EFFICACY OF SECONDARY METABOLITES AND EXTRACELLULAR LYTIC ENZYMES OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) IN CONTROLLING FUSARIUM WILT OF CHICKPEA

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Abstract

Plant growth promoting rhizobacteria (PGPR) and its metabolic products may play a pivotal role in controlling wilt disease in chickpea plants caused by *Fusarium oxysporum* f. sp. *ciceris* and promote plant growth under greenhouse and field conditions. The obtained data indicated that both PGPR strains *viz. Bacillus subtilis* and *B. megaterium* were able to produce indole acetic acid (IAA), siderophore, hydrogen cyanide (HCN), extracellular compound and volatile antibiotics *in vitro*. In addition, both PGPR strains produced mycolytic enzymes *viz.* chitinase, β-1, 3-glucanase and protease in growth media. *Bacillus megaterium* produced greater amounts of secondary metabolites than *B. subtilis*. Under laboratory condition, cell cultures, culture filtrates, metabolic precipitates with acetone, ethanol and ammonium sulfate inhibited mycelial growth of the target pathogen and the percentage of inhibition varied from 26.07 % to 48.82 %. *Bacillus megaterium* inhibited mycelial growth more than *B. subtilis*. Also, the metabolic precipitate significantly suppressed mycelial growth of pathogen more than cell cultures or culture filtrates and the ethanol precipitate was the most inhibitive. Under greenhouse and field conditions, both PGPR strains used as seed soaking in cell cultures, culture filtrates and/or metabolic precipitates significantly reduced area under wilt progress curve (AUWPC) compared with untreated seeds (control). *B. megaterium* was more efficient for controlling wilt disease in chickpea than *B. subtilis* and the metabolic precipitates reduced AUWPC more than cell cultures or its filtrates. Ethanol precipitate recorded the highest reduction of AUWPC either under greenhouse or field conditions. Using cell cultures, cultures filtrates and metabolic precipitates of both PGPR strains significantly increased growth parameters (plant height and number of branches per plant), yield components (numbers of pod and seed /plant, weight of 100 seed, total yield / feddan and protein content in seeds) compared with untreated seeds (control) during growing seasons 2013-14 and 2014-15 under field conditions. Ethanol precipitate of *B. megaterium* or *B. subtilis* recorded the highest growth parameters and yield components in both growing seasons. Generally, metabolic precipitation recorded the best results for controlling wilt disease under greenhouse and field conditions and improved plant growth and increased yield components more than cell cultures or culture filtrates of both tested PGPR strains.

Keywords: Chickpea, *Fusarium oxysporum* f. sp. *ciceris*, HCN, Growth parameters and yield components, IAA, Mycolytic enzymes, PGPR, Secondary metabolic, Siderophores

INTRODUCTION

Chickpea (*Cicer arieatinum* L.) is one of the most important legume crops growing in the world, but the yield and quality of chickpea are influenced by Fusarium wilt disease caused by *Fusarium oxysporum* f.sp. *ciceris* (Padwick) Sato & Matuo (Omar *et al*., 1999 and Abdel-Hadey *et al*., 2000). Yield losses due to Fusarium wilt estimated with 10% in India and Spain, 40% in Tunisia and 17% in Iran (Jamali *et al*., 2004). The most efficient method for the disease management is using resistant cultivars, although new races of the pathogen appear to overcome resistant genes (Omar *et al*., 1999 and Abdel-Hadey *et al*., 2000). In addition, chemical control is not satisfactory; therefore biological control is an alternative for controlling the disease (Al-Kohal *et al*., 2003). Plant growth promoting rhizobacteria (PGPR) such as *Bacillus* strains, are the major root colonizers (Zaim *et al*., 2013), and can elicit plant defense resistance. Different mechanisms have been reported for their performance such as production of antibiotics, siderophore cyanide hydrogen, competition for nutrition and space, induce resistance, inactivation of pathogen's enzymes and enhancement of root and plant development (Karimi *et al*., 2012). PGPR can prevent the proliferation of fungal and other pathogens by producing siderophores that bind most of the Fe III in the vicinity of the plant root, preventing pathogens from growing close to the plant roots. This is achieved by the PGPR out-competing the pathogens for available iron. Plants are not affected by the localized depletion of soil iron as most plants can tolerate much lower iron concentrations (~1000 fold less) than microorganisms. Similarly, cyanide is a secondary metabolite produced by Gram-negative bacteria. HCN and CO_2 are formed from glycine and catalysed by HCN synthesis. HCN production by strains of PGPR *s*uppresses disease, whereas mutant strains unable to synthesise HCN lose their ability to protect plants from disease. Also, many antifungal metabolites have been produced and shown to be effective *in vitro*. These antifungal metabolites are also suspected to have antifungal activity *in vivo*. These metabolites include ammonia, butyrolactones, 2-4-diacetylphloroglucinol, kanosamine, Oligomycin A, Oomycin A, phenazine-1-carboxylic acid (PCA), pyoluterin (Plt), pyrrolnitrin (pln), viscosinamide, xanthobaccin and zwittermycin A (Whipps, 2001). In addition, certain fungi have been shown to be sensitive to particular combinations of metabolites. The microorganisms isolated from the rhizosphere of various crop have the ability to produce indole acetic acid as secondary metabolites due to rich supply of substrates. Indole acetic acid helps in the production of longer roots with increased number of root hairs and lateral roots which are involved in nutrient uptake (Datta and Basu, 2000). IAA stimulates cell elongation by modifying certain conditions such as the increase in osmotic contents of the cell, increase in permeability of water into cell, decrease in wall pressure, an increase in cell wall synthesis and inducing specific RNA

and protein synthesis. It promotes embial activity, inhibit it promotes embial activity, inhibit or delay abscission of leaves, induce flowering and fruiting. (Narasimhan *et al*., 2013). Also, Chitinase, glucanase and other hydrolytic enzymes have many roles in a wide range of different biological systems. These enzymes are usually extracellular, of low molecular weight and high stability. In addition they may be produced in multiple forms or isozymes that differ in charge, size, regulation, stability and ability to degrade cell walls (Ashwini and Srividya, 2014).

The objectives of the present study were to test the possible role of *in vitro* production of mycolytic enzymes *viz.* chitinase, β-1, 3-glucanase, protease siderophores, HCN and IAA by PGPR strains *viz. B. subtilis* and *B. megaterium* in suppression of *F. oxysporum* f. sp. *ciceris*. In addition, to study the control wilt disease under artificial inoculation in greenhouse and under natural infection under field conditions as well as its effect on growth and yield parameters under field conditions.

MATERIALS AND METHODS

Isolation of the pathogens:

Chickpea plants showing typical wilt symptoms were collected from fields at different locations in New Valley governorate and were used for isolating the pathogen. The tissues were washed in running tap water, cut into small pieces of 5-10 mm, surface sterilized with 3% sodium hypochlorite solution (NaOCl) for 3 min then washed 3 times with sterilized distilled water, blotted dry on sterile filter paper and plated on potato dextrose agar (PDA) medium. The plates were incubated at 25±2°C for 3-5 days. Fungi were purified by single spore technique and transferred to PDA slants.

Pathogenicity test:

These experiments were carried out at New Valley Agricultural Research Station. Pathogenicity of *Fusarium oxysporum* f. sp. *ciceris* isolates (10 isolates) were tested on Giza 3 cultivar. Plastic pots (30 cm in diam.) were used and soil infestation was done using a homogenized culture of the pathogens at the rate 100 ml homogenized culture per pot.

Preparation of fungal inoculums:

Disks taken from 1- wk-old culture of *F. oxysporum* f. sp *ciceris* isolates were inoculated in 75 ml of potato dextrose broth in a 250 mL flask and incubated at 25 ± 2°C. The obtained fungal mats were collected on Whatman No. 1 filter paper, rinsed with sterile distilled water, placed in a Waring blender with a small amount of sterile water, and blended for 2 min at 3000 rpm. Sterile distilled water was then added to give a final concentration of 10^6 spore/ml that was used for soil infestation 5 days

before sowing at the rate 100 ml homogenized culture per pot. Five seeds were sown in each 30 cm in diameter pot and 5 pots were used for each isolate as replicates.

Disease assessments:

Disease reactions were assessed based on the severity of symptoms at 20 days intervals, for 100 days using a 0 to 4 rating scale based on the percentage of foliage with yellowing or necrosis in acropetal progression (0 = 0%, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant). Disease severity index (DSI) described by Liu *et al*. (1995) was adopted and calculated as follows:

$$DSI = \Sigma d/(d\ max \times n) \times 100$$

Where: (d) is the disease rating of each plant, (d) max the maximum disease rating and (n) the total number of plants examined in each replicate.

The mean of area under disease progress curve (AUWPC) for each replicate was calculated as suggested by Pandy *et al.* (1989).

$$AUWPC = D\ [1/2\ (Y1+Yk) + (Y2+Y3+\ldots\ldots..+Yk-1)]$$

Where D= Time interval; Y1= First disease severity; Yk= Last disease severity;
Y2, Y3,……Yk-1= Intermediate disease severity.

Source of the PGPR and inoculum preparation:

Plant growth promoting rhizobacteria (PGPR) namely, *Bacillus subtilis* (isolate BSM1) and *B. megaterium* (isolate BMM5) were obtained from the Lab. of Plant Pathol. Dept., New Valley Agric. Res. Station. These PGPR were previously tested against several soil borne pathogens in chickpea (Abdel-Monaim, 2010). Bacterial concentration was adjusted to approximately 5×10^8 cells ml^{-1} by measuring absorbance at 600 nm against standard curves for each bacterial isolate.

Enzymatic activities and secondary metabolites of the PGPR strains:

Production of IAA:

The production of IAA was determined by colorimetric measurement at 530 nm using Salkowski's reagent. Bacteria were grown under shaking (120 rpm) for 2 days at 30ºC in Luria Bertani broth medium (Tryptone 10.0 g , Yeast Extract 5.0 g Sodium Chloride 10.0 g) supplemented with tryptophan (1 mg ml^{-1}) as IAA precursor. After incubation, the cells were centrifuged (3,000 rpm for 10 min at 4°C) and 1 ml of supernatant was combined with 2 ml of Salkowski's reagent (150 ml of 95-98% H_2SO_4, 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$, and 250 ml distilled water) and incubated for 30 min at room temperature. The quantification of IAA was carried out using a standard curve of pure IAA (Sigma–Aldrich, Co.).

Production of HCN:

Bacterial strains were grown at 25±2 °C on a rotary shaker in Tryptic Soy Broth (TSB). Filter paper (Whatman No. 1) was cut into uniform strips of 10 cm long and 0.5 cm wide saturated with alkaline picrate solution and placed inside the conical flasks in a hanging position. After incubation at 25±2 °C for 48 h, the sodium picrate

present in the filter paper was reduced to reddish compound in proportion to the amount of hydrocyanic acid evolved. The developed colour was eluted in 10 ml of distilled water and the absorbance was measured at 625 nm.

Production of siderophore:

Bacterial strains were grown in KB broth for 3 days at 25±2 °C and centrifuged at 3000 rpm for 10 min and the supernatants were collected. The pH of the supernatant was adjusted to 2.0 with diluted HCl and equal quantity of ethyl acetate was added in a separating funnel, mixed well and ethyl acetate fraction was collected. Five milliliters of ethyl acetate fraction was mixed with 5ml of Hathway's reagent (1.0 ml of 0.1M $FeCl_3$ in 0.1 N HCl to 100 ml distilled water1.0 ml of potassium ferricyanide). The absorbance for dihydroxy phenols was read at 700 nm. A standard curve was prepared using dihydroxy benzoic acid. The quantity of siderophore synthesized was expressed as µmol benzoic acid/ml of culture filtrate.

Determination of extracellular compound:

A 0.2 µm cellophane membrane was placed on PDA plates and 200 µl of antagonistic bacterial suspension (1×10^7 cfu/ml) were inoculated in the center of plates. The plates were incubated at 25±2°C for 48 h, then the membrane with the grown bacterial strains was removed and the plate was inoculated in the middle with a five mm disk of a pure culture of *F. oxysporum* f. sp. *ciceris*. Plates were incubated at 25±2°C for 7 days and the radial growth of the pathogen was measured. Sterile double-distilled water replaced the bacterial suspension in control plates. There were four replicates for each treatment.

Production of volatile antibiotics*:*

Firstly, 200 µl of bacterial suspension (1×10^7 cfu/ml) from each isolate were spread on the surface of a Petri plate containing nutrient agar medium and incubated at 25±2°C for two days. In another Petri plate containing PDA medium, a 5 mm disk of a 7-days-old culture of *Fusarium oxysporum* f. sp. *ciceris* was placed at the centre. Then both half plates were placed face to face preventing any physical contact between the pathogen and the bacterial suspension. The plates were sealed with parafilm. In the control plates, bacterial suspension was replaced with sterile water. Plates were incubated at 25-27°C for 48 h and the percentage of inhibition was calculated for each isolate in four replicates for each treatment.

Assay of chitinases:

PGPR strains were cultured at 25±2°C for 96 h on a rotary shaker in 250 ml conical flasks containing 50 ml of chitin–peptone medium (glucose 0.5%, peptone 0.2%, colloidal chitin 0.2%, K_2HPO_4 0.1%, $MgSO_4$.$7H_2O$ 0.05% and NaCl 0.05%, pH 6.8). The cultures were centrifuged at 12,000g for 20 min at 4°C and the supernatant was used as enzyme source. Colloidal chitin was prepared from crab shell chitin according to Berger and Reynolds (1958). The reaction mixture contained 0.25 ml of

enzyme solution, 0.3 ml of 1M sodium acetate buffer (pH 5.3) and 0.5 ml of colloidal chitin (0.1%). The reaction mixture was incubated at 50°C for 4 h in a water bath. Chitinase activity was determined by measuring the release of reducing sugars by the method of Nielson and Sorensen (1999). One unit of chitinase was determined as 1 nmol of N-acetyl-β-D-glucosamine (GlcNAc) released per minute per mg of protein.

Assay of β-1, 3-glucanase:

Bacterial strains were grown at 25±2°C for 96 h on a rotary shaker in 250 ml conical flasks containing 50 ml of peptone medium containing laminarin (0.2%) (Laminaria digitata; Sigma). The cultures were then centrifuged at 12,000g for 20 min at 4°C and the supernatant was used as enzyme source. The reaction mixture contained 0.25 ml of enzyme solution, 0.3 ml of 0.1M phosphate buffer (pH 5.5) and 0.5 ml of laminarin (0.2%). The reaction mixture was incubated at 40°C for 2 h in water bath. β-1,3-glucanase activity was determined as 1 nmol of glucose released per minute per mg of protein.

Protein content:

Protein content in all the samples was determined as described by Bradford (1976) using bovine serum albumin as standard.

Protease production*:*

Bacillus subtilis and *B. megaterium* were streaked on casein agar and/or incubated at 28 ± 2 °C for four days. At the end of the incubation, the plates were observed for halo zone around the colonies, which indicates the presence of protease. The halo zone diameters were recorded.

Role of the metabolic products from PGPR on *F. oxysporum in vitro*:

Liquid Kannere medium was inoculated with *B. subtilis* and *B. megaterium* then incubated at 25±2°C for 7 days. Bacterial suspensions were centrifuged (10.000 rpm for 30 min). Culture filtrates were sterilized through millipore filter paper 25 µm [x-60.0.45 N m: Gelman sciences, Ann Arbor, MI]. 20% (v/v) culture filtrates were added to Kanner agar medium. Culture filtrates were precipitated with 80% aqueous acetone at ratio 1 culture filtrate: 4 part acetone (v/v) and ethanol 96% at ratio 1 culture filtrate: 3 ethanol (v/v), all of them were incubated at 5 °C for 24 hr. Precipitates were collected on Whatman No. 1 filter paper, air dried and kept at 5°C. Also, culture filtrate was mixed with ammonium suphate at saturations 70% then centrifuged at 10000 rpm for 15 min. Precipitates were washed with saline phosphate buffer [100 ml (KH_2PO_4+K_2HPO_4) pH7+0.85 g NaCl], and dialyzed against deionized water 4 times a day for 48 hr at 5°C for salt removal. The precipitates of acetone and ethanol, ammonium sulphate were added to Kanner agar medium at ratio 250 ppm. All media were poured into sterilized Petri dishes (15 ml medium/plate) and agar plugs from 7 days old cultures of *F. oxysporum* were placed in the center of each

plate. Four replicates per treatment were used. Kanner medium without culture filtrates was served as control and the plates were incubated at 25±2°C.

Effect of metabolic products from PGPR on wilt disease in chickpea under greenhouse conditions:

Cell cultures of *B. subtilis* and *B. megaterium* at 10^8 CFU/ml, culture filtrates at 20% (v/v) or its fractions i.e. acetone, ethanol or ammonium sulfate precipitates at 250 ppm, were tested against infection by *F. oxysporum*. Chickpea seeds (cv. Giza 3) were immersed in the tested solutions for 12 hr. then seeds were planted in soil infested with *F. oxysporum*. Chickpea seeds immersed in distilled water and planted in infested soil served as a control. Disease severity index (DSI), and area under wilt progress curve (AUWPC) were recorded as mention above.

Field experiments:

Field experiments were carried out at New Valley Res. Station Farm during 2013-2014 and 2014-2015 growing seasons, to evaluate the efficiency of cell culture, culture filtrates at 20% (v/v) or its fractions i.e. acetone, ethanol and/or ammonium sulfate precipitates at 250 ppm of the tested PGPR (*B. subtilis* and *B. megaterium*) for controlling wilt disease and its effect on growth and yield parameters under field conditions. The experimental design was a complete randomized block with three replicates. The experimental unit area was 10.5 m^2 (3.5 × 3m). Each unit included 5 rows; each row was 3.5 m in length and 60 cm width. Chickpea seeds (cv. Giza 3) were soaked in treatments described above for 12 hr. The treated seeds were sown in hills 25 cm apart on one side of row in both seasons, 2 seed per hill. In control treatment, chickpea seeds were soaked in water for 12 hr and sown at the same rate. The normal cultural practices of growing chickpea were followed. The mean of area under wilt progress curve (AUWPC) for each replicate was calculated as above. At harvest, plant height (cm), number of branches/plant, number of pods/plant, number of seeds/plant, 100-seed weight and total yield (kg/fed.) were recorded. Protein percentage content in seeds was recorded using the method of Jackson (1973).

Statistical analysis:

Analyses of variance were carried out using MSTATC, 1991 program ver. 2.10. Least significant difference was employed to test for significant difference between treatments at $p \leq 0.05$ (Gomez and Gomez. 1984).

RESULTS

Isolation of the pathogen and Pathogenicity test:

Isolation trials resulted 10 isolates belonging to the genus Fusarium. All isolates were identified as *Fusarium oxysporum* f. sp. *ciceris.*

In the pathogenicity test, all isolates showed significantly different abilities to cause wilt symptoms on artificially inoculated chickpea cv. Giza 3 (Fig. 1). *Fusarium*

oxysporum f sp. *ciceris* isolate FO3 and FO5 recorded the highest area under wilt progress curve (1142.5 and 958.26), respectively. Isolate FO8 and FO9 gave the lowest AUWPC values (254.36 and 240.74), respectively.

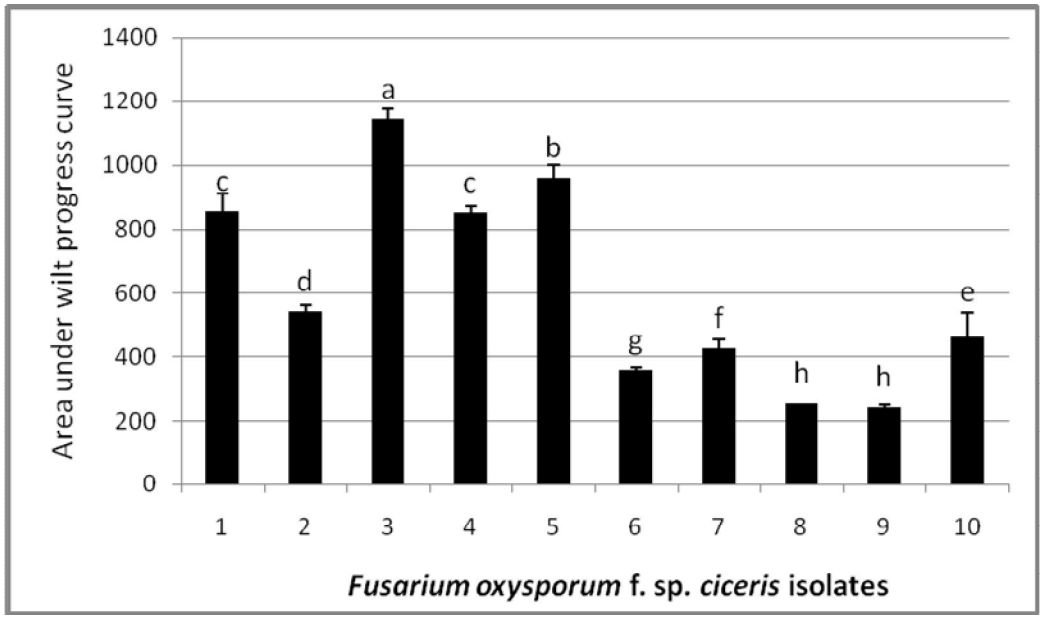

Fig. 1. Pathogenicity test of *Fusarium oxysporum* f. sp. *ciceris* isolates collected from different location in New Valley governorate to chickpea cv. Giza 3. Different letters indicate significant differences between *F. oxysporum* f. sp. *ciceris* isolates according to L.S.D. test (*P*=0.05). Bars indicate the standard deviation. AUWPC= D [1/2 (Y1+Yk) + (Y2+Y3+……..+Yk-1)]; Where D= Time interval, Y1= First disease severity, Yk= Last disease severity, Y2, Y3,……Yk-1= Intermediate disease severity.

Enzymatic activities and secondary metabolite production by the PGPR strains:

Data present in Table (1) show that both plant growth promoting rhizobacteria strains (PGPR) were able to produce IAA, HCN and siderophore in growth media. *Bacillus megaterium* produced these metabolites more than *B. subtilis*, where *B. subtilis* produced 0.259 µg ml^{-1} IAA, 0.041 OD at 625 nm and 6.912 µ mol benzoic acid / ml. While *B. megaterium* produced 0.289 µg ml-1 IAA, 0.059 OD at 625 nm and 7.894 µ mol benzoic acid / ml, respectively. Furthermore, the results indicated that *B. subtilis* and *B. megaterium* produced extracellular metabolites and volatile compound. *B. megaterium* was significantly produce extracellular metabolites and volatile compound more than *B. subtilis*. *Bacillus megaterium* and *B. subtilis* inhibited 30.14, 25.12% of mycelia growth in case of extracellular metabolites and 27.08,

22.14% inhibition of mycelia growth in case of volatile compounds, respectively. On the other hand, both PGPR strains, *B. subtilis* and *B. megaterium* produce mycolytic enzymes *viz*. chitinase β-1,3-glucanase and protease in growth media. *B. megaterium* recorded the activities of chitinase, β-1,3-glucanase and protease more than *B. subtilis*. *B. megaterium* produced 21.124 and 80.471 nmol/min/mg protein and 6.4 mm of hole zone diameter chitinase, β-1,3-glucanase and protease, respectively. While, *B. subtilis* produced 25.476 and 96.360 nmol/min/mg protein chitinase and β-1,3-glucanase and 4.2 hole zone diameter in case of protease activity, respectively.

Role of the metabolic products from PGPR on *F.* oxysporum *in vitro*:

In dual culture test, *B. subtilis* and *B. megaterium* strains, culture filtrates and its metabolic product *viz*. acetone, ethanol and ammonium sulfate precipitate showed a strong inhibitory effect of *F. oxysporum* isolate FO3 on the PDA medium (Fig.2). *Bacillus megaterium* showed more inhibition of *F. oxysporum* than *B. subtilis* in all cases. Furthermore, the results indicated that the metabolites product inhibited growth of the pathogen more than cell filtrates and culture filtrates of both PGPR strains. On the other hand, ethanol precipitate recorded the highest inhibition of *F. oxysporum* growth (42.30 and 48.82% inhibition in case of *B. subtilis* and *B. megaterium*, respectively). Ammonium sulfate precipitate recorded the least inhibition of the pathogen compared with the other precipitate in both PGPR strains.

Table 1. *In vitro* production of various metabolites , enzymes by PGPR strains and inhibition of mycelial growth of *F. oxysporum* f sp. *ciceris*.

Antifungal metabolites	PGPR Strains	
	Bacillus subtilis	*B. megaterium*
Production of IAA (µg/ml)	0.259	0.289
Production of HCN (OD at 625 nm)	0.041	0.059
Production of siderophore (µmol benzoic acid/ml)	6.912	7.894
Production of extracellular compounds (%Inhibition of mycelia growth)	25.12	30.14
Production of volatile antibiotics (%Inhibition of mycelia growth)	22.14	27.08
Chitinase activity (nmol/min/mg protein)	21.124	25.476
β-1,3-glucanase activity (nmol/min/mg protein)	80.471	96.360
Protease production (halo zone diameter with mm)	4.20	6.40

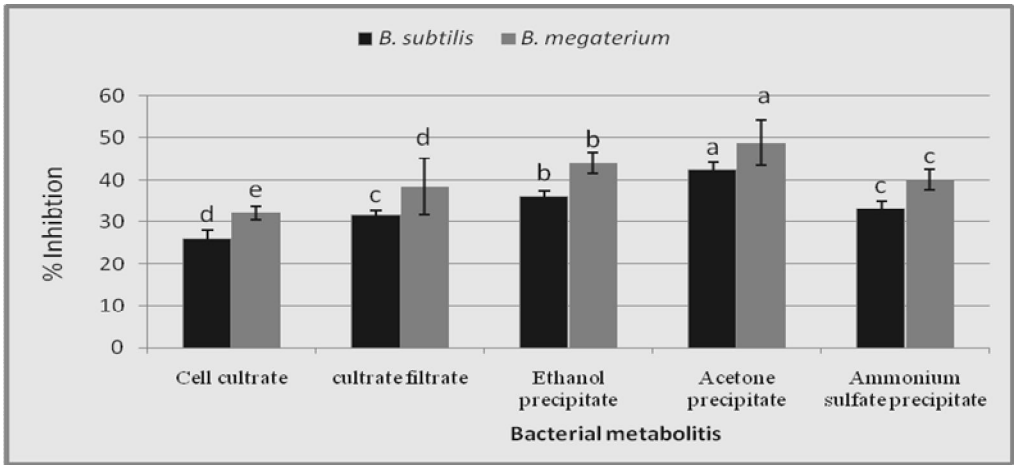

Fig 2. Effect of PGPR antagonists and its metabolites on the mycelial growth of *Fusarium oxysporum* f. sp. *ciceris* in dual culture test. The column followed by different letters indicate significant differences among treatments at $P \leq 0.05$. Bars indicate the standard deviation.

Efficacy of the bacterial metabolites from PGPR to control wilt disease under greenhouse conditions:

Data presented in Table (2) showed that chickpea plants exhibited less disease acquired resistance when grown from seeds previously treated with the cell culture, culture filtrate and metabolic products of PGPR *B. subtilis* and *B. megaterium*. *Bacillus megaterium* was more effective in protecting of chickpea plants from infection with *F. oxysporum* than *B. subtilis* in all treatments. The metabolic products protected chickpea plant against infection with *F. oxysporum* more than cell culture or culture filtrates of both PGPR strains. Ethanol precipitate recorded the lowest area under wilt progress curve in both PGPR stains (234.60 and 181.90 AUWPC, respectively) compared with control (1064.8 AUWPC). On the contrary, cell cultures of *B. subtilis* or *B. megaterium* recorded the lowest protection from the pathogen (518.5 and 428.4 AUWPC), respectively.

Efficacy of the bacterial metabolites from PGPR to control wilt disease under field conditions:

Treatment of chickpea seeds (cv. Giza 3) with cell culture, culture filtrates and/or metabolic by products of PGPR *B. subtilis* and/or *B. megaterium* significantly reduced wilt disease severity in both growing seasons compared with untreated plants (Table 3). The metabolites of both PGPR strains reduced wilt disease severity more than cell cultures or culture filtrates in both growing seasons. *Bacillus megaterium* was more efficacious for controlling wilt disease under natural infection in the field than *B.*

subtilis. The ethanol precipitate of *B. subtilis* and *B. megaterium* recorded the highest reduction of wilt reducing area under wilt progress curve from 834.00 and 892.14 in control to 211.79 and 92.09 AUWPC in season 2013-14 and 223.72 and 101.63 AUWPC in season 2014-15, respectively. On the other hand, the cell culture of *B. subtilis* and *B. megaterium* recorded the lowest protection of chickpea plants against infection in both growing seasons, while reduced AUWPC from 834.00 and 892.14 in control to 371.28 and 239.40 in first season and 436.10 and 249.90 in second season, respectively.

Table 2. Effects of chickpea seeds soaking in PGPR strains and its metabolic on area under wilt progress carve under green house conditions.

Antifungal metabolites	Area under wilt progress curve (AUWPC)	
	Bacillus subtilis	*B. megaterium*
Cell culture	518.5	428.4
Cultrate filtrate	445.4	358.7
Acetone precipitate	297.5	243.1
Ethanol precipitate	234.6	181.9
Ammonium sulfate precipitate	336.6	287.3
Control	1064.8	
LSD at 0.05 for: PGPR strains (A) = Bacterial metabolites (B) = Interaction (A×B) =	 29.87 51.73 73.16	

Table 3. Effects of chickpea seeds soaking in PGPR strains and its metabolic on area under wilt progress carve under field conditions during season 2013-14 and 2014-15.

PGPR strains	Bacterial metabolites	Area under wilt progress curve (AUWPC)	
		Season 2013-2014	Season 2014-2015
Bacillus subtilis	Cell culture	371.28	436.61
	Cultrate filtrate	318.15	335.58
	Acetone precipitate	236.36	246.09
	Ethanol precipitate	211.79	223.72
	Ammonium sulfate precipitate	308.81	349.15
	Mean	289.28	318.23
B. megaterium	Cell culture	239.4	249.9
	Cultrate filtrate	154.25	176.72
	Acetone precipitate	138.81	128.88
	Ethanol precipitate	92.09	101.63
	Ammonium sulfate precipitate	135.24	148.16
	Mean	151.96	161.06
Control		834.00	892.14
LSD at 0.05 for: Seasons (A) = PGPR strains (B) = Bacterial metabolites (C) = Interaction (A×B×C) =		 ns 19.10 35.36 46.85	

Efficacy of the bacterial metabolites from PGPR on growth and yield parameters under field conditions:

Data in Table (4) indicated that chickpea seeds treated with various treatment significantly improved growth parameters (plant height and number of branches / plant) and increased yield parameters *viz.* number of pods / plant, number of seeds /plant, seed index (weight of 100 seeds), seed yield / feddan and protein content in seeds in both growing seasons. The metabolic products of any PGPR strains were more effective in increasing growth and yield parameters than cell cultures or culture filtrates. *Bacillus megaterium* significantly improved plant growth and seed yield more than *B. subtilis* in both growing seasons. The ethanol precipitation of *B. megaterium* gave the highest plant height (110.6, 109.8 cm), number of branches /plant (5.0, 5.2 branches /plant), number of pods /plant (34.5, 35.1 pods/plant), number of seeds /plant (87.1, 88.1 seed/plant), weight of 100 seeds (85.4, 86.1 gm), total seed yield (248.9, 2472.3 Kg/ feddan) and protein content in seeds (30.2 , 30.4%), compared with 70.1, 72.5 cm; 2.9, 3.0 branches /plant; 20.0, 19.8 pods /plant; 46.0, 45.2 seeds/plant; 81.0, 81.8 gm/ 100 seeds; 1421.2, 1435.3 Kg/ feddan and 26, 26.4 % protein in seeds in control treatment during both growing seasons, respectively. On the contrary, cell cultures of *B. subtilis* and *B. megaterium* were recorded the lowest increase of plant growth and yield components.

DISCUSSION

Fusarium oxysporum f. sp. *ciceris* is an economically significant disease of chickpea. Due to the soil-borne nature of pathogen, the use of chemicals for controlling the disease is rarely successful. The use of rhizobacteria for disease control was more effective when rhizobacteria is isolated from rhizosphere of the same host plant. Inconsistencies in biocontrol under varying environmental conditions have been a common limitation in the control of soil borne pathogens (Jamali *et al.*, 2004). The present research was conducted to evaluate the efficacy of PGPR strains *viz. B. subtilis* and *B. megaterium* and its metabolic products against Fusarium wilt disease in chickpea (Abdel-Monaim, 2010).

For many PGPR, the primary mechanism of biocontrol is the production of metabolites such as antibiotics, IAA, siderophores and HCN (Karimi *et al.*, 2012). In addition, many PGPR strains can indirectly protect plants by inducing systemic resistance against various pests and diseases. They play a critical role in naturally suppressive soil to Fusarium wilt through a wide varieties of antibiotics, growth-promoting hormones, siderophores and HCN (Mazzola 2002). *Bacillus* may also

improve plant growth through the production of biologically active substances or the conversion of unavailable minerals and organic compounds into forms that are available to plants (Siddiqui and Mahmood 1999).

In the present study, it was found that *B. subtilis* and *B. megaterium* able to produce IAA, HCN, siderophores, extracellular metabolites and volatile compounds which inhibited the pathogen tested in growth media. *B. megaterium* was more active in this respect than *B. subtilis*.

Chitin and β-1,3-glucan are the main structural components of fungal cells walls, except those of the class *Oomycetes*, which contain β -1, 3-glucan and cellulose (Narasimhan, *et al*., 2013). Mycolytic enzymes (chitinase, β -1, 3-glucanase and protease) are involved in the lysis and hyperparasitism on fungal pathogens. In these mechanisms, chitin, β -1, 3- glucan and protein components of the fungal cell wall are digested by these extracellular enzymes. Many species of *Bacillus* display antagonistic activity against a number of pathogens on several different crops through produced lytic enzymes (Karimi, *et al*., 2012). In this study, both *Bacillus* species (*B. subtilis* and *B. megaterium*) were able to produce chitinase, β- 1, 3-glucanase and proteases *in vitro*. *B. megaterium* recorded to be highly activating of all mycolytic enzymes than *B. subtilis*.

On the other hand, the cell cultures and culture filtrates of both tested PGPR strains and its secondary metabolites suppressed linear growth of *F. oxysporum* f. sp. *ciceris in vitro*. The metabolites of both PGPR strains suppressed mycelial growth more than cell cultures or culture filtrates. The ethanol precipitate greatly suppressed mycelial growth of the fungus. The results were agreement with Al-Kohal *et al*. (2003).

Under field conditions, achieved cell cultures, cultures filtrates of PGPR strains and its metabolic precipitate improved plant growth and increased yield components in chickpea plants during both growing seasons. The soaking chickpea seeds in metabolic precipitate increased growth parameters' and yield components more than seed soaked in cell cultures or culture filtrates. Ethanol precipitate recorded the highest increased of plant growth and yield components in both growing seasons.

Table 4. Effect of PGPR and its metabolic on plant growth, yield components and protein content under field conditions during seasons 2013-14 and 2014-15**.**

PGPR strains	Bacterial metabolites	Plant height (cm)		No. of branches/ plant		No. of pods/ plant		No. of seeds/ plant		Weight of 100 seed		Total yield/ feddan(Kg)		% Protein	
		2013/14	2014/15	2013/14	2014/15	2013/14	2014/15	2013/14	2014/15	2013/14	2014/15	2013/14	2014/15	2013/14	2014/15
Bacillus subtilis	Cell cultrate	82.4	83.6	3.2	3.1	25.3	26.3	61.2	62.5	84.3	84.9	1715.2	1754.3	27.6	27.4
	Cultrate filtrate	90.3	92.1	3.6	3.9	28.6	28.9	71.5	73.5	86.6	87.0	1989.6	1992.5	28.3	28.6
	Acetone precipitate	93.9	93.9	3.9	3.9	26.5	27.3	67.2	68.7	88.5	87.9	2082.0	2100.3	28.6	28.3
	Ethanol precipitate	100.9	100.8	4.2	4.3	30.2	31.5	74.5	75.1	84.6	85.2	2291.7	2292.0	29.7	29.4
	Ammonium sulfate precipitate	90.38	92.4	3.8	3.9	27.4	27.8	69.5	70.5	85.5	86.7	1908.3	1934.5	27.9	28.0
	Mean	91.58	92.56	3.74	3.82	27.60	28.36	68.78	70.06	85.90	86.34	1997.36	2014.72	28.42	28.34
Bacillus megaterium	Cell cultrate	89.7	90.5	3.6	3.5	27.3	27.9	70.2	72.4	84.9	86.7	1931.0	1936.8	28.2	28.1
	Cultrate filtrate	96.0	97.3	3.9	3.8	29.6	30.0	74.3	75.6	86.9	87.6	2080.4	2105.3	28.6	28.3
	Acetone precipitate	99.3	99.2	4.4	4.5	28.3	28.8	73.8	72.5	88.0	89.2	2001.4	2009.3	29.2	29.9
	Ethanol precipitate	110.6	109.8	5.0	5.2	34.5	35.1	87.1	88.1	85.4	86.1	2468.9	2472.3	30.2	30.4
	Ammonium sulfate precipitate	98.2	97.6	4.2	4.1	30.6	32.6	76.2	75.9	85.5	85.4	2051.0	2041.9	27.9	27.9
	Mean	98.76	98.88	4.22	4.22	30.06	30.88	76.32	76.9	86.14	87	2106.54	2113.12	28.82	28.92
Control		70.1	72.5	2.9	3.0	20	19.8	46.0	45.2	81	81.8	1421.2	1435.3	26.00	26.4
LSD at 0.05 for:															
Seasons (A) =		ns		ns		ns		ns		ns		ns		ns	
PGPR strains (B) =		3.56		0.29		1.65		3.12		2.65		65.23		1.88	
Bacterial metabolites (C)=		6.25		0.65		2.75		5.12		3.25		98.25		1.52	
Interaction (A×B×C) =		9.00		1.09		3.96		ns		6.24		142.12		ns	

Plant growth-promoting rhizobacteria improve plant growth via three mechanisms: phytostimulation, biofertilization, and biocontrol. Phytostimulators enhance plant growth in a direct way, usually by the production of phytohormones (auxins, cytokinins, gibberellins). The production of plant hormones such as indole-3-acetic acid (IAA) is classified as "direct" promotion. The synthesis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase can be included in the same group: ACC deaminase cleaves ACC, the immediate precursor of ethylene, and thereby reduces its biosynthesis; ethylene inhibits growth of roots and shoots; therefore, lower levels of this plant hormone lead to plant growth promotion (Karimi *et al*., 2012).

In conclusion, this study provides further evidence that may facilitate the application of PGPR and its secondary metabolites for controlling Fusarium wilt disease in chickpea. Their low cost, low toxicity to man and environmental pollution make them ideal for seed soaking for disease control under field conditions and also for increased seed yield and seed content of protein.

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فاعلية نواتج التمثيل الغذائى والإنزيمات المحللة المنتجة من الريزوبكتريا فى مقاومة مرض الذبول الفيوزاريومى فى الحمص

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يعتبر فطر الفيوازيوم اوكسيسبورم من أهم ساكنات التربة التي تهاجم الجذور للعديد من النباتات مسببة أضرارا بالغة لها خصوصا نباتات الحمص. تم في هذه البحث دراسة إمكانية إستخدام الريزوبكتريا المشجعة للنمو ونواتج التمثيل الغذائي لها في مقاومة مرض الذبول الفيوزاريومى تحت ظروف الصوبة والحقل ومدى تأثيرها على تحسن صفات النمو في الحمص. تبين من النتائج المتحصل عليها إن كلا سلالاتي الريزوبكتريا المستخدمة والتي تضم بكتريا الباسيلس ساتلس وباسيلس ميجاتيرم تستطيع أن تنتج أندول إستيك أسد وسيديروفورز وسيانيد الإيدروجين ومركبات مضادة طيارة في بيئة نموها. كذلك تنتج الإنزيمات المحللة والتي من أهمها إنزيم الشيتينز وبيتا جلاكونيز والبروتيز . وكانت بكتريا الباسيلس ميجاتيرم أكثر إنتاجا لنواتج التمثيل الغذائي من بكتريا الباسيلس ساتلس.

وجد تحت ظروف المعمل إن استخدام خلايا الريزوبكتريا أو راشح الخلايا أو نواتج التمثيل الغذائي سواء كان راسب الأسيتون أو الايثانول او راسب سلفات الامونيا أدى إلى تثبيط النمو المسليومى لفطر الفيوزاريوم اوكسيسبوم حيث تراوحت نسبة الثبيط من ٢٦.٠٧ الى ٤٨.٨٢% وكانت بكتريا الباسيلس ميجاتيرم أكثر تثبيطا للنمو المسليومى من بكتريا الباسيلس ساتلس. كذلك وجد إن نواتج التمثيل الغذائي أكثر تثبيطا للنمو الميسليومى من إستخدام خلايا البكتريا أو راشح مزارعها. وكانت الراسب الإيثانولى أكثرها نشاطا في هذا الشأن.

أدى نقع بذور الحمص في مزارع الخلايا أو راشح المزارع أو نواتج التمثيل الغذائي لكلا سلالاتي الريزوبكتريا المستخدمة في الدراسة إلى خفض المساحة الموجودة تحت المنحنى المرضى مقارنة بالبذور الغير معاملة سواء تحت ظروف الصوبة أو الحقل. وكانت بكتريا الباسيلس ميجاتيرم أكثر فاعلية في مكافحة الذبول الفيوزاريومى من بكتريا الباسيلس ساتلس. كما أن نواتج التمثيل الغذائي كانت أفضل من إستخدام مزارع خلايا البكتريا أو رواشح مزارع تلك البكتريا. وسجل الراسب الإيثانولى أكثر انخفاضا في المساحة الموجودة تحت المنحى المرضى سواء تحت ظروف الصوبة أو الحقل.

أدى نقع بذور الحمص في مزارع خلايا الريزوبكتريا أو في راشح تلك الخلايا أو نواتج التمثيل الغذائي سواء كان الراسب الأسيتونى أوالايثانولى أو راسب سلفات الامونيوم إلى تحسين صفات النمو المتمثلة في طول النبات وعدد الأفرع لكل نبات ومكونات المحصول والتي تشمل عدد القرون لكل نبات وعدد البذور لكل نبات ووزن ١٠٠ بذرة وكمية المحصول للفدان ونسبة البروتين في البذور مقارنة بالبذور الغير معاملة (كنترول) خلال موسمي الزراعة ٢٠١٣-٢٠١٤ و ٢٠١٤-٢٠١٥م وسجل الراسب الإيثانولى التأثيرالأعلى في صفات النمو والمحصول في كلا موسمي الزراعة . عموما تعتبر نواتج التمثيل الغذائي لكلا سلالاتي الريزوبكتريا المستخدمة في الدراسة أفضل من إستخدام مزارع الخلايا أو راشح المزارع في خفض الإصابة بمرض الذبول الفيوزاريومى تحت ظروف الصوبة والحقل بالإضافة إلى تحسين صفات النمو والمحصول .