Egypt. J. Phytopathol., Vol. **40**, No. 2, pp. 95-112 (2012)

Plant Growth Promoting Rhizobacteria (PGPR) Induce Cucumber Root-Rot Protection under Plastic Tunnels Conditions

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Eighteen isolates of plant growth promoting rhizobacteria (PGPR) were isolated from samples of four crops grown in reclaimed fields at Nobariya district (Behera Governorate)**.** The isolates were *in vitro* tested for their antagonistic effect against the three fungal pathogens causing root-rot of cucumber, *i.e. Fusarium solani, Pythium ultimum* and *Rhizoctonia solani*, as well as its effect on plant growth. Nine isolates showed moderate to strong inhibition at least to one of these pathogens and increased vigour index of cucumber. Selected isolates were tested for production of indole acetic acid (IAA) and phosphorous solubilisation capability. The biocontrol ability of these antagonistic isolates was evaluated using an *in vivo* assay. Accordingly, three isolates were selected and used for soil treatment to control root-rot of cucumber in pot experiments. Bacterial isolates mixtures induced significant protection were the same as the individual isolates that significantly reduced symptoms of root-rot pathogens individual or its mixture in pot experiment. *Pseudomonas fluorescens* (P-11), *P. fluorescens* (Cu-2) and *Bacillus subtilis* (Cu-4) were effective for controlling root-rot disease, under plastic tunnels condition. Mixture of the three isolates was most effective to decrease percentage of root-rot and disease severity compared with other treatment and untreated treatment. Seeds treated with *P. fluorescens* (P-11), *P. fluorescens* (Cu-2) and *B. subtilis* (Cu-4) isolates and challenge inoculated with mixture of *P. ultimum*, *F. solani* and *R. solani* significantly reduced disease compared with check treatment and induced the plants to synthesize peroxidase and chitinase enzymes. The maximum phenolic content was observed in *P. fluorescens* (P-11) and *P. fluorescens* (Cu-2) pretreated plants and challenge inoculated with the pathogens, and the higher amounts of phenolics were noticed even on 10^{th} day after the pathogens challenge inoculation.

Keywords: *Bacillus subtilis*, biocontrol, cucumber, plant growth promoting rhizobacteria, *Pseudomonas fluorescens* and root-rot.

Cucumber (*Cucumis sativus* L.) is one of the most important and popular vegetable crops all over the world and in Egypt as well. The crop is mainly cultivated during the autumn season in open fields, while it could be grown under plastic tunnels conditions. Cucumber plants are liable to attack by several soil-borne pathogens, causing severe losses in yield and quality (Liu *et al.*, 1995 and Wei *et al.*, 1996).

Plant growth promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by providing growth promotion. PGPR reported to influence the growth, yield, and nutrient uptake by an array of mechanisms. They offer an excellent combination of traits useful in disease control and plant growth promotion. This group can produce bioactive substances to promote plant growth and/or protect them against pathogens (Harish *et al*., 2009 and Prashar *e al.,* 2013). Some PGPR may influence plant growth by synthesizing plant hormones or facilitating uptake of nutrients from the soil through different direct mechanisms such as help in increasing nitrogen fixation, solubilisation of phosphate and synthesis and synthesis of siderophores or iron sequestration, thus making nutrients more available to plant (Glick *et al*., 2007). The PGPR concept has been indicated by the isolation of many bacterial strains that fulfil at least two of the three criteria described above aggressive colonization, plant growth stimulation and biocontrol (Kloeppor *et al*., 2004 and Zarrin *et al*., 2009). In some PGPR, termed biofertilizers, plant growth promotion dominates. The mechanisms that are involved in this process can include nitrogen fixation, phosphate solubilisation, and the production of phytohormones (such as, auxin and cytokinin) and volatile growth stimulants (such as ethylene and 2, 3 butanediol) (Rezzonico *et al.,* 2007 and Zarrin *et al*., 2009). 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 from competition and environmental stresses (Bergsma-Vlami *et al.,* 2005). These PGPR which mostly belong to *Pseudomonas* spp. and *Bacillus* spp., are antagonists of recognized root pathogens. Some strains of bacteria referred to as PGPR and their effectiveness in controlling a number of plant diseases caused by soil-borne pathogens has been widely documented (Glick *et al.,* 2007 and Abdelwareth *et al*., 2012). In spite of the interest devoted to understanding the protective role of PGPR deciphering the mechanisms by which these bacteria exert their activity has remained a challenge. For instance, competition for substrate and niche exclusion (Bergsma-Vlami *et al.,* 2005), antibiosis (Benhamou *et al.,* 1996 and Ongena *et al*., 1999) or production of extra cellular enzymes (Marjan *et al*., 2003) have been described as mechanisms involved in disease suppression.

PGPR can be applied on a wide range of plants for the purpose of disease control and growth enhancement (Labuschagne *et al.,* 2011). Induced systemic resistance (ISR) has been reported as one of the mechanisms by which PGPR reduce plant disease through the manipulation of the host plant's physical and biochemical properties (Yusran *et al*., 2010).

PGPR- elicited ISR has been demonstrated in many plant species such as bean, tobacco, tomato and cucumber (Wei *et al*., 1996 and Yusran *et al*., 2010). Induced resistance may play an important role in the suppression of *Pythium ultimum* on long English cucumber by fluorescent Pseudomonad (Zhou and Paulitz, 1994). PGPR applied as seed treatments may induce systemic resistance on cucumber causing reductions in severity of several soil-borne diseases under greenhouse conditions (Liu *et al*., 1995; Raupach and Kloepper, 1998; Ongena *et al*., 1999 and Alizadeh *et al*., 2013) have showed that similar strains of PGPR protect various

plant species against diseases caused by soil borne pathogens. PGPR-mediated induce systemic resistance (ISR) is an important mechanism of biological disease control. PGPR are effective in reducing diseases under greenhouse and field conditions (Raupach *et al*., 1998). This makes it even more important to further characterize the physiological, biochemical and molecular mechanisms involved to make optimal use of ISR in plant protection.

Materials and Methods

Source of seeds:

Seeds of cucumber (*Cucumis sativus* L.) used throughout this study were obtained from commercial source in Egypt. Seeds were washed overnight in running tap water, just before sowing and were surface sterilized for 2 min in 2% sodium hypochlorite solution.

Pathogens and inoculum:

Cucumber seedlings and plants showing root-rot symptom, were collected from fields in different high tunnels locations in Nobariya district. *Pythium ultimum* (Edson) Fitzp., *Fusarium solani* Schlecht. and *Rhizoctonia solani* (Kühn) were frequently isolated and identified according to Plaats-Niterink (1981), Booth (1971) and Sneh *et al*. (1992), respectively. Pathogenicity tests of these isolates were performed and their pathogenic potentialities were proven. Purified isolates were maintained on potato dextrose agar (PDA) medium of 4°C till use. Inoculum of each fungal isolate was prepared using group, corn or barley medium in polyethylene bags, each containing 200g medium as described by Singleton *et al*. (1992). Meanwhile, inoculum was prepared as spore suspension (10^4 spore/ml) for *F. solani*, mycelial fragment suspension (10^4 cfu /ml) for *R. solani* and suspension of hyphae, sporangia and oospores (10^4 cfu /ml) for *P. ultimum*.

PGPR strains and inoculum preparation:

Several bacteria were isolated from rhizosphere of different crops grown in Nobariya district in 2007. Bacterial strains were isolated on two different media: tryptic soy agar (TSA) for isolation of heterotrophic bacteria according to Gould *et al*. (1985), King's media B (KB) for isolation of fluorescent Pseudomonads (King *et al.*, 1954). Plats were incubated at 28°C for 2-4 days when individual colonies were picked up, purified and stored at 4°C on the appropriate medium. Bacterial isolates were purified and identified according to morphological, physiological and biochemical characters recommended by Sneath (1986) at Microbiol. Lab., Desert Res. Centre.

Screening of rhizobacteria against root-rot pathogens in vitro:

The 18 obtained rhizobacteria isolates were *in vitro* screened against *P. ultimum, F. solani* and *R. solani*. The bacterial isolates were streaked on one side of a Petri dish (1cm from the edge of the plate) with PDA medium. Mycelial discs (8-mm-diam.) taken from 7-day-old cultures of *P. ultimum, F. solani* and *R. solani* were individually placed on the opposite side of the Petri dish perpendicular to the bacterial streak (Vidhyasekaran *et al.*, 1997) and incubated at room temperature (28°C) for 4 days when the zone of inhibition was measured.

In vivo screening:

Bacterial strains were *in vivo* tested against *P. ultimum, F. solani* and *R. solani* using the soil-dishes technique (Mosa *et al*., 1997). Tested Pathogens were grown for five days on 9-cm-diam. Petri dishes containing thin layer of PDA medium. Then, the fungal colony was covered by autoclaved mixture of peat moss and vermiculite (1:1 v/v). Treated cucumber seeds with rhizobacteria were sown over soil in each Petri dish using sterile tweezers to prevent cross contamination through handling. Set of dishes contained non infested soil served as control. Treatment with the fungicide Rizolex-T (2g/kg seeds) was carried out for comparison. Thereafter, seeds covered by soil mixture, watered daily by sterilized distilled water. Survived seedlings (%) were recorded 25 days after sowing date. Seedlings dry weights were also determined.

Efficacy of rhizobacterial isolates as plant growth-promoters under laboratory conditions:

Bacterial isolates were grown in flasks (250ml) containing 100ml of King's medium broth (KMB) for fluorescent Pseudomonads and tryptic soy agar (TSA) broth for bacilli, for 48h on a rotary shaker at 28±2°C. Cells were removed by centrifugation at 800g for 10 min at 4°C and washed in sterile water. The pellet was transferred in a small amount of sterile distilled water and then diluted with an adequate amount of sterile distilled water to obtain a bacterial suspension of 10^8 cfu ml^{-1} (Thompson, 1996). For bacterization of seeds of cucumber (*Cucumis sativus* L.) were surface sterilized with 2% sodium hypochlorite for 30s and rinsed in sterile distilled water and dried overnight under a sterile air steam. Ten millilitre of bacterial inoculum (10^8 cfu ml^{-1}) was put in a Petri dish. To this, 100mg of carboxymethylcellulose was added as adhesive material. One gram of seeds was soaked in 10ml of bacterial suspension for 12h and dried overnight in sterile Petri dish. Plant growth promoting activity of bacterial isolates was assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993). The vigour index was calculated using the following formula as described by Abdulbaki and Anderson (1973):

Vigour index = (Mean root length + Mean shoot length) × Germination (%).

Potential of PGPR isolates to elicit systemic resistance against root-rot diseases:

PGPR isolates were used for induction of defence reaction in cucumber plants. The following treatments were included in the experiment: (1) seeds treated with bacterial isolates; (2) seeds treated with bacterial isolates and challenge inoculated with mixture of *P. ultimum, F. solani* and *R. solani*, 15 days after planting (50 g sand-maize medium containing 10^3 cfu g^{-1} medium in each pot); (3) plants inoculated with the pathogens 15 days after sowing; and (4) non-treated plants. Seeds were sown in pots filled with sterilized potting soil at 25 seeds per pot. Three replications were maintained in each treatment: each replicate consisted of eight pots. Plants were carefully uprooted without causing any damage to root tissues at different time intervals (0.5. and 10 days after pathogens inoculation). Four plants were sampled from each replication of the treatment separately for biochemical analysis. Fresh roots were washed under tap water and homogenized with liquid nitrogen in a pre-chilled mortar and pestle, and then stored at -70°C.

Efficacy of bacterial isolates against root-rot disease in pot experiment:

A series of greenhouse experiments were conducted to assess control by PGPR isolates against multiple pathogens using the susceptible cucumber (*Cucumis sativus* L.). Treatments were arranged in a randomized complete block design with eight replications consisting of single plant per treatment. A total of eight treatments for each experiment were used for each study: three treatments consisting of single PGPR strains (1,2 and 3); three treatments consisting of strains mixtures (1 plus 2, 1 plus 3 and 1 plus 2 plus 3) and two controls, *i.e*. a nonbacterized but pathogen-challenged disease and a non treated healthy control.

PGPR were applied in one of two ways in this experiment:

For seed treatment: 1.0 ml of bacterial cell suspensions (10^8 cfu /ml) were applied to each cucumber seed. For soil drench: 30 ml of a 10^8 cfu /ml bacterial suspension was poured into each pot at 2-3 weeks after planting.

Cucumber seeds were planted to a depth of approximately 1cm in 10 cm^2 plastic pots (one seed per pot) and kept in the greenhouse with daily watering. One week later, plants were inoculated with three pathogens; *Pythium ultimum, Fusarium solani* and *Rhizoctonia solani* the causal agents of root-rot disease and incidences of the disease was recorded after 4 weeks.

Efficacy of bacterial isolates against root-rot disease under plastic tunnels conditions:

The experiment was carried out at Nobariya district, Behera Governorate under plastic high tunnels in the season 2009 to study the effect of PGPR against root-rot pathogens. Soil was sand loam pH 7.2 and drip irrigation system was applied in a complete randomized block design. In plastic tunnels were dug in the soil to prepare 5 rows. The dimensions of each row was 60m in length, 50cm in height and 50cm in width, cucumber transplants were treated by drenching with (1×10^8cfu/ml) for each of three PGPR strains (1, 2 and 3) alone and in all possible combinations, plus nonbacterized control. A fungicide treatment was also included using "Rizolex-T (50WP)", where 800ml suspensions (500µg active ingredient /ml) were sprayed directly into each seedling tray. Control plants were treated with water only. Cucumber transplants were sown at 10 seedlings within each row. Cucumber seeds were sown 15 days before transplanting in tube preformed trays containing peat/vermiculite (1:1, v:v). Incidences of root-rot disease were recorded after 4 weeks from transplantation. Meanwhile, plant height, dry weight and fruit yield were also determined.

Detection of plant growth promotion activities for antagonistic isolates:
Production of Indole acetic acid production:

Selected six isolates were investigated for their ability to produce indole acetic acid (IAA). Each isolate was grown on NAM (nutrient agar) broth medium containing tryptophan (1.0 mg/L) and incubated in shaker with 30c and 160 rpm for 48h. Next, bacterial culture was centrifuged at 10000 rpm for 15 min, and 1 ml of culture filtrate was mixed with 1ml of salkowskis reagent (1.5 ml of $Fecl_3$. $6H_2O$ 0.5M solution, in 80 ml of 60% H_2S0_4) and the mixture incubated at room temperature for 30 min, presence of pink colour indicates that isolate can produce

indole acetic acid (IAA). Meanwhile (IAA) concentration for each tested strain was quantified colourimetrically in 550 nm by spectrophotometer comparing with IAA standard curve (Gordon and Weler, 1951).

Phosphate solubilisation:

Capacity of 6 selected isolates to solubilise phosphate in form of calcium phosphate was checked qualitatively by using glucose yeast extract agar (GYA) medium containing per 1 L distilled water; 10g glucose; 2g yeast extract and 15g agar. In addition, two other solutions were prepared separately; first 5g K_2HpO_4 was dissolved in 50 ml distilled water and second 10g $CaCl_2$ in 100 ml distilled water. These two solutions were added to 1L CYA just before pouring medium to plates (Beneduzi *et al*., 2008). Each isolate was grown in GY broth for 24h., and then 10ml of bacterial culture were dropped in each plate and inculcated for 7 days at 28°C. Isolates which showed clear halos around their colonies were considered as phosphate solubilisation.

Phenols determination:

Root samples (1g) of Cucumber plants grown in pot experiment and obtained from different treatments and control (20 days after inoculation were homogenized in 10ml of 80% methanol and agitated for 15 min at 70C (Zieslin and Ben-Zaken,1993). One millilitre of the metabolic extract was added to 5ml of distilled water and 250µl of Folin-Ciocalteau reagent (1N) and the solution was kept at 25°C. The absorbance of the developed blue colour was measured using a spectrophotometer at 725nm. Catechol was used as the standard. The amount of phenolics were expressed as µg catechol mg protein-1

Enzymes activity:

1-Peroxidase:

Root samples (1g) of Cucumber plants grown in pot experiment and obtained from different treatments and control (20 days after inoculation were homogenized in 2ml of 0.1M phosphate buffer, pH 7.0 at 4C for 15min and the supernatant was used as enzyme source. The reaction mixture consisted of 1.5ml of 0.05M pyrogallol, 0.5ml of enzyme extract and 0.5ml of 1% H_2O_2. The reaction mixture was incubated at room temperature (28 ± 2°C). The changes in absorbance at 420nm were recorded at 30s intervals for 3min. The enzyme activity was expressed as changes in the absorbance min-1 mg protein-1 (Hammerschmidit *et al*., 1982).

2- Chitinase:

Root samples (1g) of Cucumber plants grown in pot experiment and obtained from different treatments and control (20 days after inoculation were homogenized with 0.2 M Tris HCl buffer, pH 7.8 containing 14 mM B-mercaptoethanol at a rate of 1/3 (w/v). The homogenate was centrifuged at 3000 rpm for 15 min. and the supernatant was used to determine the enzyme activity, according to the colourimetric method suggested by Monreal and Reese (1969) using 1% colloidal chitin. Chitinase activity was measured by the release of N-acetyl-D-glucosamine (NAG) from colloidal chitin, and expressed as acetylglucosamine released/ gram fresh weight tissue/60 min.

Statistical analysis:

Data were statistically analysed, whenever needed, according to the procedures "ANOVA" described by Snedecor and Cochran (1980). Calculated means were compared according to Duncan's multiple range test at 1% and/or 5% level of probability (Duncan, 1955).

Results

Isolation of PGPR:

Eighteen isolates of PGPR were isolated from four different plant species grown at Nobariya district in Egypt. Data in Table (1) illustrate the two isolates of *Pseudomonas fluorescens* and one *Bacillus subtilis* isolated from rhizosphere soil of cucumber, squash, pepper and maize.

Table 1. Antagonistic effects of selected rhizosphere colonizing bacterial isolates on growth of three plant pathogenic soil borne fungi and their potential for plant growth-promotion

Source crop	Isolate treatment	Inhibition zone (mm)*			Vigour ** index
		F. solani	*P. ultimum*	*R. solani*	
Cucumber	(Cu-1)	19.5 ***	13.7	14.5	1152
	P. fluorescens (Cu-2)	23.5	26.2	22.6	1435
	Cu-3	2.9	5.4	6.2	1054
	B. subtilis (Cu-4)	22.4	23.6	25.4	1233
	Cu-5	18.4	12.4	21.6	1023
Squash	Sq-6	5.3	2.4	4.7	754
	Sq-7	20.6	18.6	19.6	950
	Sq-8	21.3	18.4	16.8	1056
	Sq-9	3.8	5.2	4.6	736
	Sq-10	12.6	9.6	8.8	854
Pepper	*P. fluorescens* (P-11)	25.4	21.7	24.7	1478
	P-12	13.7	11.3	9.5	865
	P-13	3.5	2.8	4.3	765
	P-14	2.5	3.1	3.5	698
Maize	Mz-15	8.9	5.7	6.8	896
	Mz-16	4.3	3.5	4.5	696
	Mz-17	19.5	13.7	10.3	856
	Mz-18	91.3	20.5	15.4	1145
Control (check)		0.0	0.0	0.0	652

***** Inhibition of pathogens is expressed as the distance (mm) between fungal mycelium and bacteria colony on potato dextrose agar (PDA); each value is the mean of three replicates.

** Vigour index = (Mean root length + Mean shoot length) × Germination (%)

*** Significant at 0.01 level of probability.

In vitro screening of rhizobacterial isolates against root-rot pathogens:

Among the eighteen bacterial isolates tested for their efficacy to inhabit the mycelial growth, six isolates, *P. fluorescens* (cu-2), *P. fluorescens* (p-11), sq7, sq8, mz18 and *B. subtilis* (cu-4) showed higher inhibitory effect on mycelial growth of *Fusarium solani*, *Pythium ultimum* and *Rhizoctonia solani* when compared to other tested isolates. Among these three isolates, *P. fluorescens* (p-11), *P. fluorescens* (cu-2) and *B. subtilis* (cu-4) exhibited the maximum inhibition for mycelial growth *in vitro* by recording 25.4, 21.7 and 24.7 mm of inhibition zone (Table 1). *P. fluorescens* (cu-2), *P. fluorescens* (p-11) and *B. subtilis* (cu-4) showed an increased vigour index of cucumber in the roll towel assay (Table1). Based on the *in vitro* inhibition of mycelial growth against tested fungi, two isolates of *P. fluorescens*, Sq-7, Sq-8 and *B. subtilis* were screened for further studies of assessing their *in vivo* efficacy against damping-off disease.

IAA production and phosphate solubilisation:

All the isolates showed significant production of IAA (Table 2) that ranged from 5.2 to 38.5 µg/ml. The isolates *P. fluorescens* (Cu-2) and *P. fluorescens* (P-11) produced highest concentration of IAA that was 38.5µg/ml. Three out of six bacterial isolates also showed phosphate solubilisation ability.

Table 2. Determination of IAA and phosphate solubilisation for PGPR isolates

PGPR isolate	IAA (µ/mg)	Phosphate solubilisation ability
P. fluorescens (Cu-2)	35.4	+
B. subtilis (Cu-4)	30.2	+
Sq-7	5.2	-
Sq-8	19.6	-
P. fluorescens (P-11)	38.5	+
Mz-18	10.5	-

In vivo screening of selected antagonistic isolates:

Data in Table (3) indicate that three bacterial isolates, *i.e. P. fluorescens* (p-11), *P. fluorescens* (cu-2) and *B. subtilis* (cu-4) were effective in reducing damping-off of cucumber seedlings caused by *Fusarium solani*, *Pythium ultimum* and *Rhizoctonia solani*. The degree of reduction of damping-off varied according to bacterial isolate and the pathogen. However, the fungicide Rizolex-T seed treatment performed best reduced damping-off caused by the three pathogens. Data in Table (3) also indicate that, there were varied effects of the tested bacterial isolates on seedling survival in pathogen-non infested soil isolate *P. fluorescens* (cu-2) increased the seedling dry weight by 16%. Results also indicated that the cucumber seed germination test in Petri-plates could be used for evaluation the biocontrol agents against root-rot pathogen.

Table 3. *In vivo* screening of selected antagonistic isolates against three pathogenic fungi of cucumber and their effect on seedling dry weight

Tested isolate	Infested soil						Non infested soil	
	F. solani		*P. ultimum*		*R. solani*			
	Survived seedlings (%)	Dry weight (mg)	Survived seedlings (%)	Dry weight (mg)	Survived seedlings (%)	Dry weight (mg)	Survived seedlings (%)	Dry weight (mg)
P. fluorescens (Cu-2)	81 a *	15 a	85 a	10 b	83 a	12 b	84 a	16 a
B. subtilis(Cu-4)	80 a *	11 b	82 a	16 a	84 a	14a	81 a	15 a
Sq-7	70 b	8 c	78 b	13 ab	76 ab	12b	78 b	12 ab
Sq-8	72 b	9 bc	75 b	11 b	78 ab	15 a	78 b	13 ab
P. fluorescens (P-11)	86 a *	13a	84 a	13 ab	80 a	14 a	84 a	15 a
Mz-18	73 b	11 b	77 b	13 ab	74 ab	11 b	78 b	14 ab
Rizolex-T **	86 a	14 a	85 a	13 ab	87 a	14 a	78 b	12 ab
Check ***	46 c	5 c	42 c	5 c	38 b	4 c	59 c	6 b

* Significant at 0.01 level of probability.
** Seeds were treated with Rizolex-T at rate of 2 g/kg seeds.
*** Seeds were treated with 0.01% MC only.

Efficacy of bacterial isolates against root-rot disease in pot experiment:

Data in Table (4) indicate that PGPR treatments induced significant disease protection compared with the non-induced disease control to varying degrees against *F. solani, P. ultimum and R. solani.* The frequency with which various PGPR treatments induced significant protection varied with pathogen used. All three individual strain treatments significantly reduced symptoms of the three pathogens (Table 4). The number of strain mixtures that induced significant protection was the same as the number of individual strains that significantly reduced symptoms of *F. solani, P. ultimum* and *R. solani* pathogens individual or its mixture. In addition, the consistency of obtaining significant disease suppression by PGPR treatments was increased with mixture as compared with single strain treatments. Also, all three individual isolates significant increase plant height and plant dry weight as well as its mixtures.

Efficacy of bacterial isolates against root-rot disease under plastic tunnels conditions:

Results indicated that, the bacterial isolates *P. fluorescens* (Cu-2), *P. fluorescens* (P-11) and *B. subtilis* (Cu-4) were effective for controlling root-rot disease under plastic tunnels condition. These three strains were tested individually and as mixture. The number of strain mixtures that induced significant protection was the same as the number of individual strains that significantly reduced symptoms of root-rot disease incidence. Mixture of three isolates treatment was the most effective to decrease percentage of root-rot incidence compared with other treatment and check treatment (Table 5). Also, data in (Table 5) illustrate that, all treatments led to significant increase in plant growth as measured by the plant height and dry weight, but there were differences among the treatments in the magnitude of growth promotion. The least significant growth promotion occurred with *B. subtilis*

Table 4. Effect of bacterial isolates as seed treatment on the incidence of cucumber root-rot disease in pot experiment

Treatment	Disease incidence (%)				Plant height (cm)	Dry weight (g)
	P. ultimum	*F. solani*	*R. solani*	Mixture		
P. fluorescens (Cu-2)	54.4 bc*	55.5 bc	51.3 bc	57.8 c	56.7 b	0.59 b
P. fluorescens (P-11)	45.6 bc	48.5 bc	53.8 bc	52.5 c	63.4 a	0.72 a
B. subtilis(Cu-4)	71.3 a	69.7 a	67.7 a	72.4 b	50.1 b	0.53 c
P. fluorescens (Cu-2) + *P. fluorescens* (P11)	41.9 bc	45.2 bc	42.1 bc	48.6 c	65.3 a	0.68 a
P. fluorescens (Cu-2) + *B. subtilis* (Cu-4)	61.9 b	66.3 b	62.7 b	60.8 c	67.2 a	0.72 a
P. fluorescens (P11) + *B. subtilis* (Cu-4)	46.8 bc	45.4 bc	38.5 c	39.7 d	64.3 a	0.60 b
P. fluorescens (Cu-2) + *P. fluorescens* (P11) + *B. subtilis* (Cu-4).	28.7 c	22.6 c	21.5 d	27.3 e	62.8 a	0.75 a
Rizolex-T **	9.6 d	8.5 d	6.7 e	8.6 f	51.3 b	0.51 c
Healthy plants	0.0 e	0.0 e	0.0 e	0.0 f	50.5 b	0.54 c
Check ***	75.0 a	81.0 a	89.0 a	95.0 a	19.7 c	0.23 d

* Means within the same column followed by the same letter are not significantly different according to Duncan's

** Seeds were treated with Rizolex-T at rate of 2 g/kg seeds.

*** Seeds were treated with 0.01% MC only.

Table 5. Effect of PGPR isolates as seedlings soaking treatment on incidence of root-rot diseases of cucumber plants under plastic tunnels conditions

PGPR isolate	Disease incidence (%)	Plant growth		Yield (kg)
		Plant height (cm)	Dry weight (g)	
P. fluorescens (Cu-2)	54.0 c *	49.4 c	0.67 b	13.4 b
P. fluorescens (P-11)	38.2 cd	64.1 b	0.75 a	13.2 b
B. subtilis(Cu-4)	69.6 b	42.6 d	0.60 c	11.8 b
P. fluorescens (Cu-2) + *P. fluorescens* (P-11)	41.5 cd	58.9 b	0.71 a	13.5 b
P. fluorescens (Cu-2) + *B. subtilis* (Cu-4)	52.3 c	50.4 c	0.69 b	15.5 a
P. fluorescens (P11) + *B. subtilis* (Cu-4)	20.5 d	68.5 a	0.65 b	15.8 a
P. fluorescens (Cu-2) + *P. fluorescens* (P-11) + *B. subtilis* (Cu-4)	11.7 d	70.2 a	0.76 a	17.5 a
Rizolex-T **	09.6 d	45.8 c	0.55 c	17.0 a
Check ***	81.7 a	21.6 f	0.21 d	9.4 c

* Means within the same column followed by the same later are not significantly different according to Duncan's multiple range test (P≥0.05).

** Seeds were treated with Rizolex-T were 800ml suspensions (500µg active ingredient /ml) were sprayed directly into each seedling tray.

*** Seeds were treated with 0.01% MC only.

(Cu-4), which was significantly less than the growth promotion that resulted from treatment isolate *P. fluorescens* (P-11) and from mixtures of isolates (*P. fluorescens* (P11) plus *B. subtilis* (Cu-4) and *P. fluorescens* (Cu-2) plus *P. fluorescens* (P-11) plus *B. subtilis* (Cu-4)) compared with other treatments (Table 5). There was significantly increase effect on yield for the combination of isolates (Table 5).

Potential of PGPR isolates to elicit systemic resistance against root-rot diseases:

Data in Table (6) indicate that, seeds treated with bacterial isolates and challenge inoculated with mixture of *P. ultimum, F. solani* and *R. solani* significantly reduced disease compared with check treatment.

Table 6. Potentiality of bacterial isolates to elicit systemic resistance against root-rot diseases

Treatment	Infected plants (%)*
P. fluorescens (Cu-2)	43.2 bc**
P. fluorescens (P-11)	24.6 c
B. subtilis (Cu-4)	76.4 b
Check	90.0 a

* Plants inoculated with mixture of *P. ultimum, F. solani* and *R. solani*.

** Means within the same column followed by the same later are not significantly different according to Duncan's multiple range test (P≥0.05).

Biological changes associated with PGPR Treatments:

The maximum phenolic content was observed in *P. fluorescens* (Cu-2) and *P. fluorescens* (P-11) pretreated plants challenge inoculated with the pathogens and the higher amounts of phenolics were noticed even on 10^{th} day after the pathogens challenge. In plants inoculated with the pathogens alone the phenolic content decline to the initial level on the 10^{th} day after inoculation. Plants treated with bacterial isolates alone also had increased content of phenolics compared to untreated (check) plants (Table 7).

Table 7. Effect of PGPR isolate treatments on phenolic compounds in cucumber plants inoculated with mixture of pathogens; *F. solani, P. ultimum* and *R. solani*

Bacterial isolate	Total phenols (*ug* catechol mg protein^{-1})					
	Without pathogen			With pathogen		
	0*	5	10	0	5	10
Pseudomonas fluorescens (Cu-2)	50	50	50	55	88	85
Pseudomonas fluorescens (P-11)	50	52	50	58	90	86
Bacillus subtilis (Cu-4)	45	48	48	45	53	55
Check	40	40	40	42	63	40

* Data were recorded after 0, 5, 10 days.

Meanwhile, seeds treated with PGPR isolates *P. fluorescens* (Cu-2) and *P. fluorescens* (P-11) induced plants to synthesize peroxidase and chitinase, whereas an additional increase in the synthesis was observed in *P. fluorescens* (Cu-2) and *P. fluorescens* (P-11) isolates pretreated plants challenge inoculated with mixture of pathogens; *F. solani*, *P. ultimum* and *R. solani*. The activity reached the maximum level on the third day after pathogens challenge and thereafter the activity remained at higher levels throughout the experimental period of 10 days. In plants treated with the pathogens alone, increased activity of peroxidase and chitinase was observed for period of 5 days and thereafter declined drastically in plant (Table 8).

Table 8. Effect of PGPR isolate treatments on peroxidase and chitinase activity in cucumber plants inoculated with mixture of pathogens; *F. solani, P. ultimum* and *R. solani*

Bacterial isolate	Peroxidase activity * (min^{-1} mg protein $^{-1}$)						Chitinase activity (min^{-1} mg protein $^{-1}$)					
	Without pathogen			With pathogen			Without pathogen			With pathogen		
	0**	5	10	0	5	10	0	5	10	0	5	10
P. fluorescens (Cu-2)	1.8	2.1	1.8	1.6	2.4	2.2	2	2	2	2	2.8	2.5
P. fluorescens (P-11)	1.8	2.2	1.8	1.6	2.6	2.4	2	2	2	2	3	2.6
B. subtilis (Cu-4)	1.6	1.8	1.6	1.6	2	1.8	2	2	2	2	2.4	2
Check	1.6	1.6	1.6	1.6	1.6	1.6	2	2	2	2	2.4	2

* Peroxidase activity was determined in 1 g of cucumber roots and expressed as changes in the Absorbance min^{-1} mg $protein^{-1}$ (Hammerschmidit *et al*., 1982). Meanwhile, Chitinase activity was determined in 1 g of cucumber roots according to the colourimetric method suggested by Monreal and Reese (1969).

** Data were recorded after 0, 5, 10 days.

Discussion

Eighteen isolates of plant growth promoting rhizobacteria (PGPR) were isolated and tested for their antifungal potential or induction systemic resistance for the management of soil-borne diseases (Ongena *et al.*, 1999; Ramamoorthy *et al.*, 2002; Rezzonico *et al*., 2007; Zarrin *et al*., 2009 and Prashar *et al*., 2013). Out of eighteen, three isolates were selected on the basis of antifungal potential or induction of systemic resistance against *Fusarium solani*, *Pythium ultimum* and *Rhizoctonia solani*. The PGPR promote plant growth through more than one mechanism that includes secretion of variety of growth stimulating hormones and suppression of plant growth retarding agents that are pathogens. The *in vitro* test of this study showed that six PGPR isolates produced growth promoting hormone IAA ranging 5.2-35.4 μg^{-1}. Among six isolates, three showed ability to convert insoluble phosphorus (P) to soluble phosphorus (P) usable for plants. Production of growth hormone such as IAA and solubilisation phosphate by PGPR has also been reported by Dilfuza (2008). The isolates *Pseudomonas fluorescens* (*P*-11), *P. fluorescens*

(cu-2) and *Bacillus subtilis* (*cu*-4) showed the maximum inhibitory effect on mycelial growth of *F. solani, P. ultimum* and *R. solani* due to antifungal substances released by the bacteria into the culture medium. In addition, these isolates increased the vigour index and seedling survival using *in vivo* assay. Over the past decade, several strains of PGPR applied to seeds or roots of field crops have been used as elicitors of ISR, leading to reductions in disease severity in roots or in leaves of cucumber (Liu *et al*., 1995 and Raupach *et al*., 1998).

In some experiments in both the greenhouse and field, individual or mixture treatments of some PGPR strains did result in significant protection. Therefore, these mechanisms by applying a mixture of the isolates lead to more effective or at least more reliable biocontrol of root-rot of cucumber. As certain strains improve plant growth in addition to biological control; these strains are collectively called plant growth promoting rhizobacteria designated as PGPR (Kloeppor *et al*., 2004 and Glick *et al.,* 2007). The use of *P. fluorescens* and *B. subtilis* for increasing yield and crop protection are attractive approaches in the modern system of sustainable agriculture (Haas and Defago, 2005 and Dilfuza, 2008). *Pseudomonas fluorescens* and *B. subtilis* having antagonistic activity and increasing the plant growth would certainly be promising in evaluating suitable isolates in biological control (De Meyer *et al.,* 1999 and Kloeppor *et al*., 2004). The present study also indicates that application of isolates *P. fluorescens* (p-11); *P. fluorescens* (cu-2) and *B. subtilis* (cu-4) increase plant growth in pot and field experiments and reduced disease incidence. The increase in plant growth might be associated with secretion of (auxins, gibberellins and cytokinins) and solubilisation phosphate in the soil (Zarrin *et al*., 2009 and Abdelwareth *et al*., 2012) and suppression of deleterious microorganisms in the rhizosphere (Haas and Defago, 2005).

In addition to direct antagonism and plant growth promotion, *P. fluorescens* (p-11); *P. fluorescens* (cu-2) increased the activities of various defence -related enzymes and chemicals in response to infection by the pathogen. It is well known that all plants are endowed with defence genes which are quiescent in nature and appropriate stimuli or signals are needed to activate them. In the present study, it has been observed that seeds treated with *P. fluorescens* (p-11); *P. fluorescens* (cu-2) increased the activities of various defence -related enzymes which lead to the synthesis of defence chemicals in the plants (Ramamoorthy *et al*., 2002 and Alizadeh *et al*., 2013). De Meyer *et al.* (1999) reported that rhizosphere colonization by *P. aeruginosa* 7NSK2 activated PAL in bean roots and increased the salicylic acid levels in leaves. The higher peroxidase activity was noticed in cucumber roots treated with *P. corrugate* challenged with *Pythium aphanidermatum* (Chen *et al*., 2000).

Phenolic compounds may be fungitoxic in nature and may increase the mechanical strength of the host cell wall. In the present study, seed treatment with *P. fluorescens* (p-11); *P. fluorescens* (cu-2) resulted in increased accumulation of phenolic substances in response to infection by the pathogen. Pretreated seeds with *P. fluorescens* isolate Pf1 challenged with *Pythium aphanidermatum* showed higher accumulation of phenolics (Ramamoorthy *et al*., 2002 and Alizadeh *et al*., 2013). However, M'Piga *et al*. (1997) reported that *P. fluorescens* isolate 63-28 induced the

accumulation of phenolics in tomato root tissues. The hyphae of the pathogen surrounded by phenolics substances exhibited considerable morphological changes including cytoplasmic disorganization and loss of protoplasmic content. Accumulation of phenolics by prior application of *P. fluorescens* in pea has been reported against *P. ultimum* and *F. oxysporum* f.sp. *pisi* (Benhamou *et al*., 1996).

Application of mixture of three isolates *P. fluorescens* (p-11); *P. fluorescens* (cu-2) and *B. subtilis* (cu-4) has resulted in much more intensive plant growth promotion and disease reduction when compared to isolates tested singly. This might be due to different mode of action for PGPR and related to sufficient root colonization and efficiency of biocontrol (Raupach and Kloepper, 1998 and Prashar *et al*., 2013). It can be concluded that PGPR isolates from rhizosphere of some plants has potential to be used successfully for biological control of root-rot in cucumber especially under plastic tunnels conditions.

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(Received 25/06/2012;
in revised form 29/07/2012)

التحفيز الوقائى لمرض عفن جذور الخيار بإستخدام البكتريا المشجعة للنمو تحت ظروف الأنفاق البلاستيكيه

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يصاب الخيار فى مناطق نظم الزراعة تحت ظروف الأنفاق البلاستيكية للأصابة بالعديد من ممرضات التربة التى تسبب خسائر شديدة فى المحصول ،ونظرا لتباين المسببات المرضية المختلفة المسببة لتلك الأمراض فى حساسيتها للمبيدات الفطرية المستخدمة فى المكافحة وعدم جدواها أحيانا بالأضافة للتأثيرات البئية الناشئة عن إستخدامها ، فقد هدف البحث إلى دراسة إمكانيية المكافحة الحيوية لتلك الممرضات. عزلت ثمانية عشر عزلة من بكتيريا المجموع الجذرى المنشطة للنمو من جذور بعض المحاصيل النامية فى الأراضى المستصلحة فى منطقة النوبارية. وقد أظهرت العزلات (p11) *Pseudomonas fluorescens* ، (cu-2) *P. fluorescens* ، (cu-4) *Bacillus subtilis* أعلى قدرة تثبيطية للنمو الميسليومى للفطريات المختبرة الممرضة وذلك فى المعمل على أطباق الأجار كما أحدثت زيادة فى Vigour index (دليل الحيوية) لبادرات الخيار أظهرات العزلات البكتيرية المستخدمة أعلى قدرة لإفراز إندول حامض الخليك وتيسير الفوسفات .تم عمل تقيم حيوى لتلك العزلات البكتيرية بأستخدام أطباق التربة لدراسة تأثيرها كمعاملة بذور فى إختزال موت الممرضات وحيث تم إنتقاء أفضل ثلاثة عزلات مضادة أظهرت تأثيرا معنويا فى خفض الأصابة وكذلك زيادة فى نمو البادرات المتسبب عن الفطريات الممرضة *Fusarium solani* ، *Rhizoctonia solani* ، *Pythium ultimum* فى المعمل. أدت معاملة البذور بخليط من الثلاثة العزلات البكتيرية إلى إحداث تأثير وقائى معنوى متساويا للتأثير المعنوى عند إستخدامهم كلا عن حدا فى خفض أعراض عفن الجذور فى تجارب الأصص. كما أظهرت العزلات البكتيرية الثلاثة المستخدمة (p-11) ، (cu-2) ، (cu-4) فاعلية فى مقاومة مرض عفن الجذور تحت ظروف البيوت المحمية البلاستيكية. وكان خليط العزلات البكتيرية الثلاثة هو الأكثر فاعلية فى خفض نسبة وشدة الأصابة بعفن الجذور بالمقارنة بباقى المعاملات . كما أظهرت معاملة البذور بالعزلات البكتيرية (p-11) ، (cu-2) ، (cu-4) خفضا معنويا للمرض مقارنة بالغير معاملة. أدت معاملة البذور بالعزلات البكتيرية إلى زيادة نشاط لإنزيمات البروكسيديز والشيتينيز فى النباتات النامية. وتم تسجيل أعلى محتوى فينولى فى النباتات التى عوملت بالبكتريا قبل عدواها بالممرضات ، كما سجلت أعلى كمية من المواد الفينولية بعد عشرة أيام من معاملتها بالممرضات.