as m/z 226 [M+1] might be phenazine-1-carboxylic acid which has the molecular formula ($C_{13}H_8N_2O_2$). The third antibacterial compound corresponding to molecular weight 264 deduced as m/z 265[M+1] might be Methyl butyric acid glycoside which has the molecular formula ($C_5H_{10}O_2$). The fourth antibacterial compound corresponding to molecular weight 441 deduced as m/z 480 [M+1] might be Folic acids which has the molecular formula ($C_{19}H_{19}N_7O_6$). The fifth antibacterial compound corresponding to molecular weight 168 deduced as m/z 207 [M+1] might be Vanillic acid which has the molecular formula ($C_8H_8O_0$). The sixth antibacterial compound corresponding to m/z 270 might be Pyoluteorin.The seventh antibacterial compound corresponding to m/z 396,341,410,450 and 480 [M+1] might be tetracycline derivatives. Synthesis of natural antibiotics by plant growth promoting rhizobacteria (PGPR) include 2, 4 diacetalphloroglucinol, phenzine-1-carboxylic acid, phenazine-1-carboxamide, pyoluteorin, pyrolnitrin, oomycin, and antitumor antibiotics FR901463. All these antibiotics have antimicrobial effect and they are also helpful in plant growth. Each of the antibiotics have different way of work based on different action some of which attack the cellular membrane and some other have inhibitory effect on ribosome and other cellular organism (Sarmin *et al.* 2013 and Singh et al.,2017). Phenazines are synthesized by a limited number of bacterial genera

including *Pseudomonas*, *Burkholderia*, *Brevibacterium*, and *Streptomyces* . Almost all phenazines exhibit broad-spectrum activity against various species of bacteria and fungi. This activity is connected with the ability of phenazine compounds to undergo oxidation-reduction transformations and thus cause the accumulation of toxic superoxide radicals in the target cells (Xiong *et al.,* 2017 and Krishnaiah *et al.,* 2018). *Bacillus brevis* produce tyrocidine which is a constituent of tyrothricin, a mixture of polypeptide antibiotics. Tyrocidine kills bacteria by interacting with their cytoplasmic membranes and causing leakage of their intracellular content. It also affects intracellular membranes such as those of mitochondria. Tyrocidine inhibits RNA synthesis in an in-vitro transcriptional system by forming a complex with the DNA (Kasturi and john 2014).

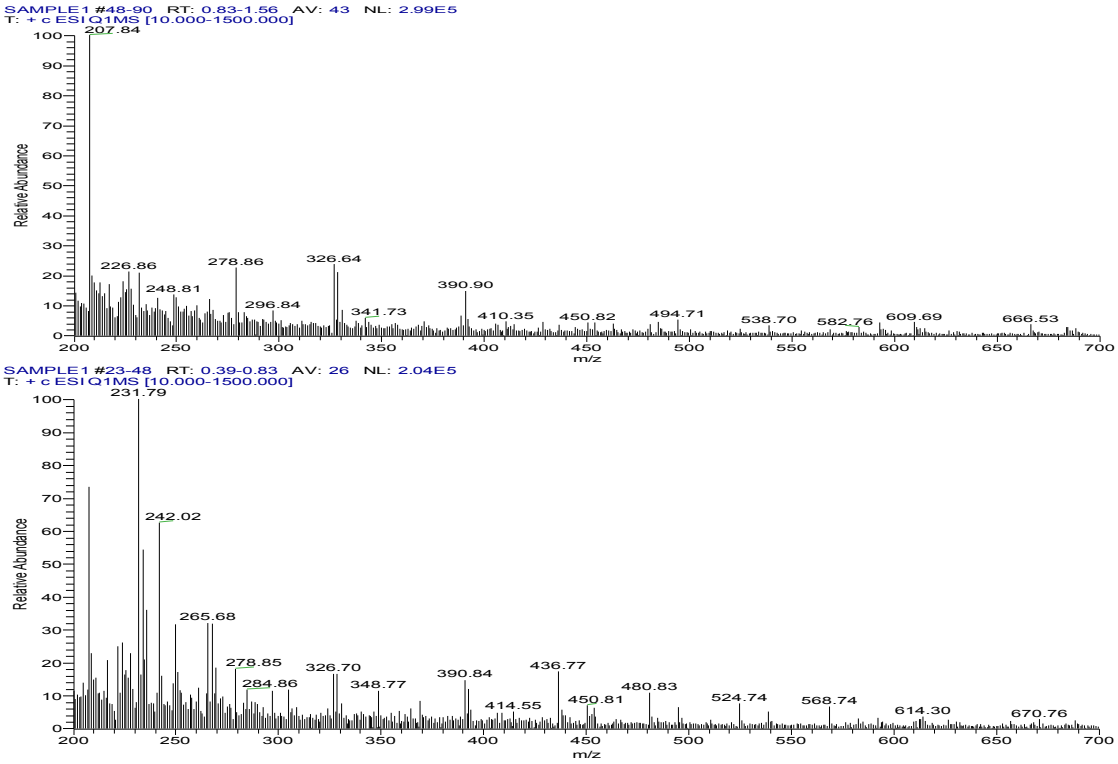


Fig. 3: Chromatogram showing mass /z and relative abundance of the compounds fractionated from *B. brevis* M4 crude extract

Table 9: List of mass, abundance, molecular formula and chemical names of isolated compounds from *B. brevis* M4 crude extract

Mass/z	Relative Abundance(%)	Molecular formula and chemical name
207	100	Vanillic acid (C_8H_8O)
248.81	17	N-acetyl-D-glucosamine ($C_8H_{15}NO_6$)
303	7	2-Methylbutyric acid glucoside
278.86	18	Bacilysin (CF3 - CO -NH: $CHCH_3$)
348.73	8	Feruloyl($C_{31}H_{44}N_7O_{19}P_3S$)
568	5	Uridine 5'-diphospho-glucose($C_{15}H_{22}N_2O_{17}P_2Na_2$).
265.68	34	Tyrocidines ($C_{66}H_{87}N_{13}O_{13}$)

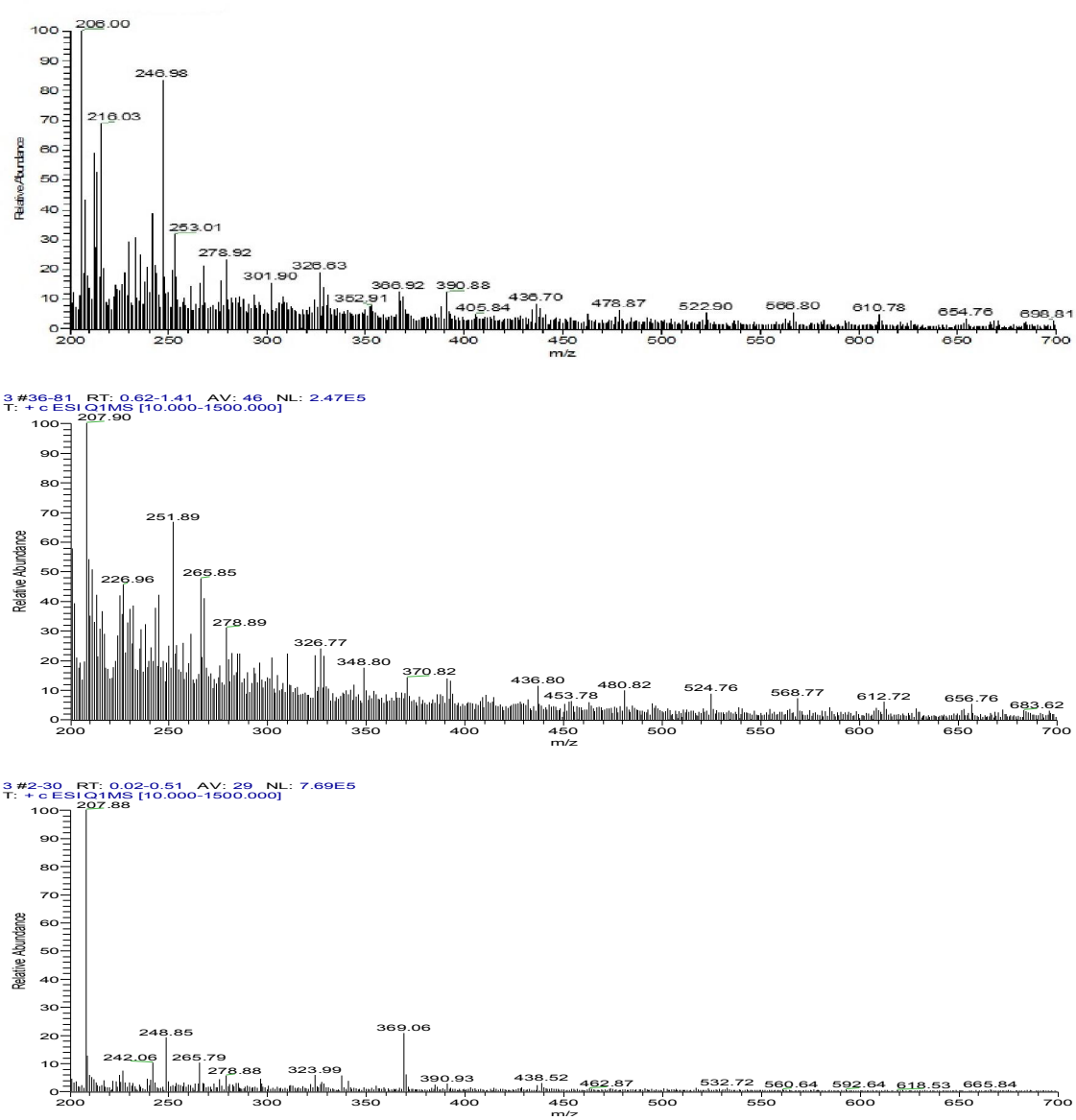


Fig. 4: Chromatogram showing mass/z and relative abundance of the fractionated compounds from *Stenotrophomonas maltophilia* BG4 crude extract

Table 10: List of mass, abundance, molecular formula and chemical names of isolated compounds from *Stenotrophomonas maltophilia* BG4 crude extract.

Mass/z	Relative abundance(%)	chemical name
231	30	Citric acid
207	100	Vanillic acid
248	20	N-acetyl-D-glucosamine
328	22	4-Hydroxyphenylpropionic glucoside

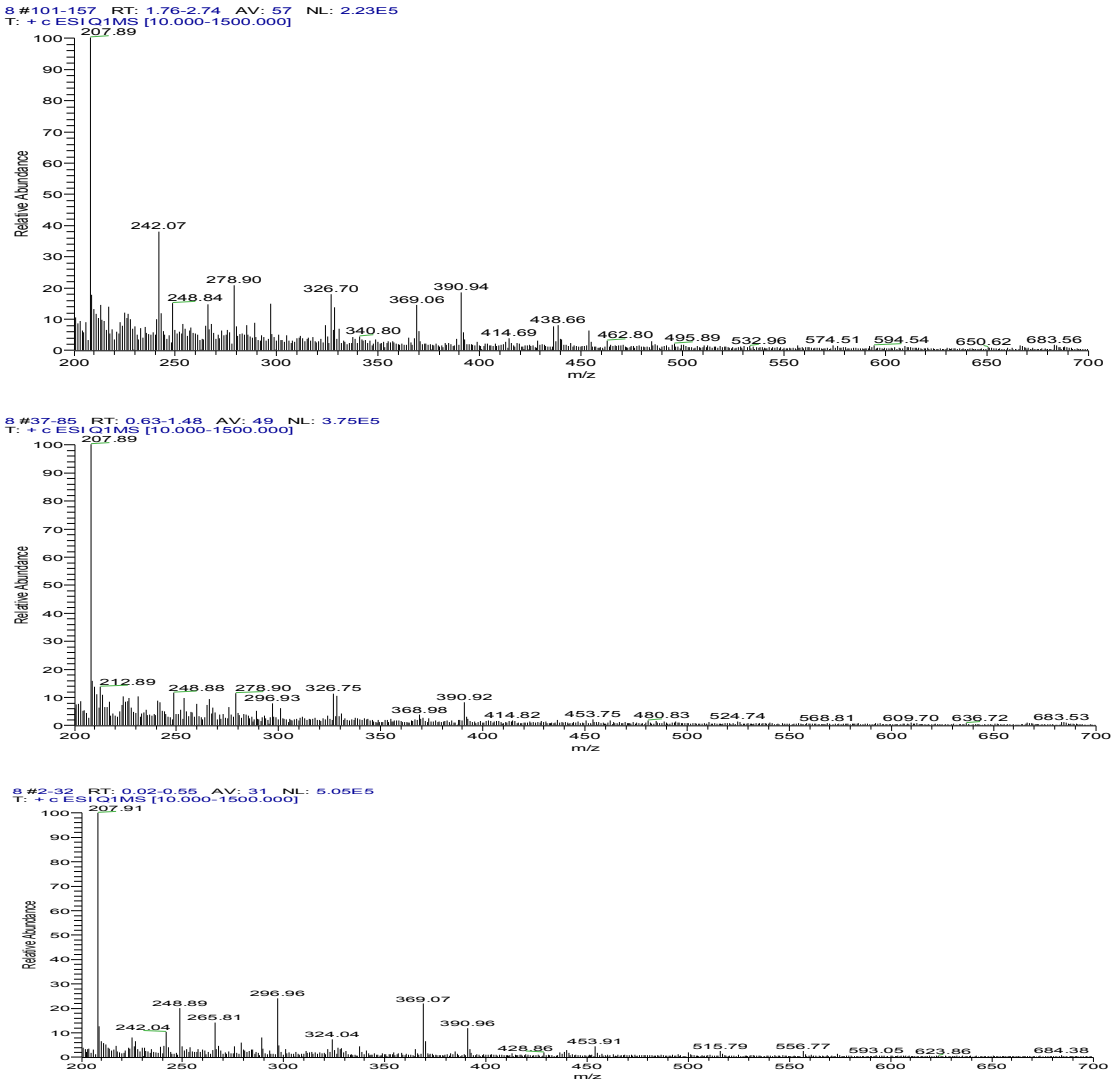


Fig. 5: Chromatogram showing mass/z and relative abundance of the fractionated compounds from *Streptomyces toxitricini* C5 crude extract

Table 11: List of mass, abundance, molecular formula and chemical names of isolated compounds from *Streptomyces toxitricini* C5 crude extract.

Mass/z	Relative abundance (%)	chemical name
231	8	Citric acid
226	12	Phenazine-1-carboxylic acid
265	17	Methyl butyric acid glycoside
480	3	Folic acids
207	100	Vanillic acid
270	15	Pyoluteorin
396	4	Tetracycline derivatives
341	8	Tetracycline derivatives
410	6	Tetracycline derivatives
450	3	Tetracycline derivatives

Conclusion

Research into the mechanisms of plant growth promotion by PGPB has provided a greater understanding of the multiple facets of bacterial wilt disease suppression by these biocontrol agents. Still, most of the focus has been on free-living rhizobacterial strains, much remains to be learned from nonsymbiotic endophytic bacteria that have unique associations and apparently a more pronounced growth-enhancing effect on host plants. Revelations about the mechanisms of PGPB action open new doors to design strategies for improving the efficacy of biocontrol agents. Particularly those that stimulate antibiotic production and activity can be exploited by targeting inoculants for soils that are more likely to support biocontrol soil borne pathogens such as *Ralstonia solanacearum* Race 3 biovar 2.

References

Abdelwareth, A., G. Almoneafy, L. Xie, W.X. Tian, L.H. Xu, G.Q. Zhang and M. Ibrahim, 2012. Characterization and evaluation of *Bacillus* isolates for their potential growth and biocontrol activities against tomato bacterial wilt. African journal of Biotechnology, 11(82): 7193-7201.

Alexander, D.B. and D.A. Zuberer, 1991. Use of Chrome Azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biology and Fertility of Soils, 12: 39-45.

Ausubell, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl,1987.Current Protocols in Molecular Biology, Greene Publishing Associates/Wiley Interscience, New York.

Champoiseau, P. G., J. B.Jones, T. M. Momol, J. Pingsheng, C. Allen, D. J. Norman and K. Caldwell, 2010. *Ralstonia solanacearum* Race 3 biovar 2 causing brown rot of potato, bacterial wilt of tomato and southern wilt of geranium .Available at American Phytopathological Society. Madison, WI. Ralstonia solanacearum. European and Mediterranean Plant Protection Organization Bulletin 34:173-178.

Duncan, D.B., 1955. Multiple range and F.test. Biometrics, 11:1-24.

Glick, B.R., B. Todorovic, J .Czarny, Z. Cheng, J. Duan and B. McConkey, 2007. Promotion of plant growth by bacterial ACC deaminase. Crit. Rev. Plant Sci., 26:227–242.

Hassan E. A., N. M. Balabel, A. E. Ahmed, N. A. Eid and E. M. Ramadan, 2017. Relationship between Ralstonia Solanacearum and bioagents recovered from different habitats. International Journal of Scientific & Engineering Research, 8 (1): 91- 103.

Hrvoje P., J. Cullum, D. Hranueli, I. S. Hunter, N. P.Concha, J. Pigac A. Thamchaipenet D. Vujaklija and P. F. Long, 2006. Genetics of Streptomyces rimosus, the Oxytetracycline Producer Microbiol Mol Biol Rev. 70(3): 704–728.

Ji, X.L., G.B. Lu, Y.P. Gai, C.C. Zheng and Z.M. Mu, 2008. Biological control against bacterial wilt and colonization of mulberry by an endophytic Bacillus subtilis strain. FEMS Microbiol Ecol., 65: 565–573.

Kasturi, R.R. and B.H. John,2014. Biosymthesis of tyrocicines by extract of tow strains of Bacillus brevis. Medecine, Research and expermintal .67:123.

Krishnaiah, M., N. Rodriguesde Almeida, V. Udumula, Z. Song, Y.S. Chhonker, M.M. Abdelmoaty, V. Aragaodo Nascimento, D.J. Murry and M. Conda-Sheridan, 2018. Synthesis, biological evaluation, and metabolic stability of phenazine derivatives as antibacterial agents. European Journal of Medicinal Chemistry, 143: 936-947.

Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, Editors. Nucleic Acid Techniques in Bacterial Systematics. Chichester: John Wiley and Sons, pp: 115-175.

Lugtenberg, B. and F. Kamilova, 2009. Plant growth promoting rhizobacteria. Ann. Rev. Microbiol. 36,541-556.

Mehjabeen, A., Z. Aanisia, A. John Suchit, Pradeep, K. Shukla and P.W. Ramteke, 2017. Suppression of Bacterial Wilt of Tomato (*Lycopersicon esculentum* Mill.) by Plant Growth Promoting Rhizobacteria. An International Journal of Plant Research, 30: 429- 429.

Mishra J. and N.K. Arora, 2017. Secondary metabolites of fluorescentpseudomonads in biocontrol of phytopathogens for sustainable agriculture. Applied Soil Ecology, https:// doi.org /10.1016 /j. apsoil.2017.12.004.

Oliveira, C., G.S. Avelino, K.T. Abe, T.C. Mariguela, R.C. Benine, G. Ortí, R.P. Vari and R.M. Castro, 2011. Phylogenetic relationships within the speciose family Characidae (Teleostei: Ostariophysi: Characiformes) based on multilocus analysis and extensive ingroup sampling. BMC Evolutionary Biology, 11:275

Saber, F.M.A., A.A. Abdelhafez, E.A. Hassan and E.M. Ramadan, 2015. Characterization of fluorescent Psudomonads isolates and their efficiency on the growth promotion of tomato plant .Annals of Agric.Sci.60:131-140.

Safdarpour F. and G. Khodakaramian, 2018. Endophytic Bacteria Suppress Bacterial Wilt of Tomato Caused by *Ralstonia solanacearum* and Activate Defense-related Metabolites. Biological Journal of Microorganism, 6(24): 39- 52.

Sarmin, N.I., G.Y. Tan, CM. Franco, R. Edrada-Ebel, J. Latip and N.M. Zin, 2013. Streptomyces kebangsaanensis sp. nov., an endophytic actinomycete isolated from an ethnomedicinal plant, which produces phenazine-1-carboxylic acid. Int. J. Syst. Evol. Microbiol. 63(Pt 10):3733-8.

Sherif, S., L. Ebada, R. A. Edrada, W. Lin and P. Proksch, 2008. Methods for isolation, purification and structural elucidation of bioactive secondary metabolites from marine invertebrates3. Nature Protocols.1820 – 1831.

Singh, H., V. Jaiswal, S. Singh, S. P. Tiwari, B. Singh and D. Katiyar, 2017. Antagonistic Compounds Producing Plant Growth Promoting Rhizobacteria: A Tool for Management of Plant Disease. Journal of Advances in Microbiology, 3(4): 1-12.

Singh, H., V. Jaiswa, S. Singh, S.P. Tiwar, B. Singh and D. Katiyar, 2017. Antagonistic Compounds Producing Plant Growth Promoting Rhizobacteria: A Tool for Management of Plant Disease. Journal of Advances in Microbiology, 3(4): 1-12.

Song, W., L. Zhou, C. Yang, X. Cao, L. Zhang and X. Liu, 2004. Tomato Fusarium wilt and its chemical control strategies in a hydroponic system. Crop Protection 23: 243-247.

Usharani, P., N. Fatima and M. Nizampatnam, 2013. Evaluation of *Terminalia arjuna* on cardiovascular parameters and platelet aggregation in patients with Type II diabetes mellitus .Research Journal of Life Sciences. 01:7-12.

Wei, G., J.W. Kloepper and S. Tuzun, 1991. Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strain of plant growth promoting rhizobacteria. Phytopathology, 81: 1508-1512.

Whipps, J.M., 2014 .Microbial interactions and biocontrol in the rhizosphere. Journal of Experimental Botany. 52 :487-511.

Xiong, Z., J. Niu, H. Liu, Z. Xu, J. Li and Q. Wu, 2017. Synthesis and bioactivities of Phenazine-1-carboxylic acid derivatives based on the modification of PCA carboxyl group. Bioorganic & Medicinal Chemistry Letters, 27(9): 2010-2013.

Yahya, N.A., Z. Ismail, K.H. Embong and S.A. Mohamad, 1995. High performance liquid chromatography (HPLC) method for confirming thin layer chromatography (TLC) findings in inborn errors of metabolism children in Malaysia. Southeast Asian J. Trop. Med. Public Health.1:130-3.

Yun, C., F. Yan, Y. Chai, H. Liu, R. Kolter, R. Losick and J.h. Guo, 2014. Biocontrol of tomato wilt disease by Bacillus subtilis isolates from natural environments depends on conserved genes mediating biofilm formation. Environ. Microbiol.15 (3): 848–864.