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Impact of Co-Inoculation with *Rhizobium leguminosarum* and some Plant Growth Promoting Rihzobacteria against *Rhizoctonia solani* and *Fusarium oxysporum* Infected Faba Bean

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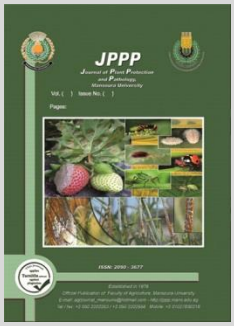

ABSTRACT

Plant Growth Promoting Rhizobacteria (PGPR) were evaluated individually or in combination and compared to the fungicide Topsin M-70® for their antagonistic activities and suppressive actions against *Fusarium oxysporum* and *Rhizoctonia solani* severally, which were deemed the causal agents of root rot and wilt diseases of faba bean plants. *In vitro*, most of the examined strains showed eminent abilities to inhibit mycelial growth of *Rhizoctonia solani* or *Fusarium oxysporum*. Strains of *Rhizobium leguminosarum* (No.9), *Azotobacter chroococcum* (No.12) and *Pseudomonas fluorescens* (No. 6) recorded the highest inhibition zone against two tested pathogenic fungi and were used in the present study. Generally, under greenhouse and field conditions, all the tested PGPR strains significantly reduced root-rot and wilt incidence and increased the percentage of survived plants compared to control (infested soil). Combination among *R. leguminosarum* + *A. chroococcum* + *P. fluorescens* was more effective in controlling root-rot and wilt diseases, increasing growth parameters, yield components, photosynthetic pigments, activities of nitrogenase, dehydrogenase and total phenols. Obtained results clarified the importance of PGPR in controlling pathogenic fungi, enhancing growth parameters of faba been plants compared to chemical fungicide and lowering chemical fertilizers application.

Keywords: PGPR, rhizobium, *Rhizoctonia solani*, *Fusarium oxysporum*, root rot, wilt, faba bean.

INTRODUCTION

Faba bean (*Vicia faba* L.) belongs to *Fabiacea* is one of the oldest domesticated food legumes (Singh *et al*., 2012). It is a major legume species because of its high nutritional value, energy, and protein content (24–30%) which is grown for human food and animal feed (Pszczółkowska *et al*., 2020). It is also one of the most important legume crops in Egypt, providing consumers with a cheap and high quality of protein (Mohamed, 2003). It is susceptibility to number of soil pathogenic fungi such as *Rhizoctonia solani*, *Fusarium oxysporum* and other *Fusarium* species that may cause serious root rot and wilt diseases which causing reduction in crop productivity and reduce seed quality (Mazen *et al*., 2008; Elwakil *et al*., 2009a). Traditionally, control of these diseases depended on chemical control, crop rotations, resistant varieties and improvement of soil quality. Modern agriculture faced challenges such as loss of soil fertility, climate fluctuation and increasing pathogen resistance. Sustainability and environmental safety of agricultural production relied on eco-friendly approaches like biofertilizers, biopesticides, recycling of crop residues and beneficial effects of microbial inoculants particularly plant growth promoters (PGP) (Son *et al*., 2014).

Biological control is an environment-friendly strategy to reduce crop damage caused by plant pathogens. There were four advantages of biological control: 1) it appeared to be ecologically safer than chemicals because control agents were not accumulated in the food chain; 2) some biological control agents could provide persistent control; 3) biocontrol agents had slight effects on ecological balance; 4) they were compatible with other control agents (Aeron *et al*., 2011). Biocontrol of plant pathogens drew a great attention of many investigators. Most researchers had been concentrated on microorganisms that played an enormously important role in plant disease control. Biocontrol agents brought about higher disease reduction when used as a component of integrated pest management (Van Lenteren *et al*., 2018). The use of microbes for crop protection and production and soil health had been practiced for centuries.

The beneficial microbes to soil and plant such as *Azotobacter*, *Azospirillum*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, *Pseudomonas*, *Rhizobium* and *Serratia* had been found to enhance soil quality and plant growth and were termed as plant growth-promoting rhizobacteria (PGPR) (Katiyar *et al*., 2016). Plant growth-promoting rhizobacteria (PGPR) were naturally occurring soil bacteria that aggressively colonize roots and benefit plants health. Application of PGPR in crop production can reduce the agro-chemical utilization and supported eco-friendly sustainable food production. Plant growth promotion by PGPR was due to increase in seedling emergence, root hair proliferation, increased in number of branches, early nodulation and nodule functioning, enhancing both leaf surface area and biomass, phytohormone production, nutrient, water and air uptake, promoted accumulation of carbohydrates and yield in many plant species (Podile and Kishore, 2006).

Likely, PGPR involved in various beneficial activities within the soil such as decomposition of crop residues, mineralization of soil organic matter, immobilization of mineral nutrients, phosphate solubilizers, synthesis of soil organic matter, nitrification, nitrogen fixation, and plant growth promoters including nutrient acquisition (biofertilizers), phytohormone production (biostimulants), and suppression of plant disease (bioprotectants) which helped in crop production and protection.

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Soil moisture content affected the colonization of the plant roots by the PGPR after inoculation (Shrivastava *et al*., 2014).

Biological nitrogen fixation, especially rhizobia-legumes symbiosis, is one of the alternative solutions and promising technology which played an important role in reducing chemical N-fertilizers, increasing soil fertility, decreasing the production cost, and eliminating the undesirable pollution impact of chemical fertilizers in the environment (Peoples *et al*., 1995). It had been reported that inoculation by indigenous *R. leguminosarum* significantly increased growth parameters and seed yield of faba bean (Khosravi and Ramezanpour 2004).

Antagonistic bacteria such as *Azotobacter* and *Rhizobium* which were present in the soil could restrain the growth of different bean pathogens. They were important for plant growth-promoting rhizobacteria that encourage plants in multiple ways including indole acetic acid, HCN, solubilization of inorganic phosphate, fixation of atmospheric nitrogen and can also compete with pathogen for iron through production of siderophores (Maheshwari *et al*., 2012and Das *et al*., 2017).

Moreover, inoculation with nitrogen-fixing *Rhizobium/ Bradyrhizobium* in combination with *Azospirillum* or *Azotobacter* led up to alterations in total content of K, P, Ca, Mg, Fe, B, Mn, Zn and Cu. It also resulted in considerable stimulatory leverages on nodulation and growth of faba bean plants (Rodelas *et al*., 1999).

Shaban and El-Bramawy (2011) found that the treatment with *R. leguminosarum*in combination with *Trichoderma harzianum*, significantly increased plant height, number of branches/ plant, number of pods/ plant, 100-seed weight and seed yield in faba bean in comparison with single inoculation. Simultaneous inoculation of faba bean (*Vicia faba* L.) with *R. leguminosarum*in and *Pseudomonas fluorescens* gave positive effects on faba bean growth and yield (El Sayed *et al.*, 2015).

Pseudomonas fluorescens was adapted to survive in soil and colonization plant roots. The microbial inoculants utilized in agriculture included biofertilizers, biocontrol agents and plant growth promoting rhizobacteria (Kiely *et al*., 2006).

Fluorescent *Pseudomonas* spp. were among the most effective rhizosphere bacteria in reducing soil-borne diseases in infected suppressive soils (Weller, 1988), where disease incidence was low, despite the presence of pathogens and environmental conditions conductive to disease prevalence. Strains of *Pseudomonas fluorescens* had elucidated the efficiency to encourage seed germination, shoot and root development of different crops including faba bean. Some strains seemed to have the ability to devastate the fungal cell wall by secreting lytic enzymes and restrain the growth of fungal pathogens by secreting hydrogen cyanide and antibiotics such as phycocyanin and phenazine. Several modes of action for antagonistic PGPR, *Pseudomonas* spp. had been reported, including production of different antimicrobial compounds and induction of plant defense mechanisms (Sindhu *et al*., 2016).

Interactions among rhizobia, PGPR and plants were affected by abiotic factors such as soil, temperature and moisture, chemical properties of the soil and soil type and biotic factors which included interactions among plant pathogens, antagonists and total microbial communities sharing the same niche (Zhang *et al*., 2010).

The aim of this work was to study the effect of combined application of *R. leguminosarum* and antagonistic PGPR towards faba bean pathogens. Furthermore, investigating the appropriateness of *Rhizobium* with PGPR and comparing their colonization competence in faba bean. The investigations were guided by the hypothesis that the co-inoculation of *Rhizobium* with compatible PGPR will reduce the impact of pathogenic fungi in faba bean cultivations and reduce the levels of soil-borne inoculum.

MATERIALS AND METHODS

Laboratory tests:

Isolation of bacteria used:

The bacteria used in this study were isolated from rhizosphere region of faba bean plants and collected from Ismailia Agricultural Research Station.

Ten grams of faba bean rhizosphere soil were suspended in 90 ml sterilized water in conical flask (250 ml), thoroughly shacked for 10 min and dilution series up to 10^{-7} were prepared. Dilutions from soil sample were plated in King's medium (King *et al*., 1954) for *Pseudomonas*, while *Azotobacter* plated in modified Ashby' medium (Abd El Malak and Ishac, 1968). Plates were incubated at 28± 2C° for 2-3 days for *Pseudomonas* plates, while *Azotobacter* plates were incubated at 28± 2C° for 5-7 days then individual colonies were picked up, purified and microscopically tested for morphological characteristics. Isolation of *Rhizobium* from the root nodules of faba bean plants were carried out. The nodules of faba bean plants were washed and surface sterilized for 30 seconds using H_2O_2 30% and in a sterile Petri-dishes rinsed completely with sterile water for 6 times, then they were washed, and finally crushed with a sterile glass rod. A loop of the nodule slurry was aseptically transferred to a Petri-dish containing yeast extract mannitol agar medium Y.E.M according to Vincent (1970), then incubated for 2-5 days at 28ºC. Pure isolates were examined morphologically by direct light microscope.

Selection of active isolates:

A. Nitrogenase enzyme activity:

Active isolates of both asymbiotic *Azotobacter* spp. and symbiotic *Rhizobium* spp. were evaluated through measuring of nitrogenase activity for each isolate. The enzyme activity was estimated using the acetylene reduction technique according to Somasegaran and Hoben (1985).

B. Antagonistic bioassay of bacteria against pathogenic fungi:

Fifteen pure isolates from *Pseudomonas* spp., *Azotobacter* spp., and *Rhizobium* spp. were tested for antagonism against the pathogenic fungi *Rhizoctonia solani* and *Fusarium oxysporum*. A disc (1 cm in diameter) of each fungal growth (7-day old) was inoculated on fifteen ml of PDA medium supplemented with 0.05% peptone and mixed thoroughly and poured in sterilized Petri dishes. Tested bacterial isolates were placed on the fungal inoculated plates (4 isolates / plate) equidistantly from each other in spots about 1 mm in diameter (three replicates were used). The plates were incubated at 28±2°C for 5 days, the inhibition zone diameters for the antagonistic isolates were measured as mm (Brock, 1973).

Identification of the selected active isolates:

Morphological characteristics of all isolates such as cell shape, color and consistency of isolates were recorded as described in Bergey's Manual of Systemic Bacteriology (Krieg *et al.*, 1994). The active isolates of *Pseudomonas* were selected and identified by Biolog Automated Microbe Identification Method at Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt, while *Azotobacter* was identified through Biolog GN2 Microplate Biolog 2000 in the VACSERA, Cairo, Egypt. On the other side, *Rhizobium* is the generic name for those bacteria forming nitrogen-fixing nodules on leguminosae. The taxonomy of the genus *Rhizobium* was under review currently, it is

subdivided according to the ability of its members to nodulate particular host plants (Fahraeus, 1957). This system of specification was inadequate and exceptions to the rule that were many. Rhizobia which nodulated faba bean (*Vicia faba* L.) belong to *R. leguminosarum* in common with other Rhizobia, is a Gram negative, motile rod (0.5-0.9 µm X 1.2 -3.0 µm).

Fungal Pathogens and preparation of inocula:

Rhizoctonia solani and *Fusarium oxysporum* fungi were isolated from naturally infected faba bean plants, showing damping off and wilt symptoms. Their pathogenicity was previously confirmed and identified on the basis of cultural properties as well as microscopic and morphological characters according to Sneh *et al*. (1991).

Rhizoctonia solani and *Fusarium oxysporum* inocula were prepared by growing the fungi in 500 ml glass bottles containing 100 gram sterilized sorghum grains medium. The bottles were inoculated with actively growing (0.5 cm) four days old for R. *solani* or seven days old for *F. oxysporum* cultures. Bottles were incubated at 25 ± 1°C for 18 days; vigorously shaken daily for the first 4 days to encourage more rapid and uniform colonization of the sorghum grains (Leslie and Summerell, 2006).

Greenhouse experiment:

Seed and soil treatments:

Faba bean (*vicea faba* L.) c.v. Sakha-1 used in this study was obtained from Legume Crop Research Department, Field Crop Res. Inst., Agric. Res. Center, Egypt.

Seeds of faba bean were surface disinfected by immersion in sodium hypochlorite 1 % for 2 min, and washed many times with sterilized water, then left to dry on screen cloth with paper towel underneath to absorb the excess water at room temperature for approximately two hours.

Seed soaking was carried out to the disinfected faba bean seeds by applying the fungicide Topsin-M 70® 70 % WP (Thiophanate- methyl), at the recommended dose.

The experiment was carried out in the greenhouse of Ismailia Agricultural Research Station, ARC. Pots of 30 cm in diameter with a bottom drainage hole were sterilized by dipping in 5 % formalin solution for 15 minutes, and left for one week until complete formalin evaporation. Pots were filled with un-sterilized sandy soil. Soil infestation was achieved by mixing the inoculum of *R. solani* or *F. oxysporum* with the soil at the rate of 2 % of soil weight (Papavizas and Davey, 1962). Sterilized un-inoculated grounded sorghum grains were added to the disinfested soil at the same rate as a healthy control. The un-inoculated pots received ammonium nitrate (33.5 % N) at a rate (150 Kg fed^{-1}) as a recommended dose, while inoculated pots with *Rhizobium* received (60 Kg fed^{-1}) as a starter N-dose. Superphosphate (15 % P_2O_5) received at a rate of 200 Kg fed^{-1}, while potassium was added in the form potassium sulfate (48 % K_2O) at rates of 50 Kg fed^{-1}.

The infested soil was mixed thoroughly and watered every 2 days for a week before planting to stimulate the fungal growth and ensured its distribution in the soil. Five seeds of treated faba bean seeds were sown in each pot and pots were irrigated. Four pots were used for each treatment. All pots were irrigated when necessary and kept in a greenhouse. *Rhizobium leguminosarum* (No. 9) was used as a specific nitrogen fixer for faba bean plants. Cell suspension inoculum was prepared on yeast extract mannitol medium (YEM) to reach to 10^8 cells/ml. *A. chroococcum* (No.12) that obtained highly nitrogenase activity and with high antagonistic effect against pathogenic fungi was prepared and cultured on modified Ashby's medium (Abd El Malak and Ishac, 1968) to reach to 10^8 cfu /ml, while *P. fluorescens* that obtained highly antagonistic effect against pathogenic fungi was grown according to King's medium (King *et al*., 1954) and each 1 ml contain 10^7 - 10^8 cfu. Seeds were coated with liquid bacteria either single or mixer strains before planting and mixed with sterilized vermiculite (20% moisture) then adhesion using Arabic gum (20%) as sticker and then planted. After a month of planting another bacterial suspension was added to each treatment as equivalent to the field rate.

The treatments were as follows: (1) *R. leguminosarum* (*R.l.*) ;(2) *R. leguminosarum* + *A. chroococcum* (*A.ch.*); (3) *R. leguminosarum* + *P. fluorescens* (*P. f.*); (4) *R. leguminosarum* + *A. chroococcum* + *P. fluorescents*; (5) Fungicide Topsin M-70®; (6) Infested control and (7) Healthy control.

Plants sampling and determination:

Three replicates for each treatment were taken after 75 days to determine some root and shoot parameters as vegetative parameters. Chlorophyll a, chlorophyll b and carotenoids were determined according to Saric *et al*. (1976). Soil biological activity such as dehydrogenase and nitrogenase activities were determined according to Skujins (1976) and Dilowarth (1970), respectively. Total phenol contents in plant leaves were estimated with the procedure given by Zilesin and Ben-Zaken (1993). Pots were arranged in the in a greenhouse completely randomized design with four replicates.

Disease assessment:

Disease incidence (DI%) was determined by recording pre-emergence damping-off, post-emergence damping off, root-rotted plants, wilted plants and the percentage of survived plants after 15, 30, and 90 days of sowing respectively, according to the following formula:

$$\textbf{Pre-emergence (\%)} = \frac{\text{Total No. of un-germinated seeds} \times 100}{\text{Total No. of planted seeds}}$$

$$\textbf{Post-emergence (\%)} = \frac{\text{Total No. of rotted seedlings} \times 100}{\text{Total No. of planted seeds}}$$

$$\textbf{Survived seedlings} = \text{Total No. of planted seeds - (pre +post emergence + root rotted and/ or wilted plants)}$$

Reduction or increasing (%) over the infected control was also calculated according to the following formula :

$$\textbf{Reduction or increasing (\%)} = \frac{\text{(DI of Control - DI of treatment)} \times 100}{\text{DI of Control}}$$

Percentages of early and late wilt were recorded after 30 and 90 days of sowing respectively, while the number of the survived plants was recorded at 120 days after sowing. Survived plants were examined by cutting longitudinally stem and root; survived plants are considered healthy when no visual evidence of the disease is observed.

Field experiments:

The field experiments were carried out during the two winter growing seasons (2016/2017) and (2017/2018) at Ismailia Agricultural Research Station, Egypt, in field known to have root rot history. The disinfected faba bean seeds were treated by the same manner as in the greenhouse experiment. In the control treatment, seeds were soaked in distilled water. The field trial (18 plots) was designed in complete randomized block with three replicates 2×3 m2. The field plot with four rows, each row contained 10 hills on the eastern side. 100 seeds of c.v. Sakha-1 were sown in each plot. All the recommended agricultural practices were applied. The treatments were as follows: 1) *R.leguminosarum* (*R.l.*); (2) *R.leguminosarum*+ *A.chroococcum* (*A.ch.*); (3) *R. leguminosarum* + *P.fluorescens* **(***P. f.*); (4) *R. leguminosarum*+ *A.chroococcum* + *P. fluorescens* ; (5) Fungicide Topsin M-70® ; (6) Control without any addition. Disease incidence (DI %) and

percentage of wilt were determined as mentioned before. Also, chemical fertilizers and biofertilizers were performed as described before with faba bean pot experiment.

Three replicates for each treatment were up rooted after 75 days of planting to determine some root and shoot parameters as vegetative parameters. Chlorophyll a, chlorophyll b and carotenoids were determined according to Saric *et al.* (1976), while soil biological activity such as dehydrogenase and nitrogenase activities were determined according to Skujins (1976) and Dilowarth (1970), respectively. At harvest, faba bean plants were collected from each plot to determine plant height (cm), number of branches, number of pods per plant, weight of one hundred seed and seed weight per plant were recorded as well as seed yield (ton/Fadden) were calculated.

Statistical analysis:

Completely randomized design (CRD) and randomized block design (RBD) were conducted in greenhouse and field experiments, respectively. The obtained data were subjected to computer statistical software (COSTAT) originated by Silva and de Azevedo (2009). Data analyzed using analysis of variance (ANOVA), and mean values were compared using Duncan's multiple range test at a significance level of $P \leq 0.05$.

RESULTS AND DISCUSSION

Results:

Laboratory tests:

Isolation, identification and selection of active isolates of nitrogen fixing bacteria from the rhizosphere and roots of faba bean plants:

Fifteen isolates of each N_2-fixer were isolated on selective media from the rhizosphere region and from the root nodules of faba bean plants. All isolates were identified to genus level as *Azotobacter* spp. and *Rhizobium* spp. Ability of the isolates to fix atmospheric nitrogen varied from one to another according to their activity of nitrogenase. Nitrogenase activity of *Azotobacter* and *Rhizobium* spp. isolates is found in Table 1. In the case of *Azotobacter* spp., the acetylene reducing activity recorded the highest value with isolates No. 12 and No. 8, which gave 46.0 and 44.5 µmol C_2H_4 $ml^{-1,}$ respectively, while in the case of *Rhizobium* spp. isolates No.9 and No.7 which gave 24.5and 20.6 µmol C_2H_4 ml^{-1}.

Table 1. Acetylene reducing activity (µmol C_2H_4 ml^{-1}) of some isolated nitrogen fixing bacteria.

Isolate number	Nitrogenase activity µmol C_2H_4 ml^{-1}	
	Azotobacter	*Rhizobium*
1	8.0	12.0
2	25.0	3.2
3	29.0	9.3
4	2.0	14.3
5	19.0	7.3
6	35.0	13.2
7	11.0	20.6
8	44.5	2.4
9	10.2	24.5
10	13.3	1.0
11	40.0	1.2
12	46.0	17.2
13	9.5	3.8
14	31.0	9.2
15	21.2	15.2

Antagonistic activity of *Azotobacter*, *Rhizobium* and *Pseudomonas* isolates against the tested pathogenic fungi:

All bacterial isolates were tested to determine their inhibition potency against the growth of pathogenic fungi by measuring the inhibition zone. Among the 15 isolates of *Azotobacter* spp., three isolates as shown in Table 2 were active against the tested fungi either *F. oxysporum* or *R. solani.* The isolate No.12 was very active to inhibit the growth of *F. oxysporum*, which recorded 34.0 mm in diameter, while it recorded 22.0 mm with *R. solani*. Results in Table 2 also showed that the fifteen isolates of *Rhizobium* subdued the growth of the tested pathogenic fungi at disparate levels. Isolate No.9 was very active to inhibit the growth of *F. oxysporum* and *R. solani* severally, which recorded 27.0 and 22.0 mm in diameter, respectively as a highest clear zone. Isolates of *Pseudomonas* inhibited most tested pathogenic fungi with variable activities as indicated with measured inhibition zone. Isolate No.6 was superior in potency against the tested pathogenic fungi, which recorded 31.0 and 29.0 mm in diameter against *F.* oxysporum and *R. solani*, respectively as highest inhibition zone. The active isolates were selected and identified by different identification method, the isolate No.12 of *Azotobacter* was classified as *A. chroococcum*, while isolate No. 9 of symbiotic N_2-fixing *Rhizobium* was characterized as *R. leguminasarum* as it has obligate symbiosis on faba bean. The active isolate of *Pseudomonas* No.6 was considered as *P. fluorescens.*

Table 2. Antagonistic activity of *Azotobacter*, *Rhizobium* and *Pseudomonas* isolates against the tested pathogenic fungi.

Isolate number	Diameter of inhibition zone (mm)					
	Rhizoctonia solani			*Fusarium oxysporum*		
	Azotobacter	*Rhizobium*	*Pseudomonas*	*Azotobacter*	*Rhizobium*	*Pseudomonas*
1	19.0	7.0	28.0	8.0	0.0	26.0
2	9.0	0.0	4.0	15.0	0.0	16.0
3	31.0	19.0	9.0	21.0	20.0	0.0
4	14.0	9.0	0.00	9.0	0.00	0.0
5	8.0	0.0	2.0	2.0	13.0	9.0
6	9.0	2.0	31.0	7.0	5.0	29.0
7	0.0	13.0	29.0	0.0	7.0	19.0
8	28.0	14.0	6.0	18.0	8.0	10.0
9	0.0	27.0	0.0	1.0	22.0	0.0
10	25.0	8.0	13.0	16.0	9.0	25.0
11	8.0	18.0	0.00	10.0	16.0	10.0
12	34.0	0.0	22.0	22.0	0.0	25.0
13	16.0	25.0	6.0	10.0	8.0	0.0
14	12.0	0.0	6.0	13.0	6.0	10.0
15	11.0	22.0	16.0	8.0	18.0	14.0

Greenhouse experiment:

A pot experiment was conducted to study the impact of the selected nitrogen fixing bacteria, *A. chroococcum* No.12 and *R. leguminasarum* No.9 as highly active strains in both nitrogen fixation and as biocontrol agents as well as the selected *P. fluorescens* No.6 on growth of faba bean plant infected with pathogenic fungi.

Effect of co-inoculation with *R. leguminosarum* and some plant growth promoting rihzobacteria on the incidence of faba bean *Rhizoctonia* damping- off or *Fusarium* wilt diseases.

Data in Table 3, A & B indicated that all treatments induced reduction in the percentages of pre-emergence damping-off and root rot caused by *R. solani* or percentage of wilt caused by *F. oxysporum* compared to untreated control. All treatments significantly increased survived plants compared to infested control, while, the fungicide Topsin M-70® gave the highest reduction effect followed by the combined treatment of *R. leguminosarum* + *A. chroococcum* + *P fluorescens* in the presence of *R. solani* or *F. oxysporum.*

Table 3. Effect of co-inoculation with *R. leguminosarum* and some plant growth promoting rihzobacteria on the percentage of root- rot and wilt diseases of faba bean plants grown in infested soil by *R solani* (A) or *F. oxysporum* (B) under greenhouse conditions.

(A) *R. solani*

Treatments	Damping- off				Root rot		Survived plants (%)	Increase (%)
	Pre-emergence		Post- emergence					
	Incidence (%)	Reduction (%)	Incidence (%)	Reduction (%)	Incidence (%)	Reduction (%)		
R. leguminosarum (*R. l.*)	15.0 b	70.0	5.0 a	0.0	10.0 a	0.0	70.0 c	100.0
(*R. l.*) +*A. chroococcum* (*A. ch.*)	15.0 b	70.0	0.0 a	100.0	10.0 a	0.0	75.0 bc	114.28
(*R. l.*) +*P. fluorescens* (*P. f.*)	15.0 b	70.0	0.0 a	100.0	10.0 a	0.0	75.0 bc	114.28
(*R. l.*) + (*A. ch.*) + (*P. f.*)	10.0 b	80.0	0.0 a	100.0	10.0 a	0.0	80.0 bc	128.57
Topsin M-70®	15.0 b	70.0	0.0 a	100.0	0.0 a	100.0	85.0 b	142.85
Infested control	50.0 a	-	5.0 a	-	10.0 a	-	35.0 d	-
Healthy control	0.0 b	-	0.0 a	-	0.0 a	-	100.0 a	-

(B)*F. oxysporum*

Treatments	Damping- off				Wilted plants		Survived plants (%)	Increase (%)
	Pre-emergence		Post- emergence					
	Incidence (%)	Reduction (%)	Incidence (%)	Reduction (%)	Incidence (%)	Reduction (%)		
R. leguminosarum (*R. l.*)	15.0 ab	40.0	5.0 a	0.0	10.0 ab	33.33	70.0 cd	16.70
(*R. l.*) +*A. chroococcum* (*A. ch.*)	10.0 bc	60.0	5.0 a	0.0	5.0 ab	66.66	80.0 bc	33.40
(*R. l.*) +*P. fluorescens* (*P. f.*)	5.0 bc	80.0	0.0 a	0.0	10.0 ab	33.33	85.0 b	41.70
(*R. l.*) + (*A. ch.*) + (*P. f.*)	5.0 bc	80.0	0.0 a	0.0	5.0 ab	66.66	90.0 ab	50.00
Topsin M-70®	0.0 c	100.0	5.0 a	0.0	5.0 ab	66.66	90.0 ab	50.00
Infested control	25.0 a	-	0.0 a	-	15.0 a	-	60.0 d	-
Healthy control	0.0 c	-	0.0 a	-	0.0 b	-	100.0 a	-

- Means in each column followed by the same letter are not significantly different according to Duncan's multiple range test, (p = 0.05).

Effect of different biocontrol agents on some root parameters of faba been plants in infested soil with pathogenic fungi:

Data in Table 4 showed the essential role of biocontrol agents on root parameters of faba bean plants. The biocontrol agents recorded enhancing growth of faba bean plants particularly in rhizosphere area, where the significant increase in root length was recorded by great extent in the case of mixture of *R. leguminasarum* + *A. chroococcum*+ *P. fluorescens* which reached to 17.6 cm plant^{-1} with *F.* oxysporum, whereas it gave 14.3 cm plant^{-1} with *R. solani*. Increase percentage reached to 20.5 and 39.6 % up the treatment supplemented with *R. leguminasarum*+ *A. chroococcum* and treatment with *R. leguminasarum*+ *P. fluorescens*, respectively in presence of *F. oxysporum* but the increase percentage reached to 7.5 and 26.5%, respectively in presence of *R. solani*. The tested pathogenic fungi inhibited the growth of faba been plants and reduced root length in infested control which gave 4.6 and 4.3 cm plant^{-1} with *F.* oxysporum and *R. solani*, respectively.

Regarding to root size, the treatment supplemented with *R. leguminasarum, A. chroococcum* and *P. fluorescens*, exhibited striking effect on root development and led to increase of root size in presence of *F. oxysporum* or *R. solani* compared to other treatments. Inoculation with *R. leguminasarum* either with *A. chroococcum* or *P. fluorescens* recorded gradually increase in root size than the individual treatment with *R. leguminasarum* in presence tested pathogenic fungi.

Table 4. Effect of some biocontrol agents on some root parameters of faba been plants infected with pathogenic fungi.

Treatments	Root parameters									
	Fusarium oxysporum					*Rhizoctonia solani*				
	Root length (cm plant^{-1})	Root size (cm^3 plant^{-1})	Root dry weight (g plant^{-1})	Number of nodules plant^{-1}	Dry weight of nodules (g plant^{-1})	Root length (cm plant^{-1})	Root size (cm^3 plant^{-1})	Root dry weight (g plant^{-1})	Number of nodules plant^{-1}	Dry weight of nodules (g plant^{-1})
R. leguminosarum (*R l.*)	9.3 d	9.3 d	3.0 c	77.3 d	0.173 c	9.3 d	9.3 d	2.6 c	66.0 d	0.168 c
(*R. l.*) +*A. chroococcum* (*A. ch.*)	14.6 b	11.9 c	3.5 b	89.3 b	0.193 b	13.3 a	12.6 b	3.2 b	75.6 b	0.174 b
(*R. l.*) +*P. fluorescens* (*P. f.*)	12.6 c	14.0 b	3.1 bc	82.3 c	0.190 b	11.3 b	12.3 b	2.9 c	71.0 c	0.171 b
(*R. l.*) + (*A. ch.*) + (*P. f.*)	17.6 a	16.3 a	4.0 a	94.0 a	0.240 a	14.3 a	15.3 a	3.6 a	82.0 a	0.211 a
Topsin M-70®	10.6 d	11.3 c	3.1 c	10.6 f	0.070 e	9.6 d	11.3c	2.8 c	9.7 f	0.074 e
Infested control	4.6 e	7.3 e	1.1 d	8.3 g	0.051 f	4.3 e	5.3 e	1.0 d	7.2 g	0.052 f
Healthy control	12.3 c	14.3 b	3.2 bc	30.3 e	0.112 d	10.6 c	13.0 b	3.1 b	26.6 e	0.097 d

The treatment that inoculated with *R. leguminasarum, A. chroococcum* and *P. fluorescens* was superior in the root dry weight which recorded 4.0 and 3.6 g plant^{-1} in presence of *F. oxysporum* and *R. solani*, respectively. On the other hand, the inoculation with asymbiotic N_2 fixers as *A. chroococcum* and symbiotic N_2 fixers as *R. leguminasarum* led to increase of root dry weight to 17 % and 23% in presence of *F. oxysporum* and *R. solani*, respectively compared to the treatment that supplemented with *R. leguminasarum* only.

Concerning the number and dry weight of nodules were positively affected especially when inoculation with *R. leguminasarum* either with *A. chroococcum* or with *P. fluorescens* in the presence of *F. oxysporum* or *R. solani* compared to inoculation by *R. leguminasarum* alone. Meantime, the treatment amended with all tested bacteria was considered superior ones occurring resistance against the tested pathogenic fungi and recorded highest number and dry weight of nodules compared to other treatments where it gave 94.0 nodules plant^{-1} and 0.240 g plant^{-1} for number of nodules and dry weight respectively against *F. oxysporum*, while it gave 82.0 nodules plant^{-1} and 0.211 g plant^{-1} for nodules number and dry, respectively against *R. solani*.

Effect of different microorganisms on some shoot parameters of faba been plants infected with pathogenic fungi:

Data presented Table 5 showed that use of biofertilizers as biocontrol agents enhanced the plant growth and improved development in the presence of pathogens. Consequently, the

combined treatment with *R. leguminasarum, A. chroococcum* and *P. fluorescens* exhibited significant increase in shoot height by great extent compared to other treatments which gave 70.0 cm plant^{-1} for *F. oxysporum,* and 60.0 cm plant^{-1} for *R. solani*.

Similarly, inoculation by *R. leguminasarum* either with *A. chroococcum* or *P. fluorescens* recorded best shoot height in presence of *F. oxysporum* or *R. solani* compared to *R. leguminasarum* only.

Table 5. Effect of some biocontrol agents on some shoot parameters of faba been plants infected with pathogenic fungi.

Treatments	Shoot parameters					
	F. oxysporum			*R. solani*		
	Shoot height(cm plant^{-1})	Number of branches plant^{-1}	Shoot dry weight(g plant^{-1})	Shoot height (cm plant^{-1})	Number of branches plant^{-1}	Shoot dry weight(g plant^{-1})
Rhizobium leguminosarum (*R. l.*)	50.3d	2.3d	15.6 f	40.3 c	2.0 c	14.3d
(*R. l.*)+*A. chroococcum* (*A. ch.*)	65.0 b	2.3 d	22.6 c	55.0 a	3.3 a	20.6 b
(*R. l.*) +*P. fluorescens* (*P. f.*)	61.2 b	4.1 a	24.3 b	60.0 a	3.2b	20.0 b
(*R. l.*) + (*A. ch.*) + (*P. f.*)	70.0 a	4.3 a	28.3 a	60.6 a	3.6 a	25.5 a
Topsin M-70®	55.6 c	3.3 c	17.3 e	46.6 b	2.8 b	16.0 c
Infested control	30.6 e	1.3 e	8.3 g	23.0d	1.3 d	8.3e
Healthy control	56.9c	3.8 b	20.6 d	50.3 b	3.2 b	17.6 c

Regarding number of branches on plant, the highest values of faba been plants were 4.3 and 3.6 branches plant^{-1} obtained from combined treatment of *R.leguminasarum, A. chroococcum* and *P. fluorescens* against *F. oxysporum* or *R. solani*, respectively and the increase percent reached to 13.2 and 12.5%, respectively up healthy control. The combination of *R.leguminasarum, A. chroococcum* and *P. fluorescens* exhibited positive effect on dry weight of shoot plants infected with tested pathogenic fungi which recorded 28.3 and 25.5 g plant^{-1} in the presence of *F. oxysporum* or *R. solani*, respectively. Furthermore, combined applications of *R.leguminasarum* with *A. chroococcum* or *P. fluorescens* were more effective compared to individual treatment of *R.leguminasarum*.

Role of individual or combined biocontrol agents on chlorophyll a, chlorophyll b and carotenoids content of faba bean plants grown in infested soil:

Table 6 illustrated the effect of different treatments on chlorophyll a, chlorophyll b and carotenoids (mg g^{-1}) of faba bean leaves. The infested control treatment exhibited the lowest chlorophyll a, chlorophyll b and carotenoids content in comparison to other treatments where recorded 0.143, 0.052 and 0.214 mg g^{-1}, respectively in the presence of *F. oxysporum*, while its values were 0.155, 0.053 and 0.216 mg g^{-1}, respectively in the presence of *R. solani*.

Application of biofertilizers as biocontrol agents exhibited striking effect on chlorophyll and carotenoid contents. The best values of chlorophyll a, chlorophyll b and carotenoids were recorded with the treatment that inoculated by *R. leguminasarum, A. chroococcum* and *P. fluorescens* with *F. oxysporum*, where the values were 1.052, 0.420 and 0.671 mg g^{-1}, respectively, whilst they gave 0.932, 0.402 and 0.553 mg g^{-1}, respectively with *R. solani*. It is worth to mention that the pathogenic fungi had no negative effect on faba bean plants in the presence of biofertilizers. Addition of mixture *R. leguminasarum* either with *A. chroococcum* or *P. fluorescens* recorded better chlorophyll and carotenoids content of faba bean leaves than individual treatment with *R. leguminasarum* alone.

Table 6. Effect of some biocontrol agents on chlorophyll a, chlorophyll b and carotenoids content of faba been plants infected with pathogenic fungi.

Treatments	*F. oxysporum*			*R. solani*		
	Chlorophyll a (mg g^{-1}fresh weight)	Chlorophyll b (mg g^{-1}fresh weight)	Carotenoids (mg g^{-1}fresh weight)	Chlorophyll a (mg g^{-1}fresh weight)	Chlorophyll b (mg g^{-1}fresh weight)	Carotenoids (mg g^{-1}fresh weight)
R. leguminosarum (*R. l.*)	0.5o1 e	0.204 d	0.353 d	0.516 e	0.196 e	0.312 d
(*R. l.*) +*A. chroococcum* (*A. ch.*)	0.816 c	0.376 c	0.412 c	0.802 c	0.346 c	0.372 c
(*R. l.*) +*P. fluorescens* (*P. f.*)	0.933 b	0.402 b	0.523 b	0.882 b	0.383 b	0.397 b
(*R. l.*) + (*A. ch.*) + (*P. f.*)	1.052 a	0.420 a	0.671 a	0.932 a	0.402 a	0.553 a
Topsin M-70®	0.592 d	0.213 e	0.383 cd	0.583 f	0.223 f	0.386 d
Infested control	0.143 f	0.052 f	0.214 e	0.155 g	0.053 g	0.216 e
Healthy control	0.611 d	0.276 d	0.395 cd	0.636 d	0.265 d	0.398 d

Impact of microorganisms on some biological parameters of faba been plants infected with pathogenic fungi:

Table 7 showed the role of microbial treatments on nitrogenase activity, dehydrogenase activity and total phenol. All biocontrol treatments had a positive role in increasing the activities of nitrogenase and dehydrogenase as well as total phenol. Generally, combination between *R. leguminosarum* with *A. chroococcum* and the mixture of *R. leguminosarum, A. chroococcum* and *P. fluorescens* gave the highest values of nitrogenase activity in presence of *F. oxysporum* or R. *solani*. The maximum activity of nitrogenase were 24.6 and 24.7 µmol C_2H_4 g^{-1} dry nodules, respectively with *F. oxysporum*, while with *R. solani* recorded 26.0 and 27.6 µmol C_2H_4 g^{-1} dry nodules, respectively.

Rhizobium leguminosarum individually or in combination with *A. chroococcum* or *P. fluorescens* recorded significant increase in dehydrogenase activity compared to non-inoculated treatments in the presence of tested pathogenic fungi. The mixture of *R. leguminosarum* + *A. chroococcum* + *P. fluorescens* was superior treatment where it recorded the highest values of dehydrogenase activity (186.6 and 177.6 ugTPF g^{-1} soil) in presence of *F. oxysporum* and R. *solani*, respectively.

Generally, faba bean plants inoculated with *R. leguminosarum* either alone or in combination with another application attained significant increase in total phenol compared to control and treatment with fungicide Topsin M-70®.

Co-inoculation with *R. leguminosarum* with *A. chroococcum* and *P. fluorescens* played an important role in enhancing total phenol which recorded 0.821 and 0.806 mg g^{-1} fresh weight in presence of *F. oxysporum* and *R. solani*, respectively.

Field experiment:

Field experiments were carried out in two seasons 2016/2017 and 2017/ 2018 to evaluate the effect of *Rhizobium leguminosarum* and some plant growth promoting rhizobacteria on growth and productivity of faba bean plants grown in naturally infected soil with pathogenic fungi.

Table 7. Effect of some biocontrol agents on some biological parameters of faba been plants infected with pathogenic fungi

Treatments	*F. oxysporum*			*R. solani*		
	Nitrogenase activity (µ mole $C_2H_4g^{-1}$ dry nodules)	Dehydrogenas activity (µg TpF g^{-1} soil)	Total phenol (mg g^{-1} fresh weight)	Nitrogenase activity (µ mole $C_2H_4g^{-1}$ dry nodules)	Dehydrogenas activity (ug TPF g^{-1} soil)	Total phenol (mg g^{-1} fresh weight)
R. leguminosarum (*R. l.*)	20.3 b	145.1 d	0.670 d	21.0 c	141.0 d	0.639 c
(*R. l.*) +*A. chroococcum* (*A. ch.*)	24.6 a	160.2 c	0.732 c	26.0 b	157.0 c	0.701 b
(*R. l.*) +*P. fluorescens* (*P. f.*)	21.3 b	173.6 b	0.772 b	20.3 c	165.2 b	0.705 b
(*R. l.*) + (*A. ch.*) + (*P. f.*)	24.7 a	186.6 a	0.821 a	27.6 a	177.6 a	0.806 a
Topsin M-70®	6.3 d	85.0 f	0.224 e	7.50 d	80.0 f	0.207 d
Infested control	4.3 e	45.2 g	0.116 f	4.20 e	47.20 g	0.057 e
Healthy control	9.3 c	131.6 e	0.059 g	8.90 d	125.3 e	0.048 f

Effect of co-inoculation with *Rhizobium leguminosarum* and some plant growth promoting rihzobacteria on the incidence of faba bean damping- off or percentage of *Fusarium* wilt diseases:

Field experiments demonstrated the effect of co-inoculation with *Rhizobium leguminosarum* and some plant growth promoting rihzobacteria on damping- off, root rot, percentages of wilted plants and survived plants of faba bean under field conditions. Table 8 exhibited that all treatments significantly increased the percentages of survived plants compared to untreated control with variable degrees in the two seasons. The highest increase percent over control was obtained from treatments with the fungicide Topsin M-70® followed by the combined treatment of *R. leguminosarum* + *A. chroococcum* + *P. fluorescens* compared to the control.

Table 8. Effect of co-inoculation with *Rhizobium leguminosarum* and some plant growth promoting rihzobacteria on the percentage of root-rot and wilt diseases of faba bean plants grown under field conditions during the growing seasons 2016/2017 (A) and 2017/2018 (B).

A- Season 2016/2017

Treatments	Damping- off				Root rotted plants (%)	Wilted plants (%)	Survived plants (%)	Increasing (%)
	Pre-emergence		Post- emergence					
	Incidence (%)	Reduction (%)	Incidence (%)	Reduction (%)				
R.leguminosarum (*R. l.*)	7.40 a	0.0	3.0 a	25.00	0.60 bc	1.40 cd	87.60 bc	4.78
(*R. l.*) +*A. chroococcum* (*A. ch.*)	4.0 abc	29.82	1.60 a	60.00	2.40 ab	3.40 a	88.60 bc	5.98
(*R. l.*) +*P. fluorescens* (*P. f.*)	6.70 ab	0.0	2.30 a	42.50	0.30 bc	0.70 d	90.0 ab	7.65
(*R. l.*) + (*A. ch.*) + (*P. f.*)	3.40 bc	40.35	1.0 a	75.00	0.0 c	3.0 ab	92.60 ab	10.76
Topsin M-70®	2.40 c	57.90	1.0 a	75.00	0.0 c	1.60 bcd	95.0 a	13.63
Control	5.70 abc	-	4.0 a	-	4.0 a	2.70 abc	83.60 c	-

B- Season 2017/2018

Treatments	Damping- off				Root rotted plants (%)	Wilted plants (%)	Survived plants (%)	Increasing (%)
	Pre-emergence		Post- emergence					
	Incidence (%)	Reduction (%)	Incidence (%)	Reduction (%)				
R.leguminosarum (*R. l.*)	6.70 bc	59.88	2.40 b	44.18	2.30 ab	1.60 ab	87.0 c	19.66
(*R. l.*) +*A. chroococcum* (*A. ch.*)	8.40 bcd	49.70	1.60 b	62.80	1.60 b	1.40 ab	87.0 c	19.66
(*R. l.*) +*P. fluorescens* (*P. f.*)	5.70 bcd	65.86	3.0 b	30.23	1.30 b	1.30 ab	88.70 bc	22.00
(*R. l.*) + (*A. ch.*) + (*P. f.*)	4.40 cd	73.65	2.0 b	53.48	1.30 b	0.60 b	91.70 ab	26.13
Topsin M-70®	3.0 d	82.00	2.0 b	53.48	1.0 b	0.60 b	93.40 a	28.47
Control	16.70 a	-	4.30 a	-	3.30 a	3.0 a	72.70 d	-

- Means in each column followed by the same letter are not significantly different according to Duncan's multiple range test, (p = 0.05).

Root parameters of faba bean plants affected by different bioagents:

A positive influence of *R. leguminosarum* in combination with *A. chroococcum* or *P. fluorescens* was observed in root parameters of faba bean plants. Table 9 showed that a significant increase in root length and root size were recorded with the mixture of *R. leguminosarum*, *A. chroococcum* and *P. fluorescens* at the two seasons which gave 28.3 and 27.2 cm $plant^{-1}$ for root length at the first and second season, respectively, while the root size gave 30.3 and 27.0 cm^3 $plant^{-1}$ at the first and second season, respectively.

Significant increase in root dry weight was recorded with the mixture of three biological agent-used compared to the treatment of *R. leguminosarum* alone and the treatment with fungicide Topsin M-70® which reached to 6.8 and 6.6 g $plant^{-1}$ at the first and second season, respectively. The increase percent reached to 17.2 and 13.3 %, respectively compared to *R. leguminasarum* alone and treatment with fungicide Topsin M-70® at the first season, while the increase percent reached to 27.0 and13.7% at the second season, respectively.

On the other hand, the highest number of nodules and dry weight of nodules per plant during the two season were 203.3 and 177.0 for number of nodules $plant^{-1,}$ while 0.388 and 0.372 g $plant^{-1}$ for dry weight of nodules at the first and second season respectively, were obtained from plants which treated with *R. leguminosarum*, *A. chroococcum* and *P. fluorescens*. Generally, the first season gave the best results of root parameters than in the second season.

Shoot parameters of faba bean plants affected by different bioagents.

Tables 10 revealed that shoot parameters of faba bean plants were significantly improved due to the use of combined treatments. Faba bean plants treated with the selected strains of *R. leguminosarum*, *A. chroococcum* and *P. fluorescens* gave significantly increased in shoot height and number of branches during two seasons compared to the individual treatment of *R. leguminosarum* or treatment with fungicide Topsin M-70®. Use of mixture of all microbes was considered to be more efficient than using dual inoculation with *R. leguminosarum* + *A. chroococcum* or with *R. leguminosarum* + *P. fluorescens.*

Furthermore, the mixture of *R. leguminosarum*, *A. chroococcum* and *P. fluorescens* was the most effective treatment on shoot dry weight compared to other treatments which recorded 35.0 and 35.6 g plant^{-1}on shoot dry weight at the first and second season, respectively.

Table 9. Effect of some biocontrol agents on some root parameters of faba been plants.

Treatments	Root parameters									
	First season (2016/2017)					Second season (2017/2018)				
	Root length (cm plant^{-1})	Root size (cm^3 plant^{-1})	Root dry weight (g plant^{-1})	Number of nodules plant^{-1}	Dry weight of nodules (g plant^{-1})	Root length (cm plant^{-1})	Root size (cm^3 plant^{-1})	Root dry weight (g plant^{-1})	Number of nodules plant^{-1}	Dry weight of nodules (g plant^{-1})
R. leguminosarum (*R. l.*)	23.6 c	22.3 c	5.8 b	130.6 d	0.273 d	22.6 b	19.9 c	5.2 c	132.3 d	0.252 d
(*R. l.*) +*A. chroococcum* (*A. ch.*)	26.0 b	26.3 b	6.33 a	141.3 c	0.302 c	26.6 a	25.1 b	6.43 a	147.2 c	0.271 c
(*R. l.*) +*P. fluorescens* (*P. f.*)	25.6b	26.6 b	6.1 a	183.0 b	0.368 b	26.3 a	24.3 b	6.2 a	163.6 b	0.355 b
(*R. l.*) + (*A. ch.*) + (*P. f.*)	28.3 a	30.3 a	6.8 a	203.3 a	0.388 a	27.2 a	27.0 a	6.6 a	177.0 a	0.372 a
Topsin M-70®	24.3 c	18.2 d	6.0 b	13.2 f	0.071 f	20.6 b	16.3 c	5.8 b	14.6 e	0.077 f
Control	9.3 d	8.3 e	2.5 c	18.6 e	0.083 e	9.0 c	10.5 d	2.7 d	16.3 e	0.076 e

Table 10. Effect of some biocontrol agents on some shoot parameters of faba been plants

Treatments	Shoot parameters					
	First season (2016/2017)			Second season (2017/2018)		
	Shoot height (cm plant^{-1})	Number of branches plant^{-1})	Shoot dry weight(g plant^{-1})	Shoot height (cm plant^{-1})	Number of branches plant^{-1}	Shoot dry weight(g plant^{-1})
R.leguminosarum (*R. l.*)	65.3 d	3.0 b	24.00 d	60.7 d	2.6 b	20.3 d
(*R. l.*) +*A. chroococcum* (*A. ch.*)	81.6 a	4.0 ab	31.20 b	80.2 a	4.3 a	29.3 b
(*R. l.*) +*P. fluorescens* (*P. f.*)	77.8 b	4.2 ab	33.32 ab	72.3 b	3.6 ab	32.6 a
(*R. l.*) + (*A. ch.*) + (*P. f.*)	85.3 a	4.5 a	35.0 a	82.6 a	4.4 a	35.6 a
Topsin M-70®	70.6 c	3.33 ab	28.66 c	68.7 c	3.6 ab	24.3 c
Control	43.3 e	2.0 c	11.66e	40. 3 e	2.1 c	12.6 e

Impact of some microorganisms on chlorophyll and carotenoids content of faba bean plants.

Table 11 demonstrated the viability and activity of nitrogen fixing bacteria especially in the presence of *P. fluorescens* reflected the positive effect on chlorophyll and carotenoids contents than treatment received with fungicide Topsin M-70® and the individual treatment with *R. leguminosarum*. The highest values of these parameters were 1.112, 0.532 and 0.721 mg g^{-1} fresh weight for chlorophyll a, chlorophyll b and carotenoids through first season respectively, while at the second season the values were 1.166, 0.496 and 0.703 mg g^{-1}fresh weight, respectively. Furthermore, treatment that supplemented with *R. leguminosarum* and *P. fluorescens* gave the best values of chlorophyll a, chlorophyll b and carotenoids compared to dual treatment with *R. leguminosarum* and *A. chroococcum* through two season.

Table 11. Influence of bio-agent's treatments on chlorophyll and carotenoids content through two seasons of faba bean planting.

Treatments	First season (2016/2017)			Second season (2017/2018)		
	Chlorophyll a content (mg g^{-1} fresh weight)	Chlorophyll b content (mg g^{-1} fresh weight)	Carotenoids content (mg g^{-1} Fresh weight)	Chlorophyll a content (mg g^{-1} fresh weight)	Chlorophyll, b content (mg g^{-1} fresh weight)	Carotenoids content (mg g^{-1} fresh weight)
R. leguminosarum (*R. l.*)	0.771 d	0.273 d	0.492 d	0.733 d	0.224 d	0.456 c
(*R. l.*) +*A. chroococcum* (*A. ch.*)	0.903 c	0.352 c	0.566 c	0.871 c	0.316 c	0.562 b
(*R. l.*) +*P. fluorescens* (*P. f.*)	1.082 b	0.431 b	0.612 b	1.053 b	0.401 b	0.622 b
(*R. l.*) + (*A. ch.*) + (*P. f.*)	1.112 a	0.532 a	0.721 a	1.166 a	0.496 a	0.703 a
Topsin M-70®	0.763 d	0.283 d	0.452 e	0.751 d	0.283 d	0.416 c
Control	0.412 e	0.192 e	0.243 f	0.401 e	0.193 e	0.213 d

Impact of some microorganisms on nitrogenase and dehydrogenase activities of faba bean plants through two seasons:

Table 12 revealed the influence of bio-agent's treatments on nitrogenase and dehydrogenase activities in the soil through two seasons of faba bean plants. Results indicated that faba bean plants supplemented with *R. leguminosarum* alone or in combination with *A. chroococcum* or P. *fluorescens* gave significant increase in nitrogenase activity (21.50, 28.30 and 24.30 µmole C_2H_4 g^{-1} dry nodules) at the first season respectively, while through the second season they recorded 20.6, 27.3 and 23.3 µ mole C_2H_4 g^{-1} dry nodules, respectively compared to non-inoculated treatments. The maximum nitrogenase activity was 33.3 and 31.6 µmole C_2H_4 g^{-1} dry nodules recorded with both N_2 fixers and *P. fluorescens* through first and second season, respectively. The lowest activity of enzyme was recorded with treatment received with fungicide Topsin M-70®.

Table 12. Influence of bio-agent's treatments on nitrogenase and dehydrogenase activities through two seasons of faba bean planting.

Treatments	First season (2016/2017)		Second season (2017/2018)	
	Nitrogenase activity (µ mole C_2H_4g^{-1} dry nodules)	Dehydrogenase activity (µ TPF g^{-1} soil)	Nitrogenase activity (µ mole C_2H_4g^{-1} dry nodules)	Dehydrogenase activity (µ TPF g^{-1} soil)
R. leguminosarum (*R. l.*)	21.5 d	202.0 d	20.6 d	200.6 c
(*R. l.*) +*A. chroococcum* (*A. ch.*)	28.3 b	219.0 b	27.3 b	216.4 b
(*R. l.*) +*P. fluorescens* (*P. f.*)	24.3 c	214.0 c	23.3 c	215.3 b
(*R. l.*) + (*A. ch.*) + (*P. f.*)	33.3 a	253.6 a	31.6 a	257.0 a
Topsin M-70®	5.6 f	98.0 f	5.3 e	96.0 e
Control	8.8 e	104.0 e	8.3 e	100.6 d

The same trend was occurred in dehydrogenase activity, where the highest values of dehydrogenase activity recorded 253.6 and 257.0 µgTPFg^{-1} soil for *R. leguminosarum*, *A. chroococcum* and *P. fluorescens* at the two seasons, respectively. The increase present reached to 15.7 and 18.5% compared to treatment that supplemented with *R. leguminosarum* + *A. chroococcum* and treatment that supplemented with *R. leguminosarum* + *P. fluorescens*, respectively during the first season, while in the second season the present were 18.7 and 19.3%, respectively.

Effect of co-inoculation with *R. leguminosarum* and some plant growth promoting rihzobacteria on some yield parameters of faba bean during the growing seasons.

Under field condition, the co-inoculation with *R. leguminosarum* and some plant growth promoting rihzobacteria treatments significantly improved growth parameters and yield compared to the untreated control treatment in the two seasons. Table (13) indicated that all treatments significantly increased shoot height, number of branches per plant, number of pods per plant, seed weight per plant, the weight of one hundred seed and seed yield as compared with untreated control at the two seasons. The maximum shoot height and number of branches per plant were arranged by the combined treatment of *R. leguminosarum* + *A. hroococcum* + *P. fluorescens* followed by *R. leguminosarum* + *P. fluorescens*. The dual and triple inoculation gave the highest number of pods per plant compared to other treatments. The combination of *R. leguminasarum*, *A. chroococcum* and *P. fluorescens* exhibited significant increase in number of pods compared to all treatments which recorded 20.0 and 15.0 pods plant^{-1} through two seasons, respectively.

Combination with the tested bacterial strains enhanced seed yield (g plant^{-1}) and 100 seed weight (g) compared to inoculation with *R. leguminosarum* alone. The mixture of tested microbes (R. *leguminosarum* + *A. chroococcum* + *P. fluorescens*) recorded the highest values of seed yield per plant were 60.88 and 55.90 g plant^{-1} through the two seasons, respectively, while 100 seed weight were 102.90and 102.60 g plant^{-1} through the two seasons, respectively.

Using triple inoculation of *R. leguminosarum*, *A.chroococcum* and *P.fluorescens* caused significant increases in seed yield weight(Kg feddan^{-1}) over single inoculation (*R. leguminosarum*) and dual inoculation (*R. leguminosarum*+ *A.chroococcum* or *R. leguminosarum*+ *P. fluorescens*). Combined treatment with *R. leguminosarum*, *A.chroococcum* and *P. fluorescens* was more effective and recorded 1005.68 and 1055.6 Kg feddan^{-1} through two seasons, respectively.

Table 13. Effect of co-inoculation with *Rhizobium leguminosarum* and plant growth promoting rihzobacteria on growth parameters and yield of faba bean plants at harvest through two seasons.

Treatments	Shoot height (cm plant^{-1})	Branches number plant^{-1}	Pods number plant^{-1}	Seed yield (g plant^{-1})	100 seed weight (g)	Seed yield weight (Kg feddan^{-1})
Season 2016/2017						
R. leguminosarum (*R. l.*)	82.93 c	3.40 d	12.20 c	35.30 c	92.60 bc	944.46 d
(*R. l.*) +*A. chroococcum* (*A. ch.*)	87.00 bc	4.40 b	15.26 b	45.56 b	98.68 ab	977.80 c
(*R. l.*) +*P. fluorescens* (*P. f.*)	88.70 ab	4.53 b	16.46 b	47.93 b	99.60 ab	988.86 b
(*R. l.*) + (*A. ch.*) + (*P. f.*)	92.40 a	5.00 a	20.00 a	60.88 a	102.90 a	1055.6 a
Topsin M-70®	85.70 d	3.8 c	10.70 d	36.30 c	91.26 bc	943.00 d
Control	46.60 e	2.5 e	5.53 e	14.40 d	67.40 c	766.70 e
Season 2017/2018						
R. leguminosarum (*R. l.*)	81.00 b	3.20 d	10.80 b	30.64 c	88.40 d	920.00 d
(*R. l.*) +*A. chroococcum* (*A. ch.*)	89.00 b	4.20 b	13.00 a	40.00 b	90.60 c	950.40 c
(*R. l.*) +*P. fluorescens* (*P. f.*)	86.00 b	4.30 b	13.60 a	44.12 b	94.80 b	976.60 b
(*R. l.*) + (*A. ch.*) + (*P. f.*)	101.00 a	4.80 a	15.00 a	55.90 a	102.60 a	1005.68 a
Topsin M-70®	80.00 c	3.60 c	9.70 b	29.34 c	85.00 e	922.80 d
Control	44.00 d	2.30e	6.80 c	13.98 d	64.40 e	750.60 e

- Means in each column followed by the same letter are not significantly different according to Duncan's multiple range test, (p = 0.05).

Discussion

Recently, root rot and wilt diseases caused by a number of pathogenic fungi (*R. solani* and different species of *Fusarium*) have become a common disease of faba bean in Egypt. They cause considerable loss in the crop productivity and quality of seeds (Mazen *et al*., 2008; Elwakil *et al*., 2009a). Until now, traditional methods for controlling the disease by crop rotation, resistant varieties and fungicides are economically limited. Many researchers oriented their efforts for applying recent methods such biological control as alternative approach instead chemical treatments (Elwakil *et al*., 2009b).

The objective of this study was to investigate the efficiency of three strains of *R. leguminosarum*, *A. chroococcum* and *P. fluorescens* as biocontrol agents compared to the fungicide Topsin M-70® to suppress the root rot and wilt diseases of faba bean and to select the bacteria which have the beneficial role in viability of faba bean.

Data *in vitro* revealed that most isolates of Plant Growth Promoting Rhizobacteria (PGPR) caused linear growth reduction of the two tested pathogenic fungi, *F. oxysporum* and *R. solani* but with variable degrees. Active isolates were selected and identified as *A. chroococcum*, *R. leguminasarum* and *P. fluorescens*. Analogous results displayed that *Rhizobium* spp. significantly diminished growth of *Fusarium* spp. by 54% *in vitro* (Sharif *et al*., 2003). Akhtar *et al*. (2011) found that *Rhizobium* spp. significantly reduced the *Fusarium* wilt on lentil plant. Jan *et al*. (2011) reported that many strains of *Pseudomonas* suppressed the growth of pathogenic *F. oxysporum* and had the capability to produce HCN, siderophores and antibiotics.

In addition, all treatments caused significant reduction of infection by damping-off and root rot/wilt diseases of faba bean and increased the plant survival either in the pots or field experiments compared to control treatments. In pot experiment the mixture of *R. leguminosarum*, *A. chroococcum* and *P. fluorescens* followed by *R. leguminosarum* and *P. fluorescens* were more effective to reduce damping-off, root rot/wilt severity and increased survived plants than using *R. leguminosarum* alone. The results were in coincidence with those reported by Samavat *et al*. (2011) who found that the combined treatments of common bean seeds with rhizobia cultural filtrates and *P. fluorescens* decreased root rot and damping-off intensity. Moreover, Kumar *et al.* (2001) showed that seed treatment with

P. fluorescens alone and compined with a *Rhizobium* reduced the number of infected pea plants grown in *F. oxysporum* infected soil. Thus, treatment of faba bean with effective strains of *R. leguminosarum* alone or in combination with other beneficial microorganisms may be preferred versus the fungicides because of their multiple possibilities to fix nitrogen, disease control, enhance of soil fertility, and increase crop productivity besides reducing the negative environmental impact associated with chemical use (Huang and Erickson, 2007). *Azotobacter chroococcum* was regarded as free-living aerobic nitrogen fixer present in soils, *Azotobacter* synthesises and secreted considerable amount of biologically active substances like B-vitamins, nicotinic acid, pentothenic acid, biotin, heteroauxins, gibberellins, etc., which were reported to enhance the growth of plants and their tolerance to pathogenic diseases (Van Loon, 2007). *Azotobacter* spp. were capable to produce siderophore which bind to the available form of iron Fe^{+3} in rhizosphere region, so it became unavailable to the phytopathogens and protecting the plant health (Althaf and Srinivas, 2013). *Azotobacter* also secreted an antibiotic-like structure similar to anisomycin, which registered as fungicidal antibiotic. Sufficient numbers of *Azotobacter* will out-compete pathogens for food. Some of the pathogens that have been controlled by *Azotobacter* in the soil and on the leaf involved some genus of *Fusarium*, *Rhizoctonia*, *Macrophomina* and *Cephalosporium* (Jnawali *et al*., 2015).

On the other hand, results exhibited that faba bean plants infected with either *F. oxysporum* or *R. solani* decreased both chlorophyll a, b and carotenoids contents of plant tissue which was common visible symptoms following infection with phytopathogenic fungi. The results were deemed as a consequence of either a) photo oxidative destruction of existing pigments, or b) inhibition of pigment synthesis. It was possible that the effect of the phytopathogenic fungi on chlorophyll and carotenoids was an attenuation of the biosynthetic rate rather than a breakdown of pigments already formed (Elwakil *et al.,* 2009a). This reduction might be attributed to the consequence of the degradation of leaf pigments of plant tissues (Abd El-Hai *et al*. 2017).

Kern (1972) reported that the adverse effect of fungal pathogen on chlorophyll pigments may be due to the fact that the fungal toxins form iron-chelate, transforming iron to become unavailable to participate in chlorophyll synthesis. The results coincided with Kosslak and Bohlool (1984), who detected that photosynthetic capacity of the host plant, may be influenced by number of successful infections. Advantages to plants from PGPR have been shown to include increase in seed germination rate, root growth, yield, leaf area, chlorophyll content, nutrient uptake, protein content, hydraulic activity, tolerance to abiotic stress, shoot and root weights, biocontrol, and delayed senescence (Yang *et al*.,2009). Plant Growth Promoting Rhizobacteria isolates individually or in combination appeared to stimulate chlorophyll synthesis or at least eliminate the adverse effect of the phytopathogens on pigment formation. (Heidari *et al*., (2011).

Generally, combined treatment of *R. leguminosarum* + *A. chroococcum* and the mixture of *R. leguminosarum, A. chroococcum* and *P. fluorescens* gave the highest values of nitrogenase activity in presence of *F. oxysporum* and R. *solani*. Tajini *et al*., (2012) and Byan and El-Shimi (2014) reported that the combined application of *Rhizobium* conjugated with tested rhizobacteria or arbuscular mycorrhizal (AM) fungi showed a significant amelioration in nodulation and N_2-ase activity of faba bean roots in relative to the uninoculated plants or plants inoculated with *Rhizobium* alone. These results might be due to that biofertilizers which produced some growth regulators that might enhance the nodulation status by hormonal stimulation besides N_2-fixation.

Also, the dual inoculation with *R. leguminosarum* +*A. chroococcum* or application of *R. leguminosarum* +*P. fluorescens* recorded significant increases in dehydrogenase activity (DHA-ase) compared to individually treatment with *R. leguminosarum* in presence of tested pathogenic fungi. Mixture of *R. leguminosarum, A. chroococcum* and *P. fluorescens* was the leading treatment where it recorded highly dehydrogenase activity in presence of *F. oxysporum* or R. *solani*. The *Rhizobium* inoculation might improve plant growth through soil nutrient enrichment, enhanced enzymatic activity and increased of resistance versus plant pathogens (Gopalakrishnan *et al*., 2015). Siczek and Lipiec (2016) showed that *Rhizobium* inoculation promoted a significant increase in a majority of enzymatic activities in the rhizosphere region throughout the vegetative period of faba bean. However, the maximal rates of DHA-ase activity, alkaline and acidic phosphatase were get by a *Rhizobium* conjugated mixture of PGPR's and AM fungal treatment. This might have been due to increased microbial and root activities. In fact, the microbiological properties of rhizosphere soil which expressed by dehydrogenase activity displayed higher response to applied biofertilizers. These results were in conformity with those of Hashem *et al*. (2014) and Vafadar *et al*. (2014) who affirmed that dual inoculation of PGPR and AM-fungi led to higher biological N_2 fixation (BNF) and DHA-ase activity.

Furthermore, co-inoculation between *R. leguminosarum* with *A. chroococcum* + *P. fluorescens* treatment led to increase the total phenolic content compared to the untreated control. The role of phenolic compounds in disease resistance was postulated by many authors (Nicholson and Hammerschmidt, 1992). They indicated that phenols were oxidized to quinones or semi-quinones which were more toxic and played a great role as antimicrobial substances on the invaded pathogen (Farkas and Kiraly, 1962).

Regarding application with *R. leguminasarum* individually or in combination with some PGPR evaluated in field trials during the winter growing seasons 2016-2017 and 2017-2018 at Ismailia Research Stations The experiment showed a significant increase in faba bean growth parameters and yield components. The combination of PGPR strains recorded high increase in all growth and yield parameters more than using *R. leguminosarum* alone. This was due to the specific studies showed that PGPR either directly or indirectly promote plant growth and yield. The direct growth promoting mechanisms includes (i) N_2 fixation; (ii) solubilization of mineral phosphate and zinc; (iii) sequestration of iron by production of siderophores; (iv) production of phytohormones such as auxins, cytokinins and gibberellins; (v) production of the enzyme 1-aminocyclopropane -1- carboxylate (ACC) deaminase which hydrolyses ACC, the immediate precursor of ethylene in plants. Lowering of ethylene concentration in seedlings promoted the stimulation seedling root length (Tsegaye *et al*., 2019). Plant Growth Promoting Rhizobacteria also support plant growth indirectly, by improving growth restricting conditions via (i) production of antibiotics; (ii) depletion of iron from the rhizosphere; (ii) production of fungal cell wall lysing enzymes ß-(1, 3) - glucanase and chitinase; (iii) synthesis of antifungal metabolites such as cyanide ion; (iv) competition for infection sites on roots; (v) induction of systemic resistance (Aeron *et al*., 2011 and Saraf *et al*., 2014). In this concern, Woyessa and Assefa (2011) reported that inoculation of teff crops with *P. fluorescent* increases the mean of root dry

weight by 39%, root shoot ratio by 42%, and grain yield by 28%. Several reports demonstrated significant improvement of yield and yield components in faba bean by Rhizobium inoculation (Denton *et al*., 2013 and Ismael *et al*., 2018). The results showed that faba bean inoculation could effectively reduce the need of applied inorganic N-fertilizers while achieved higher grain yield. The results agree with those published by (Tena *et al*., 2016) who reported that in a field experiment, inoculation of lentil by *Rhizobium* strains Lt29 increased seed yield by 59% while N fertilizer enhanced yields by 40% over the un-inoculated plants. Also, El-Wakeil and El-Sebai (2009) found a significant positive effect of *Rhizobium* strains on fresh and dry weight of leaves and stems, root/ shoot ratio, pods/flowers ratio, as well as, the number and weight of nodules compared to NPK fertilizer plots. Ögutcu *et al*. (2008) found significant differences among Rhizobia strains for various parameters such as nodule dry weight and shoot dry weight. Deshwal *et al*. (2003) reported that Rhizobia are known to increase nodulation and nodule dry weight in legumes along with increased in host plant growth and development. This result agreed with Fatnassi *et al*. (2015), who found that *Vicia faba* inoculated with *R. leguminosarum* appeared significant increase in the number and the weight of nodules by 50%. Whereas, co-inoculation between *Rhizobium* and *Pseudomonas* positively influence the growth and seed yield per plant. Dashadi *et al*. (2011) found that co-inoculation of *Rhizobium* and *Azotobacter* increased total nitrogen content, nodulation, seed yield and biological yield under water deficit condition. However, El-Wakeil and El-Sebai (2009) found that the highest number of pods was achieved in treatment of Rhizobia mixed with Mycorrhiza or *Pseudomonas*. Several workers have reported that seed inoculation with *Rhizobium* had significantly increased the growth and yield of legume crops (Pathak *et al*., 2001).

The increase in dry-matter and nitrogen content of co-inoculated plants may be attributed to increase nodulation, higher N_2-fixation and improvement of root development under controlled or field conditions (Dashti *et al*., 1998). PGPR bacteria can increase P availability to plants through solubilizing insoluble phosphate and this may improve biological nitrogen fixation and availability of other nutrients (Gyaneshwar *et al*., 2002).

CONCLUSION

From the aforementioned results, it can be concluded that co-inoculation with the mixture of *R. leguminosarum*, *A. chroococcum* and *P. fluorescens* treatment significantly increased plant resistance than using each microbe alone against *R. solani* and/or *F. oxysporum* that infected faba bean plants. The results also showed the role of PGPR in improvement of plant growth, yield components, accumulation of some antimicrobial substances such as total phenols contents, stimulate chlorophyll synthesis and increasing activities of nitrogenase, dehydrogenase enzymes. Such these treatments can be suggested as a part of integrated disease management for field crops and for achieving better crop yields with reduced usage of chemical fertilization.

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تأثير التلقيح المشترك ببكتريا العقد الجذرية وبعض السلالات البكتريا المشجعة لنمو النبات ضد *F.oxysporum* و *R. solani* اللذان يصيبان الفول البلدى

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فى هذه الدراسة تم تقييم بعض السلالات البكتريا المشجعة لنمو النبات بشكل منفرد او المخلوط بمقارنة باللميد الفطرى توبسين ام-70 وذلك لما تحدثه من نشاط تضادى وتأثير مثبط لفطرى *Rhizoctonia solani* و *Fusarium oxysporum* المسببات المرضية لأمراض اعفان الجذور والذبول فى الفول البلدى. وقد استعملت بعض سلالات البكتريا *Rhizobium leguinosarum* (No.9) , *Azotobacter chroococcu* (No.12) و *pseudomonas fluorescens* (No. 6) وقد اظهرت هذه الدراسة ان معظم عزلات البكتريا المشجعة لنمو النبات لها القدرة على تثبيط النمو الميسليومى لفطريات *R. solani* و *F. oxysporum* تحت ظروف المعمل وبشكل ملحوظ .وبشكل عام تحت ظروف الصوبة والحقل، فإن كل عزلات البكتريا المشجعة لنمو النبات قللت بشكل معنوى شدة الإصابة بأمراض اعفان الجذور والذبول وزادت من نسبة النباتات السليمة المتبقية مقارنة بالكنترول . وقد كان التلقيح المشترك ما بين *R. leguminosarum* و *A. chroococcum* و *P. Fluorescens* الأكثر فاعلية فى مقاومة امراض اعفان الجذور والذبول ، وزيادة مقاييس النمو ، مكونات المحصول ، صبغات البناء الضوئى ، ونشاط كل من النيتروجينيز والديهيدروجينيز والفينولات الكلية. وقد بينت النتائج المتحصل عليها مدى اهمية البكتريا المشجعة لنمو النبات فى مقاومة الفطريات اللمرضة و تحسين مقاييس نمو نباتات الفول البلدى مقارنة بالمبيد الكيميائى وتقليل استخدام التسميد الكيميائى.