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Potential Plant Growth-promoting Activity of *Pseudomonas* spp. and *Bacillus* spp. as Biocontrol Agents Against Damping-off in Alfalfa

[1]Ehab A.D. Sarhan and [2]Heba Sh. Shehata
[1]Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt
[2]Soil, Water and Environmental Research Institute, Agricultural Research Center, Giza, Egypt

Abstract: Four strains of plant growth promoting rhizobacteria (*Bacillus subtilis*, *Paenibacillus polymyxa*, *Pseudomonas fluorescens* and *Pseudomonas putida*) and *Sinorhizobium meliloti* were tested for their antibiosis toward damping-off disease of growth and yield of the alfalfa crop during 2010-2011 and 2011-2012 seasons. *In vitro* the four PGPR strains produced hydrogen cyanide (HCN), indole-3-acetic acid (IAA), siderophore, solubilized insoluble phosphate and showed protease and β-1,3-glucanase activities, whereas *S. meliloti* produced IAA and solubilized insoluble phosphate only. PGPR strains strongly inhibited growth of *Fusarium oxysporum*, *Fusarium solani* and *Rhizoctonia solani in vitro*. Furthermore, *Ps. fluorescens* was the most effective followed by *P. polymyxa*, *B. subtilis* and *Ps. putida* respectively. Seed treatment with the tested PGPR strains significantly reduced alfalfa damping-off diseases incidence compared to untreated control under greenhouse and field conditions. *Ps. fluorescens* and *P. polymyxa* combined with *S. meliloti* recorded the highest reduction in diseases incidence. Under greenhouse conditions, uninoculated plant recorded the lowest nodule number and biomass, while co-inoculation with *S. meliloti* and mixture of the four tested strains recorded the highest values. Same results were obtained when alfalfa seeds were treated by PGPR combined with *S. meliloti* on Number of nodules, dry weights of nodules, as well as plant height, tillers/m^2 fresh, dry weight and protein content of alfalfa plants under field conditions. *Ps. fluorescens* and *P. polymyxa* combined with *S. meliloti* recorded the highest values followed by *B. subtilis* and *Ps. putida* respectively.

Key words: Alfalfa, plant growth promoting, rhizobacteria, damping-off

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is one of the most important forage legume crops in the world as a source of high quality feed for livestock. This forage legume has unique characteristics including high yield and quality and tolerance to drought and other environmental stresses. Their symbiotic association with rhizobia makes the atmospheric nitrogen available for themselves and other crops. Alfalfa considered one of the major forage legume crops in Egypt, covers the shortage of green feed in the country, particularly in the summer. (Hassouna *et al*., 1994; Lamb *et al*., 2006; Yanes *et al*., 2012).

Alfalfa is attacked by more than 70 fungi in addition to bacteria, mycoplasmas, viruses and nematodes as well as weeds. The fungal diseases affect all the plant parts seed, leaves, crowns and roots (Hancock, 1985). Seedling diseases caused by soilborne fungi, i.e., *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *medicaginis*, *Fusarium solani*, *Fusarium semitectum*, *Verticillium* spp., *Sclerotium bataticola*, *Sclerotium rolfsii*, *Macrophomina phaseolina*, *Phytophthora* sp. and *Pythium* spp. were reported as the critical factors which limit alfalfa establishment causing plant death and decreased yield quantity and quality (Seif El-Nasr and Leath, 1983; Omar and Rammah, 1992; Quagliotto *et al*., 2009; Morsy *et al*., 2011).

Biological control using antagonistic microorganism(s) against alfalfa pathogenic fungi is ideal and safe approach unlike chemical fungicides application. Besides, their detrimental effects on the biological nitrogen fixation by rhizobia (Carlton, 1993; Haas and Defago, 2005; Quagliotto *et al*., 2009).

Pseudomonas spp. and *Bacillus* spp. are the most promising groups of rhizospheric inhabitants able to control pathogenic soilborne microorganisms. They showed antagonistic activity against *Rhizoctonia* spp., *Fusarium* spp., *Verticillium* spp., *Sclerotium bataticola*, *Sc. rolfsii*, *Macrophomina phaseolina*, *Phytophthora* sp. and *Pythium* spp. caused legume diseases. However, little research has been previously carried out on the use of rhizospheric microorganisms, *Pseudomonas* spp. and *Bacillus* spp. to control diseases caused by soil-borne

Corresponding Author: Ehab A.D. Sarhan, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt

pathogens fungi of alfalfa (Kazmar *et al*., 2000; Spadaro and Gullino, 2005; Quagliotto *et al*., 2009; Morsy *et al*., 2011).

The objectives of the present research were to evaluate the efficacy of *Rhizobium* and four promising plant growth promoting rhizobacteria (PGPR) i.e., *Bacillus subtilis*, *Paenibacillus polymyxa*, *Pseudomonas fluorescens* and *Pseudomonas putida* for controlling *Fusarium oxysporum* f. sp. *medicaginis*, *Fusarium solani* and *Rhizoctonia solani* associated with pre- and/or post-emergence seedling damping-off in alfalfa. Growth and yield parameters of alfalfa under field conditions were determined for two successive seasons at Giza governorate.

MATERIALS AND METHODS

Inocula used: *Sinorhizobium meliloti* (*Rhizobium*) and four PGPR strains i.e., *Bacillus subtilis*, *Paenibacillus polymyxa*, *Pseudomonas fluorescens* and *Pseudomonas putida* were kindly provided by Biofertilizer Production Unit, Soils, Water and Environment Research Institute, ARC, Giza, Egypt. The soil-inhabiting bacterium *Paenibacillus polymyxa* (previously *Bacillus polymyxa* but reclassified by Ash *et al*. (1994).

Pathogenic fungi: The used pathogenic fungi *F. oxysporum*, *F. solani* and *R. solani* were isolated from alfalfa diseased plant and previously tested and identified as a pathogenic caused alfalfa seedling damping-off were kindly provided by legume and forage diseases research department, Plant Pathology Research Institute, ARC, Giza, Egypt.

Production of antifungal metabolites and plant growth promoting substances by rhizobacteria: Strains were tested for their capabilities to produce siderophore, hydrogen cyanide (HCN), β-1,3 glucanase, protease as well as Phosphate solubilization and indole-3-acetic acid (IAA).

Antifungal metabolites assay: Siderophore production was semiquantified using chrome azurol S (CAS) medium (Schwyn and Neilands, 1987). Glycerol (5% v/v) was used as the sole carbon source and the Pseudomonas selectivity was improved by the addition of 10 mg L^{-1} cetrimide, 10 mg L^{-1} fucidin and 50 mg L^{-1} cephaloridine. When the iron (III) is removed from the chrome azurol S complex by high affinity siderophore, the color change from the blue to orange. The diameters of orange halos around the colonies after incubation at 28°C for 2 days were indicative of the relative level of siderophore production. Production of hydrogen cyanide (HCN) was detected by growing bacteria for 16 h at 24°C on plates containing nutrient agar with an indicator paper inoculated with 5 mg of copper(II) ehtylacetoacetate, 5 mg of methylene bis(-n-n-dimethylaniline) and 2 mL chloroform, as described by Castric and Castric (1983). β-1,3-glucanase was assayed by incubating isolates on KB broth containing 1 mL 0.2% laminarin (w/v) in 50 mM sodium acetate buffer (pH = 4.8) with 1 mL enzyme solution at 50°C for 1 h and by determining the reducing sugars with DNS (Nelson, 1944). The amount of reducing sugars released was calculated from standard curve for glucose. One unit of β-1, 3-glucanase activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of glucose equivalents min^{-1}. Protease activity (casein degradation) was determined from clearing zone in Skimmed Milk Agar (SMA) according to Nielsen *et al*. (1998).

Plant growth promoting substances assay: Production of indole-3-acetic acid (IAA) was determined according to the method of Bano and Musarrat (2003). Isolates were grown on king's B medium and incubated at 28°C for 5 days, then transferred to 5 mL KB broth containing 2 mg mL^{-1} L-tryptophan. Cultures were incubated at 28°C with shaking at 125 rpm for 7 days then harvested by centrifugation at 11,000xg for 15 min. One milliliter of the supernatant was mixed with 2 mL of Salkowski reagent; the appearance of a pink color indicated IAA production. Optical density (OD) was read at 530 nm. The level of IAA produced was estimated against the IAA standard. Plant growth promoting properties such as phosphate solubilization was performed by spot inoculation on Pikovskya's medium (Pandey *et al*., 2005).

Screening for antagonistic effect: Agar assay method was used to screen the selected bacterial isolates for their effects on target pathogens, namely *Fusarium oxysporum, F. solani* and *Rhizoctonia solani* on nutrient agar plates. One disc from fresh culture of each pathogen was inoculated at the periphery of the agar plate and the bacterial isolates were streaked at the opposite side of the plate. Five replicates were used for each treatment. All plates inoculated with *Fusarium oxysporum*, *F. solani* and *Rhizoctonia solani* only were used as control. Plates were incubated at 25±1°C, until the control plates were fully covered by fungal growth. Linear growths of the target fungi were measured. Inhibition percent of growth was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{\text{Growth in control-Growth in treatment}}{\text{Growth in control}} \times 100$$

Preparation of fungal pathogen: Fungal inocula were prepared by inoculating sorghum-sand medium in 500 mL glass bottles with 5 mm disk of a 7-day-old culture of the pathogen tested then incubated at 27±1°C for 15 days.

Preparation of the *Rhizobium* inoculum: Seven day old culture of *Rhizobium* (*Sinorhizobium meliloti*) grown on yeast extract mannitol broth medium (Vincent, 1970) was suspended in sterilized water and shaken vigorously to obtain homogenous cell suspension of 5×10^9 cell mL^{-1}. Vermiculite supplemented with 10% Irish peat was packed into polyethylene bags each containing 180 g to serve as inoculum carrier. Prior to be injected with bacteria, the bags were sterilized by gamma irradiation (5×10^6 rads) then bags were injected with rhizobia to insure 50% of the maximal water holding capacity.

Preparation of the biocontrol agents: Plant growth promoting rhizobacterial (PGPR) strains i.e., *B. subtilis*, *P. polymyxa*, *Ps. fluorescens* and *P. putida* were grown on king's B medium (King *et al*., 1954), incubated for 24 h at 28°C to ensure a population of 10^9 cell mL^{-1} and injected in sterilized vermiculite as previously mentioned.

Greenhouse experiments: This experiment was carried out using pots 25 cm in diameter. Pots were then sterilized by immersing in 5% formalin solution for 15 min then air dried for 5 days. The soil was supplemented with superphosphate (15.5% P_2O) at the rate of 1 g pot^{-1} (250 kg fed^{-1}) prior to seed sowing. Soil infestation was carried out by adding the fungal inoculum to the sterilized soil at the rate of 3% of soil weight. Fungal inocula were thoroughly mixed with the soil and regularly watered every day for a week before planting to ensure even distribution and growth of each particular fungus. Soil mixed alone with the same amount of autoclaved sorghum-sand medium served as a check treatment. Each pot was planted by 20 seeds of alfalfa and five pots were used for each treatment. Seeds were treated with 16% Arabic gum solution as a sticking agent and thoroughly mixed with the previously prepared vermiculate carrier containing either *Sinorhizobium meliloti*, *Bacillus subtilis*, *Paenibacillus polymyxa*, *Pseudomonas fluorescens*, *Pseudomonas putida* or dual combinations all of them at the rate 400 g 30 kg^{-1} seeds. The planted non-infested soil was served as control. The fungicide Rhizolex-T [Rhizolex-T 50%: Common name (Tolclofos-methyl and Thiram)-Chemical name (O-2,6-dichloro-p-tolyl O, O-dimethyl phosphorothioate and Tetramethylthiuram di sulfide)] was used at the rate of 2.5 g kg^{-1} seeds to comparison. In control treatment, seeds were soaked in water.

The treatments with five replicates were arranged in randomized complete block design, these were:

- Control (untreated seeds). Full dose of N = 75 kg fed^{-1}
- Inoculated with *Rhizobium* (*Sinorhizobium meliloti*)
- *Sinorhizobium meliloti*+*Bacillus subtilis*
- *Sinorhizobium meliloti*+*Paenibacillus polymyxa*
- *Sinorhizobium meliloti*+*Pseudomonas fluorescens*
- *Sinorhizobium meliloti*+*Pseudomonas putida*
- *Sinorhizobium meliloti*+Rhizolex-T

Nitrogen fertilizer was added two weeks after sowing as ammonium sulphate (20.5% N) and potassium sulphate (48% K_2O) at the recommended dose. Plants were observed daily and percentages of pre- and post-emergence damping-off as well as seedling survival were determined after 15, 30 and 60 days, respectively, as indicators of disease incidence. For symbiotic parameters after 60 and 120 days of planting, plants were uprooted to estimate number and dry weight of nodules according to Page *et al.* (1982).

Field trails: Field trails were carried out at the Experimental Farm of Giza Agric. Res. Station, Giza, during the successive seasons 2010/2011 and 2011/2012, to study the effect of *Sinorhizobium meliloti* and the PGPR bacterial strains; *Bacillus subtilis*, *Paenibacillus polymyxa*, *Pseudomonas fluorescens* and *Pseudomonas putida* to protect alfalfa plants from natural infection with seedling diseases. The experimental layout was split split plot design with three replications. The field plot size was 10.5 m^2 and the seeds were prose in the plot at the rate of 20 kg fed^{-1}. Alfalfa seeds of Ismailia-1 cv. were used. The effects on nodulation status, forage yield components and protein contents in leaf tissues were determined as well. Seeds were sown on the 10th and the 16th of October 2010 and 2011 seasons, respectively. The field experiments treatments were those previously applied in greenhouse experiments. Seed coating was made by mixing seeds with gamma irradiated vermiculite-Irish peat based inocula of *Sinorhizobium meliloti*, *Bacillus subtilis*, *Paenibacillus polymyxa*, *Pseudomonas fluorescens* or *Pseudomonas putida*, each inoculant was applied at the rate of 400 g 30 kg^{-1} seeds using 16% Arabic gum solution. In case of over head soil infestation, fresh suspensions of Ca. 3×10^8 cell mL^{-1} of either biocontrol agent was spread on soil surface

adjacent to seeds. The fungicide Rhizolex-T was added as 2.5 g kg^{-1} seeds. In control treatment, seeds were soaked in water. The treated seeds were broadcasted at the rate of 30 kg fed^{-1}. All plots received 30 kg P_2O_5 fed^{-1} in the form of superphosphate 15.5% P_2O_5 ammonium sulphate, prior to seed sowing. All recommended agricultural practices were applied. The first cut was obtained at 60 days after sowing and the subsequent cuts were carried out at an interval of 30 days. Six cuts were obtained through the growing season. At the end of each cut, percentages of infection as well as some forage yield i.e., fresh and forage dry yields and growth parameters, i.e., plant height (cm), tillers number in square meter were recorded. No. of nodules $plant^{-1}$, dry weighs of nodules were recorded after 70 days. At the end of the sixth cut, protein contents in leaf tissues were determined according to the method of Jackson (1973).

Statistical analysis: Analysis of variance was carried out using MSTAT-C program version 2.10 (1991). Least significant difference (LSD) was employed to test the significant differences between treatments at $p \leq 0.05$ (Gomez and Gomez, 1984).

RESULTS

Plant growth promoting rhizobacteria properties

Production of antifungal metabolites: The four tested strains of plant growth promoting rhizobacteria (*B. subtilis*, *P. polymyxa*, *Ps. fluorescens* and *Ps. putida*) produced HCN (Table 1). Siderophore production, as determined by the color change of chrome azurol S in agar medium, was variable among the strains, *Ps. fluorescens* produced the highest amount of siderophore followed by *B. subtilis* and *Ps. putida*, while *P. polymyxa* produced the lowest amount siderophore on CAS agar medium. The strains also showed β-1,3 glucanase activities, with highest amount in case of *Ps. fluorescens* strain. Production of protease, was detected with *B. subtilis*, *Ps. fluorescens* and *Ps. putida* strains, while β-1, 3 glucanase and protease were detected for all strains except *P. polymyxa*. Antifungal metabolites, siderophore,HCN, β-1, 3-glucanase and protease were not observed in case of *S. meliloti*.

Plant growth promoting attributes: All the tested plant growth promoting rhizobacteria and *S. meliloti* (Table 1) successfully solubilized inorganic phosphate on Pikovskya's agar medium, indicated by forming a clear halo around their spot inoculation. Phosphate solubilization after 24 h for the used strains was the maximum with *Ps. fluorescens* on day 7 of incubation, followed by *P. polymyxa, Ps. putida* and *S. meliloti* respectively while *B. subtilis* was the inferior (Table 1). Meanwhile, all the four tested strains and *S. meliloti* produced IAA as evidenced by development of pink color with and without the addition of tryptophan into the culture medium.

Plant growth promoting rhizobacteria co-inoculation effects: Result of pot experiment (Table 2) showed that nodulation of alfalfa by *Sinorhizobium meliloti* (nodule number and nodule biomass per plant) were significantly affected by the co-inoculated with the tested four PGPR strains. The average numbers of nodules and nodule biomass were significantly higher in the co-inoculated treatments than in the control (inoculated singly with rhizobia). Interestingly both of numbers of nodules and nodule biomass significantly affected by increase the days after inoculation 60 and 120 day. Co-inoculation by *Ps. Putida* and *B. subtilis* with *Rhizobium* exhibited the highest values of nodule number followed by *B. subtilis* and *Ps. fluorescens*. While Co-inoculation by *Ps. Putida* and *P. polymyxa* with *Rhizobium* exhibited the highest values of nodule biomass followed by *B. subtilis* and *Ps. fluorescens*. Plant treated with the fungicide Rhizolex-T in presence of *Rhizobium* recorded the lowest nodules number and nodule biomass.

Antibiosis of PGPR towards pathogenic fungi: The four tested strains of the PGPR were screened for their antagonistic activity against *F. oxysporum*, *F. solani* and *R. solani*. Data in Table 3 show that all the tested strains did strongly inhibit the mycelial growth of the three pathogenic fungi tested. The highest values of inhibition (88.89, 88.22 and 84.44%) were recorded with *F. oxysporum*, *F. solani* and *R. solani* respectively by *Ps. fluorescens* followed by *P. polymyxa*, *B. subtilis* and *Ps. putida*. *F. solani* was the most sensitive fungus, the highest values of inhibition (66.67, 73.33, 71.11 and 88.89 %) were recorded, followed by *R. solani* and *F. oxysporum* respectively.

Effect of PGPR and the fungicide Rhizolex-T on damping-off disease of alfalfa under greenhouse conditions: Data presented in Table 4 show that all treatments highly significantly reduced percentages of pre- and post- emergence damping-off and increased survival plants of alfalfa artificially infected with tested pathogens (*Fusarium oxysporum*, *Fusarium solani* and *Rhizoctonia solani*) compared to untreated treatment. The Fungicide Rhizolex-T was the superior treatment against all tested fungi followed by *P. fluorescens*

Table 1: Plant growth promoting (PGPR) and antifungal properties of *Sinorhizobium meliloti*, *Bacillus subtilis*, *Paenibacillus polymyxa*, *Pseudomonas fluorescens* and *Pseudomonas putida*

Bioagent	Siderophore	HCN**	β-1,3 glucanase	Protease	Phosphate solubilization	IAA*
Bacillus subtlis	++	+	+	+	+	+
Paenibacillus polymyxa	+	+	-	-	++	+
Pseudomonas fluorescens	+++	+	++	+	+++	+
Pseudomonas Putida	++	+	+	+	++	+
Sinorhizobium meliloti	-	-	-	-	++	+

-: Negative, +: Positive, +: Small halos>0.5 cm wide surrounding colonies, ++: Medium halos, +++: Large halos >1.0 cm wide surrounding colonies, *Indole-3-acetic acid, **Hydrogen cyanide

Table 2: Effect of co-inoculation with *Sinorhizobium meliloti* and PGPR strains on nodulation status of 60 and 120 day old alfalfa plants under greenhouse condition

	Nodule No. (10 plant^{-1})		Nodule biomass (mg 10 plant^{-1})	
Treatment	60 day	120 day	60 day	120 day
Control (*Sinorhizobium meliloti*)	87.0^{a}	112.0^{b}	79.33^{b}	116.33^{b}
Rhizolex-T+*S. meliloti*	66.0^{b}	99.0^{a}	43.67^{a}	74.33^{a}
Pseudomonas fluorescens+*S. meliloti*	104.3^{c}	130.7^{c}	122.33^{c}	166.67^{c}
Pseudomonas putida+*S. meliloti*	115.3^{d}	146.7^{e}	133.67^{d}	183.33^{d}
Paenibacillus polymyxa+*S. meliloti*	108.7^{cd}	136.3^{cd}	131.67^{d}	178.67^{d}
Bacillus subtilis+*S. meliloti*	109.7^{cd}	142.0^{de}	120.67^{c}	169.33^{c}

Different letter indicate significant differences among treatments within the same column according to least significant difference test ($p \leq 0.05$)

Table 3: *In-vitro* antagonism of PGPR against *Fusarium oxysporum*, *Fusarium solani* and *Rhizoctonia solani*

	Inhibition of linear growth (%)		
Bioagent	*Fusarium oxysporum*	*Fusarium solani*	*Rhizoctonia solani*
Bacillus subtilis	71.11^{c}	66.67^{d}	68.89^{c}
Paenibacillus polymyxa	77.78^{b}	73.33^{b}	77.78^{b}
Pseudomonas Putida	66.67^{d}	71.11^{c}	66.89^{c}
Pseudomonas fluorescens	82.22^{a}	88.89^{a}	84.44^{a}
Sinorhizobium meliloti	+	+	-

+: Positive effect, -: Negative effect, different letter indicate significant differences among treatments within the same column according to least significant difference test ($p \leq 0.05$)

P. polymyxa, *B. subtilis*, *P. putida* and *S. meliloti* in reducing damping-off incidences respectively. Moreover, all tested treatments showed similar effect for both pre- and post-emergence damping off.

Effect of PGPR on alfalfa damping-off under field conditions: The efficacy of plant growth promoting rhizobacteria as seed treatment against disease incidence of damping-off of alfalfa was evaluated under field conditions for both 2010-2011 and 2011-2012 growing seasons. Data in Table 5 clearly demonstrated that all treatments significantly reduced damping-off disease compared with the control. Alfalfa seeds treatmed by Rizolex-T was more effective than PGPR. Also, the obtained data show that alfalfa seed treated by any of the tested PGPR were effective to reduce Pre-and/or Post-damping-off compared to untreated treatment. *Ps. Fluorescens* recorded the highest redaction of Pre- and/or Post-damping-off in both seasons, it's recorded 94.33 and 95.33% survival plants compared with 78.77 and 80.77% in control plants in both seasons, respectively. In the contrary, alfalfa seeds treated by *Ps. Putida* and *S. meliloti* were recorded the lowest values in both seasons (89.03 and 87.50% survival plants in first seasons and 90.40 and 89.67% in second season, respectively).

Effect of PGPR on alfalfa root nodulation under field conditions: As shown in Table 6 alfalfa seeds inoculated with the tested rhizobacteria showed significant increases in number and dry weight of nodules as compared to uninoculated ones this was confirming in both growing seasons. Treatment with *Pseudomonas fluorescens* recorded the highest averages nodulation, 21.35 for No. of nodules and 8.93 for dry weight of nodules in both seasons (2010-2011) and (2011-2012) followed by *Paenibacillus polymyxa*, *Bacillus subtilis* and *Pseudomonas putida*, respectively compared with both check treatments inoculated or uninoculated with rhizobia. Seed treated with Rhizolex reduced both number and dry weight of nodules compared to other all treatments. Results of the first season were more obvious than those of the second one.

Table 4: Effect of PGPR and the fungicide Rhizolex-t on percentages of damping-off caused by *Fusarium oxysporum*, *Fusarium solani* and *Rhizoctonia solani* of alfalfa under greenhouse conditions

	Fusarium oxysporum			*Fusarium solani*			*Rhizoctonia solani*		
Treatment	*Pre-emergence (%)	Post-emergence (%)	Survival plants (%)	Pre-emergence (%)	Post-emergence (%)	Survival plants (%)	Pre-emergence (%)	Post-emergence (%)	Survival plants (%)
Bacillus subtlis	8.3^{bc}	6.7^{b}	85.0^{d}	4.3^{cb}	4.3^{c}	91.3^{c}	8.0^{b}	5.0^{c}	87.0^{d}
Paenibacillus polymyxa	6.7^{b}	5.0^{ab}	88.3^{d}	3.0^{bc}	2.7^{abc}	94.3^{d}	4.3^{b}	4.3^{b}	91.3^{e}
Pseudomonas fluorescens	7.3^{b}	5.3^{ab}	87.3^{d}	2.7^{b}	1.7^{ab}	95.7^{d}	6.3^{a}	1.0^{bc}	92.7^{e}
Pseudomonas putida	10.3^{c}	10.0^{c}	79.7^{c}	5.7^{d}	3.3^{bc}	91.0^{c}	14.3^{c}	10.0^{d}	75.7^{c}
Rhizolex-T	3.7^{a}	3.3^{a}	93.0^{e}	0.7^{a}	1.3^{a}	98.0^{e}	0.7^{a}	1.3^{a}	98.0^{f}
Sinorhizobium meliloti	21.3^{d}	39.7^{d}	39.0^{b}	7.7^{e}	10.7^{d}	81.7^{b}	28.7^{d}	15.7^{e}	55.7^{b}
Control	37.3^{e}	53.3^{e}	9.3^{a}	15.3^{f}	20.7^{e}	64.0^{a}	49.7^{e}	36.7^{f}	13.7^{a}

*: Incidence of pre- or post-emergence damping-off and survival plants (%), different letter indicate significant differences among treatments within the same column according to least significant difference test ($p \leq 0.05$)

Effect of seeds treatment by PGPR on yield component and protein content under field conditions: Effect of alfalfa seed treated by PGPR agents and Rizolex-T on growth parameters, yield components and Protein contents under field conditions during seasons 2010-2011 and 2011-2012 were studied. Data in Table 7 indicate that plant growth parameters; plant height, tillers, fresh and dry weight of alfalfa significantly increased as a result of PGPR strains treatments. Also, *Pseudomonas fluorescens* was the more effective in this respect than the other PGPR strains or the fungicide Rizolex-T. *Pseudomonas fluorescens* was the most effective treatment; that recorded the highest plant height (70.33 and 71.17 cm) and number tillers (423.67 and 417.00 Tillers m^{-2}) in both seasons, respectively. Also, this treatment recorded the highest yield components, i.e., fresh weight (9.17 and 8.93 ton fed^{-1}) and dry weight (2.17 and 2.27 ton fed^{-1}) in both seasons, respectively. On the contrary, alfalfa seed treatment by *Pseudomonas putida* was the less effective in both seasons. On the other hand, the obtained data show that all treatments significantly increased protein content in the forage compared with untreated control. *Pseudomonas fluorescens* was recorded the highest protein content (23.7 and 23.73%) followed by Rizolex-T (22.83 and 23.0%) in both seasons, respectively. While, *Pseudomonas putida* recorded the lowest protein content (20.63 and 22.10%) in first and second season, respectively.

DISCUSSION AND CONCLUSION

Plant Growth-Promoting Rhizobacteria (PGPR) has vital role in agriculture. This positive effects of PGPR have direct or indirect performance on plants, direct promotion of growth by production of metabolites that enhances plant growth, indirect growth promotion occurs via the removal of pathogens by the production of secondary metabolites. The application of PGPR as crop inoculants for biofertilization and biocontrol would be an attractive alternative approaches to decrease and substitute the excessive use of chemical fertilizers which effect environmental pollution (Vargas *et al*., 2000; Spadaro and Gullino, 2005).

It is well known that PGPR the colonize plant roots when added to seeds, roots or tubers in a wide range of crop specie play an important role in maintaining crop and soil health through versatile mechanisms, understanding that wide biological mechanisms of the beneficial PGPRs: Nutrient cycling and uptake, direct stimulation of plant growth, induction of resistance in plant host, suppression of plant pathogens, are long-standing goal in agricultural sustainability for enhance plant growth and improved crop production (Kloepper *et al*., 1980, 2004; Haas and Defago, 2005; Yadav *et al*., 2010).

On the basis of physiological and biochemical characteristics, *S. meliloti* and the four plant growth promoting bacterial strains were found as effective strains when screened for plant growth promoting properties due to their phosphate solubilization and IAA production and the ability of production of antifungal metabolites as Production of Siderophore, Protease, β-1,3-glucanase and Hydrogen Cyanide (HCN). As well as all the four PGPR isolates inhibited the pathogen in the dual culture assay, whereas *Ps. fluorescens* isolates showed the maximum percent inhibition of radial growth (88.22, 88.89 and 84.44%, respectively) these similar result with the previously obtained by (Kumar *et al*., 2010; Srividya *et al*., 2012) as well as the previous observations of Yanes *et al*. (2012) rhizospheric fluorescent Pseudomonas isolates from alfalfa plants strong in vitro antagonistic activity toward *Pythium debaryanum* and confirm the ability for the tested PGPR isolates to produce fungistatic metabolites secreted.

Table 5: Effect of alfalfa seed treatment with PGPR on alfalfa pre-and post-emergence damping-off severity under field conditions during (2010-2011) and (2011-2012) seasons

	Season 2010-2011			Season 2011-2012		
Treatment	Pre-emergence	Post-emergence	Survival plants	Pre-emergence	Post-emergence	Survival plants
Bacillus subtilis	*5.03^{bc}	1.77^{ab}	93.20^{c}	4.20^{bc}	3.53^{bc}	92.27^{cd}
Paenibacillus polymyxa	4.17^{ab}	2.83^{bc}	93.00^{c}	3.07^{ab}	3.10^{abc}	93.83^{de}
Pseudomonas fluorescens	3.97^{ab}	1.70^{ab}	94.33^{c}	2.47^{a}	2.20^{ab}	95.33^{ef}
Pseudomonas putida	6.97^{cd}	4.00^{cd}	89.03^{b}	5.10^{cd}	4.50^{c}	90.40^{bc}
Rizolex-T	2.17^{a}	0.77^{a}	97.07^{d}	1.77^{a}	1.83^{a}	96.40^{f}
Sinorhizobium meliloti	7.57^{d}	4.93^{d}	87.50^{b}	6.33^{d}	4.00^{c}	89.67^{b}
Non treated (Control)	14.77^{e}	6.47^{e}	78.77^{a}	11.47^{e}	7.77^{d}	80.77^{a}

*Values are presented as percentage, different letter indicate significant differences among treatments within the same column according to least significant difference test ($p \leq 0.05$)

Table 6: Effect of alfalfa seeds treatment by PGPR on No. of nodules plant^{-1} and dry weight of nodules plant^{-1} (mg) under field conditions during (2010-2011) and (2011-2012) seasons

	No. of nodules plant^{-1}			D.W. of nodules plant^{-1} (mg)		
Treatment	2010-2011	2011-2012	Mean	2010-2011	2011-2012	Mean
Bacillus subtilis	21.43^{d}	20.67^{d}	21.05	9.03^{d}	8.50^{d}	8.77
Paenibacillus polymyxa	20.83^{d}	21.53^{de}	21.18	8.37^{cd}	8.87^{d}	8.62
Pseudomonas fluorescens	20.60^{d}	22.10^{e}	21.35	8.67^{cd}	9.20^{d}	8.93
Pseudomonas putida	19.47^{d}	21.27^{de}	20.37	7.47^{c}	8.40^{d}	7.93
Rhizolex-T	8.53^{a}	8.97^{a}	8.75	2.73^{a}	3.07^{a}	2.90
Sinorhizobium meliloti	15.27^{c}	15.63^{c}	15.45	5.23^{b}	5.93^{c}	5.58
Non treated (Control)	10.63^{b}	11.17^{b}	10.90	3.53^{a}	4.13^{b}	3.83

Different letter indicate significant differences among treatments within the same column according to least significant difference test ($p \leq 0.05$)

Table 7: Effect of alfalfa seeds treatment by PGPR on alfalfa plants plant height, tillers m^{-2} fresh, dry weight and protein content under field conditions during (2010-2011 and 2011-2012) growing seasons

	Plant height (cm)		Tillers m^{-2}		Fresh weight (Ton fed^{-1} cut^{-1})		Dry weight (Ton fed^{-1} cut^{-1})		Protein (%)	
Treatment	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12
Bacillus subtilis	66.17^{d}	63.33^{c}	415.33^{cd}	396.33^{bc}	8.64^{d}	8.23^{cd}	2.03^{cd}	2.18^{de}	22.50^{e}	22.40^{cd}
Paenibacillus polymyxa	62.33^{c}	58.33^{b}	407.33^{bc}	400.00^{c}	8.33^{c}	8.07^{c}	2.00^{c}	2.03^{cd}	21.77^{d}	22.23^{c}
Pseudomonas fluorescens	70.33^{e}	71.17^{e}	423.67^{d}	417.00^{d}	9.17^{e}	8.93^{e}	2.17^{e}	2.27^{e}	23.07^{e}	23.73^{e}
Pseudomonas putida	60.83^{bc}	59.33^{b}	405.33^{bc}	395.33^{bc}	8.10^{bc}	7.93^{c}	1.80^{b}	1.95^{c}	20.63^{c}	22.10^{c}
Rizolex-T	71.00^{e}	68.67^{d}	414.67^{cd}	411.67^{d}	8.82^{d}	8.47^{d}	2.13^{de}	2.23^{e}	22.83^{e}	23.00^{d}
Sinorhizobium meliloti	59.67^{b}	57.17^{b}	400.67^{ab}	386.00^{b}	7.95^{b}	7.57^{b}	1.70^{b}	1.65^{b}	19.10^{b}	19.90^{b}
Non treated (Control)	52.67^{a}	51.33^{a}	389.00^{a}	368.67^{a}	7.27^{a}	7.17^{a}	1.19^{a}	1.25^{a}	17.27^{a}	18.03^{a}

Different letter indicate significant differences among treatments within the same column according to least significant difference test ($p \leq 0.05$)

Results obtained in this investigation indicated that application of *Sinorhizobium meliloti* and four PGPR strains i.e., *Bacillus subtilis*, *Paenibacillus polymyxa*, *Pseudomonas fluorescens* and *Pseudomonas putida* as seed treatment significantly reduced alfalfa seedling damping-off diseases and varied in their effectiveness under greenhouse and field conditions. These results are in agreement with those obtained by Kloepper *et al.* (2004), Haas and Defago (2005), Quagliotto *et al.* (2009) and Yanes *et al.* (2012) who asserted the efficiency of PGPR on reducing root-rot and/or wilt diseases.

The obtained results are in agreement with those reported by Hammerschmidt (1999) when a pathogen has been successfully recognized and inducible defence mechanisms. A more rapid and stronger activation of basal defence mechanisms occurs upon pathogen attack in such induced plants, as, the resistance-inducing agent predisposes the plant to react more effectively against a wide spectrum of pathogens (Verhagen *et al*., 2004). The direct mechanisms involve nitrogen fixation, phosphorus solubilization, HCN production and production of phytohormones such as auxins, cytokinins and gibberellins and lowering of ethylene concentration (Glick, 1995; Glick *et al*., 1999) and confirm the previous observations of Yanes *et al.* (2012) native Pseudomonas isolates showed significant effects on alfalfa by increasing plant biomass and/or protection against pathogen infection.

Moreover, our results are in agreement with earlier reports on the production of different antifungal metabolites (hydrolytic enzymes) including siderophore, hydrogen cyanide, organic acids, IAA, solubilized insoluble phosphate, besides producing chitinase, protease and β-1,3-glucanase by the PGPRs *Bacillus* sp. and *Pseudomonas* sp. suggests the plant growth promotion and broad spectrum biocontrol potential of this

isolate and confirm the ability of indirect mechanism of PGPR to suppress plant diseases like wilt disease (*Fusarium oxysporum*) and root rot (*Rhizoctonia solani*) by *Bacillus* sp. and *Pseudomonas* spp. (Idris *et al*., 2008; Kumar *et al*., 2010; Singh *et al*., 2010; Srividya *et al*., 2012).

The observed promotion in root nodulation of plant in this study could be attributed to the cumulative effects of these rhizobacteria. Similar results were obtained by Hassouna *et al*. (1994), Quagliotto *et al*. (2009) and Wani *et al*. (2007). They showed that multiple inoculation with *rhizobium* and phosphate-solubilizing rhizobacteria increased the nodule number and biomass. In general, the lowest rate of N concentration in shoots was shown in control plants that were in a class alone. In other words the application of all bacterial inoculants studied in this experiment resulted in significant promotion of N concentration in shoots as compared with uninoculated control. Results obtained here confirm the observations of Hassouna *et al*. (1994), Wani *et al*. (2007) and Yanes *et al*. (2012).

In the present investigations, the application of PGPR and Rizolex-T to alfalfa seed treated. Table 7 revealed significantly increased plant growth parameters, plant height and tillers meanwhile yield components fresh and dry weight of alfalfa and protein contents under field conditions compared with untreated control during the two successive seasons 2010-2011 and 2011-2012. *Pseudomonas fluorescens* was the more effective in this respect than the other PGPR strains or the fungicide Rizolex-T. It's attributing for positive root colonization ability of *Pseudomonas* spp. and *Bacillus* spp. and proved the successful colonization in the rhizosphere and in alfalfa roots, meanwhile, increase the growth and yield promotion. As well as reported by Hassouna *et al*. (1994) the increased yields of alfalfa inoculated with N_2-fixing bacteria in northwestern Egypt, bacterial inoculants with *Rhizobium meliloti*, improved alfalfa (*Medicago sativa* L.) cultivar growth as measured by seven cuts per year over a 2 year experiment period, whereas increased fresh and dry weights and protein content of plants over the control. Thus, the finding supports that a composite application of symbiotic N_2-fixing organisms and plant growth promoting rhizobacteria could improved plant growth and nutrient uptake, leading to significant increases in the grain yield and protein content of field-grown chickpea (Wani *et al*., 2007). Also, Yadav *et al*. (2010) showed that isolates of PGPR induced production of plant hormones (indole acetic acid), phosphate solubilization and ammonia production to enhanced plant growth. Most of isolates resulted in a significant increases in shoot length, root length and dry matter production of shoots and roots of chickpea seedlings.

The results of the present investigation suggest that the use of PGPR as inoculants biofertilizers is an efficient approach to replace chemical fertilizers and pesticides for sustainable alfalfa cultivation in Egypt, as they exhibited synergism in promoting crop growth and yield of alfalfa besides controlling damping-off diseases. Further investigations, including efficiency test under green house and field conditions are needed to clarify the role of PGPR as biofertilizers that exert beneficial effects on plant growth and development.

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