Combination of Biocontrol Agents for Controlling Soybean Damping-off Caused by *Rhizoctonia solani*

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Soybean seeds (cv. Giza 22) were treated with *Paenibacillus polymyxa*, *Pseudomonas fluorescens* and Mycorrhizeen compound (contains mainly endospores of *Glomus* sp.) as biocontrol agents either individually or in different combinations to study their effect on the infection with *Rhizoctonia solani* under greenhouse and field conditions. Treatment of Rizolex-T 50% was also applied to compare the output data. Under greenhouse conditions, all the treatments significantly reduced the percentages of pre and post-emergence damping-off and increased survived plants compared to untreated control grown in infested soil by *R. solani*. The highest percentage of survived plants 92% was achieved from the triple combination treatment (Mycorrhizeen plus *P. polymyxa* and *P. fluorescens*) and Rizolex-T treatment as compared to the untreated control, which achieved 48%. The significant increases in plant height, shoot dry weight and root dry weight of soybean plants were recorded with the triple combination treatment followed by the fungicide treatment. Meantime, all treatments significantly enhancing both number of nodules/plant and total dry weight of nodules/plant compared with untreated control grown in infested soil by *R. solani*. The maximum increase was observed with triple combination treatment followed by the dual combination of (Mycorrhizeen plus *P. fluorescens*) then Mycorrhizeen. In addition, the results showed that combined treatments enhanced the percentage of colonization of mycorrhizae in the roots over Mycorrhizeen sole treatment as 18.7% for the triple combination treatment. Under field conditions at two locations, Giza and Etai El-Baroud Agric. Res. Stat. during the summer of 2017 growing season, the triple combination treatment resulted in high values of disease control near to that obtained with Rizolex- T treatment. Such an effect was true for pre and post-emergence damping-off as well as the percentage of survived plants. Meantime, all treatments significantly showed stimulatory effect for crop parameters of soybean plants *i.e.* plant height, number of branches/plant, number of pods/plant, seed weight (g)/plant and seed yield (kg/feddan) compared to the untreated control treatment in the two locations. The activity of oxidative enzymes and phenolic compounds contents were determined in soybean plants treated with

the different bio-agents either individually or in different combinations. All treatments increased the peroxidase (PO), polyphenol oxidase (PPO) and phenolic content in the treated soybean plants over the untreated control plants sown in infested soil with *R. solani*. The maximum increase in the activity of both enzymes and phenolic contents was recorded with the triple combination treatment followed by the two dual treatments of the Mycorrhizeen.

Soybean (*Glycine max* L. Merril) is one of the most important pulse crops in the world. However, soybean is subjected to infection by *Rhizoctonia solani* Kühn which causes seedling blight (pre- and post-emergence damping-off) and root rot (Xue *et al.,* 2007 and Chang *et al.,* 2015). This soil-borne pathogen is difficult to control because of its long persistence in the soil and wide host range (Bradley *et al.,* 2001).

Some chemicals are effective in controlling soil-borne diseases, but they are expensive and not environmentally friendly. Recently, biological control has intensively been studied by many researchers throughout the world. Bio-inoculants containing living cells of microorganisms could be an effective alternative for chemicals in food production (O´Callaghan, 2016). Rhizosphere organisms provide an initial barrier against pathogens attacking the roots (Akhtar and Siddiqui, 2010 b) and microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents. Arbuscular mycorrhizal fungi (AM fungi) associated with the roots of many crop plants can enhance plant growth and nutrient uptake (Rouphael *et al.,* 2015) also reduce the severity of several plant diseases (Tripathi *et al.,* 2017) and induce plant systemic resistance against root pathogens (Pérez-de-Luque *et al.,* 2017). At present, PGPR inoculation constitutes a promising alternative strategy to reduce the use of chemical pesticides and fertilizers (Adesemoye *et al.,* 2009 & O´Callaghan, 2016).

However, some PGPR has a strong stimulatory impact on the growth of AM fungi (Linderman, 1997). It is worthy to mention that plant co-inoculation with either *Pseudomonas fluorescens*, *Bacillus subtilis* or *Paenibacillus polymyxa* can further promote the colonization of plant roots by AM fungi (Arthurson *et al.*, 2011 & Priyadharsini and Muthukumar, 2016). Also, support the mycorrhizal symbiosis as mycorrhization helper bacteria which stimulates root development (Garbaye, 1994 & Choudhary *et al.,* 2017). Likewise, the root AM fungi association constitutes the major factor influencing the community structure and the biomass of soil microorganisms leading to the establishment of the so-called mycorrhizosphere (Priyadharsini *et al.,* 2016).

The majority of strategies for biocontrol of soil-borne plant pathogens rely on a single microbial biocontrol agent for pathogen suppression (Larkin *et al.,* 1998). This strategy must be changed because biocontrol agents applied individually are not likely to perform consistently against all pathogens of the crop or under diverse rhizosphere and soil environmental conditions. The combination between more than

one bio-agent in a single preparation is an approach to overcoming this inconsistent performance. A combination of biocontrol agents is more likely to have a greater variety of traits responsible for the suppression of one or more pathogens and, also, is likely to have these traits expressed over a wide range of environmental conditions. Several works suggest the combination between several biocontrol agents has shown to improve the efficacy and reduce the variability of efficacy (Guetsky *et al.,* 2002; Roberts *et al.,* 2005 and Pertot *et al.,* 2017).

So, the current study aims to investigate the effects of biopriming soybean seeds with Mycorrhizeen compound (mainly *Glomus* sp.) *Paenibacillus polymyxa* and *Pseudomonas fluorescens* either individually or in different combinations to suppress the infection by Rhizoctonia damping-off with regards to promotion of plant growth under greenhouse and field conditions.

M a t e r i a l s   a n d   M e t h o d s

Plant materials:
Soybean seeds (*Glycine max* L. Merrill) cultivar Giza 22 was obtained from the Legume Res. Dept., Field Crops Res. Inst., ARC, Giza, Egypt.

Source of the pathogen and inoculum preparation:
The fungus *Rhizoctonia solani* Kühn isolated from naturally infected soybean plants, showing damping-off symptoms was kindly provided by legume and forage dis. Res. Dept., Plant Path. Res. Inst., ARC, Giza, Egypt. Inoculum of *R. solani* was prepared according to Atwa *et al.*, (2014).

Biotic agents: Two PGPR isolates and the bio- product Mycorrhizeen were used.

A) PGPR isolates:
Both *Paenibacillus polymyxa* (isolate 9D14), and *Pseudomonas fluorescens* were previously isolated and identified using Biolog system according to the recommended procedure of Shehata *et al.* (2006). *P. polymyxa* was activated on fresh slants of nutrient yeast dextrose agar (NYDA) medium and after 24 hrs transferred to flasks contained 50 ml of broth (NYDB) medium (per liter: nutrient broth 8 g, yeast extract 5 g and dextrose 10 g) and *P. fluorescens* was grown in flask contained King's medium B (KMB) broth (per liter: Proteose peptone 20 g, Glycerol 10 mL, K_2HPO_4 1.5 g and $MgSO_4$ 1.5 g). The flasks were placed on a rotary shaker to grow at 120 rpm for 66 hrs. at 24±1°C.

B) Mycorrhizeen:
Mycorrhizeen formulation contains mainly endospores of *Glomus* sp. and its carrier free from Mycorrhizae were kindly obtained from Agric. Microbiol. Dept., Soils, Water, and Environ. Res. Inst., Agric. Res. Cent., Giza, Egypt. The carrier composed of peat moss and vermiculite 1:1 (w/w) and dried at 70°C for 24 h, then milled to pass through 200 μm mesh sieves; pH of the peat moss and vermiculite was neutralized by adding calcium carbonate.

Compatibility among bacterial strains:

The isolates of *P. fluorescens* and *P. polymyxa* were tested for their compatibility among each other following the method of Fukui *et al.* (1994).

Seed and soil treatments:

Apparently, healthy uniformity seeds of soybean (cv. Giza 22) were surface disinfested by immersing in sodium hypochlorite (1%) for 2 min, and washed several 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.

A) Biopriming treatments:

For single treatment, 300 ml of bacterial culture broth was used per one Kg of the carrier material, while for the mixed cultures, 150 mL of each bacterial broth was mixed with the carrier. In the case of bacterial cultures mixed with Mycorrhizeen, 300 mL of the bacterial culture broth in the case of single isolate or 150 mL from each bacterial broth in the case of mixed cultures were mixed with one Kg of Mycorrhizeen formulation. Healthy seeds of soybean were coated with the tested bacterial and Mycorrhizeen formulation either solely or in different combinations, using sucrose saturated solution to cover the seeds as adhesive 15 hrs. before sowing time. The coated seeds were left to air-dry on screen cloth.

B) Fungicide treatment:

Seed dressing was carried out by applying the Rizolex-T 50% WP (Tolclofos methyl+thiram) at the recommended dose (3 g/kg), using 1% methyl cellulose as adhesive 15 hrs. before sowing. The dressed seeds were left to dry on screen cloth.

C) Control:

The disinfected soybean seeds were coated with the peat moss and vermiculite-based formulation and the coated seeds were left to air-dry on screen cloth 15 hrs. before sowing time.

Greenhouse experiments:

The trials were carried out in the greenhouse of Plant Pathol. Res. Inst., Agric. Res. Cent., Giza. Plastic pots (25 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 steamed disinfested sandy clay soil 1:2 (v/v). Soil infestation was achieved by mixing the inoculum of *R. solani* with the soil at the rate of 2% of soil weight. Sterilized un-inoculated ground sorghum grains were added to the disinfested soil at the same rate as control. The infested soil was mixed thoroughly and watered every 2 days for a week before planting to stimulate fungal growth. Five grams of *Bradyrhizobium* sp (*rhizobium* formulation), obtained from Biofert. Prod. Unit, Soils Water and Environ. Res. Inst., Agric. Res. Centre, Giza, Egypt) were added to each pot during sowing. Five seeds of pre-treated soybean seeds were sown in each pot and pots were irrigated directly. Twelve replicated pots were used for each particular treatment. All pots were irrigated when it was necessary and fertigated one month after sowing and every week until the end of the experiment to near field capacity with a 0.1% 15:15:15 (N: P: K) fertilizer solution. The serial number (S.) and treatments are as shown in Table (1).

Table 1. Serial number (S.) and treatments

S. number	Treatment	S. number	Treatment
1	Mycorrhizeen	6	Mycorrhizeen + *P. fluorescens*
2	*P. polymyxa*	7	Mycorrhizeen + *P. polymyxa* + *P. fluorescens*
3	*P. fluorescens*	8	Rizolex-T
4	*P. polymyxa* + *P. fluorescens*	9	Untreated control (*R. solani* infested soil)
5	Mycorrhizeen + *P. polymyxa*	10	Untreated control healthy (non-infested soil)

Twelve plants (four replicates each of three plants) were uprooted 60 days after sowing. Roots were washed using slow running water to remove soil particles and organic debris. Then, the number of nodules per root system was counted after detaching nodules from main and secondary (lateral) roots. Shoots length was measured and cut at the soil line. Shoots, roots and nodules were placed in paper bags and oven-dried at 70°C for 48h, then weighed and the average were recorded. Nitrogen, phosphorus and potassium contents were estimated in the shoots. Where the dry plant samples were ground and prepared for wet digestion using H_2SO_4 and H_2O_2 methods as described by Page *et al.* (1982). The digests were then subjected to the measurement of nutrients *i.e*, nitrogen, phosphorus and potassium (Cottenie *et al.*, 1982).

Disease assessment:

Disease incidence (DI) % was determined by recording pre-emergence and post-emergence damping-off, as well as the percentages of survived plants 15, 30 and 45 days after sowing respectively. Reduction or increasing % over the infected control was also calculated according to the following formula:

$$\text{Reduction or Increasing \%} = \frac{\text{DI of Control - DI of treatment x 100}}{\text{DI of Control}}$$

Mycorrhizae colonization of soybean root:

The percentages of soybean root colonization by Vesicular Mycorrhizal fungus were determined 60 days after sowing. Roots staining technique was applied according to Koske and Gemma (1989). The root system was washed with tap water several times to remove the adhering soil particles. Roots were cut into small segments and cleared in potassium hydroxide (KOH) 5% (w:v) at 90°C for 30 min. After treatment with KOH, the roots were then rinsed in several changes of water and acidified by immersing in HCl (1%). The volume of acid equal to ca 25x the volume of the roots was used in an overnight soak. Acidified roots were stained in an acidic glycerol solution (500 ml glycerol, 450 ml H_2O, 50 ml 1% HCl) containing 0.05 % trypan blue for 20 min in a 90°C water bath. The roots detained in acidic glycerol at room temperature, in roots whose cells retain excessive stain. Root colonization levels were estimated on 5 groups of 10 root segments (1 cm long) randomly chosen and examined under the light microscope for the presence of

fungal structures. Root colonization percentage was calculated according to the following formula:

Root colonization% = No. of colonized root fragments / No. of total root fragments × 100.

Field experiments:

Field experiments were carried out during the summer growing season of 2017 at two locations, Giza Agric. Res. Stat., Giza governorate and Etai El-Baroud Agric. Res. Stat., El- Behira Governorate in fields known to have *Rhizoctonia* root rot history, in order to investigate the effect of biotic and chemical treatments on controlling damping-off diseases. The disinfected soybean seeds were treated in the same manner of the greenhouse experiment. The disinfected soybean seeds were sown in the field on May 22 & 23, 2017 for both locations respectively. The field trial was designed in a complete randomized block with four replicates. The area of each plot was 10.5 m^2 consisted of five rows; each row was 3.5 m length and 0.6 m width. The seeds were sown in hills at 20 cm apart on both sides of the row ridge, with one seed per hill. Eight hundred grams of Bradyrhizobium formulation (rhizobium) were mixed with approximately 50 kg of moistened fine sandy soil and added to field soil (feddan). All agricultural practices *i.e.*, irrigation, fertilization, hoeing weed and pests control were carried out as the recommendation of min. of Agric. and land. Recl. for soybean.

The treatments were implemented as mentioned in Table 1 and disease incidence (DI) % was determined as mentioned before. Random samples of ten soybean plants were collected (from the inner rows) at the harvest stage from each plot. Crop parameters *i.e.*, plant height, number of branches, number of pods per plant, the weight of one hundred seeds and seeds yield per plant were recorded as well as seeds yield (Kg)/feddan were estimated.

Effect of biopriming soybean seed with biotic treatment on the activity of oxidative enzymes and phenol content:

An experiment was carried out to determine the activity of oxidative enzymes and phenolic compounds. Soybean plants were grown as mentioned before in the greenhouse experiment under biocontrol treatments and untreated control in infested soil as well as untreated control for non-infested soil (Table 1). Fifteen days after sowing, the activity of peroxidase (PO), polyphenol oxidase (PPO) and phenolic contents were determined in tissue extracts of soybean plants.

Assay of enzymes activity:
A) Assay of peroxidase (PO):
Extraction and assay of peroxidase (PO) activity were carried out according to Chakraborty & Chatterjee (2007).

B) Assay of polyphenol oxidase (PPO):
Extraction and assay of polyphenol oxidase enzyme (PPO) were carried out according to Sadasivam & Manickam (1996).

Determination of phenolic compounds:

The extraction of phenolic compounds was carried out according to Sutha *et al* (1998). Phenolic compounds were determined using methods of analysis described by Snell & Snell (1953).

Statistical analysis:

Completely randomized design (CRD) and randomized blocks design (RBD) were implemented in the greenhouse experiment and field experiment, respectively. The obtained data were subjected to computer statistical software (ASSISTAT) originated by Silva & 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 ≤ 0.05.

R e s u l t s

1-Compatibility among bacterial strains:

Bacterial isolates of *P. polymyxa* and *P. fluorescens* were tested for their compatibility *in vitro*. None of the antagonistic bacteria inhibited each other, suggesting that these bacterial antagonists were compatible with each other.

2-Greenhouse experiment:

1-Effect of the bioagents and Rizolex-T on the incidence of Rhizoctonia damping-off:

Results in Table 2 show that all the treatments significantly reduced the percentages of pre- and post-emergence damping-off with significant increasing of the survived plants compared to control grown in infested soil by *R. solani*. The triple combination treatment (Mycorrhizeen plus *P. polymyxa* and *P. fluorescens*) and Rizolex-T treatment have highly reduced the pathogenesis of *R. solani* on germinated soybean seeds pre-emergence as they occurred 4% dead seeds as control healthy grown in non-infested soil. Such curative effect extended to post-emergence data and the results of survived plants after 45 days of cultivation. However, all of the biocontrol treatments were less insignificant efficiency compared with Rizolex-T and triple combination treatment. It is obvious that mixed compatible bioagents were highly effective than single bio-treatments, but they resulted in the same effect at post-emergence.

Table 2. Effect of the tested bio-agents and Rizolex-T as seed treatments on the percentage of damping-off disease of soybean plants grown in artificially infested soil by *R. solani* under greenhouse conditions

Treatment	% Damping-off				% Survived plants	% Increasing
	Pre-emergence		Post- emergence			
	% Incidence	% Reduction	% Incidence	% Reduction		
Mycorrhizeen	12.0 b	70.0	4.0 bc	66.7	84.0 ab	75.0
P. polymyxa	12.0 b	70.0	4.0 bc	66.7	84.0 ab	75.0
P. fluorescens	12.0 b	70.0	8.0 b	33.3	80.0 b	66.6
P. polymyxa + *P. fluorescens*	8.0 b	80.0	4.0 bc	66.7	88.0 ab	83.3
Mycorrhizeen +*P. polymyxa*	8.0 b	80.0	4.0 bc	66.7	88.0 ab	83.3
Mycorrhizeen +*P.fluorescens*	8.0 b	80.0	4.0 bc	66.7	88.0 ab	83.3
Mycorrhizeen +*P. polymyxa* + *P. fluorescens*	4.0 b	90.0	4.0 bc	66.7	92.0 ab	91.7
Rizolex–T	4.0 b	90.0	4.0 bc	66.7	92.0 ab	91.7
Untreated control (infested soil)	40.0 a	0.0	12.0 a	0.0	48.0 c	0.0
Untreated control (non-infested soil)	4.0 b	-	0.0 c	-	96.0 a	-

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

II- Effect of the tested bioagents and Rizolex-T on some crop parameters of soybean plants grown in artificially infested soil by R. solani under greenhouse conditions

Results in Table 3 indicate that all treatments caused significant increases in crop parameters of soybean plants grown in infested soil with *R. solani*. For plant height, there were significant differences between all treatments and control. Meanwhile there were no significant differences among sole treatment Mycorrhizeen, *P. polymyxa* and dual treatment (Mycorrhizeen plus *P. polymyxa*), or (Mycorrhizeen plus *P. fluorescens*) treatments. The maximum height was observed for triple combination treatment (Mycorrhizeen plus *P. polymyxa* and *P. fluorescens*) followed by Rizolex-T treatment as 63.3 and 60.4 cm respectively, however, *P. fluorescens* as an individual treatment showed the lowest increase in plant height compared with other treatments. As for shoot dry weight, the significant maximum value was observed with triple combination treatment, healthy untreated plants, Mycorrhizeen, and dual treatment of Mycorrhizeen plus *P. fluorescens*, respectively. Meantime, the other treatments have resulted in gained values near the Rizolex-T treatment. However, for root dry weight the triple combination treatment showed the highest value. Similar effect of bioagents was obtained for both the number of nodules/plant or total dry weight (mg) of nodules/plant. Nodulation was varied among treatments, with significant increase over infested control, the maximum

increase was observed with triple combination treatment followed by the dual combination (Mycorrhizeen plus *P. fluorescens*) and Mycorrhizeen, respectively. The levels of N, P and K contents were increased in all treatments compared with infected control, and the maximum increase observed with triple combination treatment.

III- Effect of the bacterial bioagents on mycorrhizae colonization of roots of soybean plants grown in artificially infested soil by Rhizoctonia solani under greenhouse conditions:

The results showed that combined treatments enhanced the percentage of colonization of mycorrhizae in the roots over Mycorrhizeen sole treatment as 18.7, 13.8 and 8.8% for the triple combination treatment (Mycorrhizeen plus *P. polymyxa* and *P. fluorescens*), the dual combination treatments (Mycorrhizeen plus *P. fluorescens*) and (Mycorrhizeen plus *P. polymyxa*), respectively.

Table 3. Effect of the tested bio-agents and Rizolex-T as seed treatments on some crop parameters and nodulation as well as nutrient contents of soybean plants grown in artificially infested soil with *R. solani* under greenhouse conditions

Treatment	Plant height (cm)	Shoot dry weight (g/plant)	Root dry weight (g/plant)	No. of nodules/ plant	Nodules dry weight mg/plant	NPK contents		
						N mg/g	P mg/g	K mg/g
Mycorrhizeen	59.9 cd	4.10 abc	1.41 b	145.3 b	415.1 b	3.58	0.301	2.26
P. polymyxa	58.5 cd	3.80 cde	1.30 cd	131.7 c	394.9 bc	3.49	0.277	1.79
P. fluorescens	55.2 e	3.67de	1.27 d	130.3 c	381.4 bc	3.53	0.285	1.87
P. polymyxa+ P. fluorescens	57.8 d	3.90 de	1.30 cd	128.0 c	390.7 bc	3.62	0.291	1.89
Mycorrhizeen+ *P. polymyxa*	58.1 cd	3.65 e	1.31 cd	134.7 c	384.5bc	3.62	0.318	2.20
Mycorrhizeen +*P. fluorescens*	59.3 cd	4.10 abc	1.39 bc	149.0 b	420.1 b	3.76	0.316	2.40
Mycorrhizeen+ *P. polymyxa+ P. fluorescens*	62.3 b	4.40 a	1.76 a	175.3 a	460.1 a	3.90	0.332	2.66
Rizolex –T	60.4 bc	3.90 cde	1.40 b	128.5 d	340.7 c	3.45	0.284	2.17
Untreated control (infested soil)	35.3 f	2.20 f	0.76 e	32.7 e	82.2 d	2.80	0.237	1.58
Untreated control (non-infested soil)	65.2 a	4.20 ab	1.35 bc	181.0 a	471.9 a	3.54	0.286	2.28

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

3-Field experiments
I- Effect of the bioagents and Rizolex-T on the incidence of soybean damping-off disease

Results in Table 4 indicate that all treatments significantly decreased the percentage of pre and post-emergence damping-off as well as survived plants compared with untreated control in the two locations. However, the triple combination treatment (Mycorrhizeen plus *P. polymyxa*, and *P. fluorescens*) resulted in high values of disease control near to that obtained with Rizolex-T treatment. Such an effect was true for pre and post-emergence as well as the percentage of survived plants. It is obvious that mixed compatible bioagents were highly effective for reducing pre-emergence damping-off than single bio-treatments, but they resulted in the same effect at post-emergence. It is evidently observed that values of post-emergence damping-off were clearly lower than those of pre-emergence damping-off in all treatments such effect was true in the untreated control treatment.

II- Effect of the tested bioagents and Rizolex-T on some crop parameters and yield of soybean plants

Results in Table 5 exhibited that under field conditions all treatments significantly showed stimulatory effect for crop parameters of soybean plants and yield compared to the untreated control treatment in the two locations when treated separately or in combination.

Plant height:

Among all treatments, the triple combination treatment (Mycorrhizeen plus *P. polymyxa*, and *P. fluorescens*) as well as Rizolex-T treatment showed significantly the maximal effect at Giza and Etai-El-Baroud Rese. Stat. It is obvious that there was no significant difference among all the dual combination treatments in two locations.

The number of branches:

At two locations, all treatments significantly increased the number of branches as compared with the untreated control, and the maximum increase was recorded with the triple combination treatment. Also, there were no significant differences between the dual combination treatments and Rizolex-T treatment.

Number of pods per plant:

The number of pods per plant was significantly increased in all treatments compared with untreated control. The maximum increase was recorded with the triple combination treatment, Rizolex-T treatment and the dual combination treatment (Mycorrhizeen plus *P. fluorescens*) respectively with no significant difference among them at two locations.

Seed weight per plant:

In the two locations, all treatments significantly increased seed weight per plants as compared with untreated control. The maximum figures were recorded with the triple combination treatment as well as Rizolex-T treatment. There is no significant difference among all the dual combination treatments at two locations.

Table 4. Effect of the tested bio-agents and Rizolex-T as seed treatments on the percentage of soybean damping-off under field conditions (natural infection) at Giza Agric. Res. Stat. (A) and Etai El-Baroud Agric. Res. Stat. (B) during the summer growing season 2017

Treatment	% Damping-off				% Survived Plants	% Increasing
	Pre-emergence		Post- emergence			
	% Incidence	% Reduction	% Incidence	% Reduction		
(A): Giza Res. Stat.						
Mycorrhizeen	10.8 de	66.9	2.3 b	60.3	86.9 c	41.4
P. polymyxa	14.5 bc	55.7	2.3 b	60.3	83.2 de	35.3
P. fluorescens	16.1 b	50.8	2.3 b	60.3	81.6 e	32.7
P. polymyxa + *P. fluorescens*	13.5 bcd	58.7	1.9 b	67.2	84.6 cd	37.6
Mycorrhizeen + *P. polymyxa*	11.5 cde	64.8	3.3 b	43.1	85.2 cd	38.5
Mycorrhizeen + *P. fluorescens*	10.3 de	68.5	3.5 b	39.7	86.2 bc	40.2
Mycorrhizeen + *P. polymyxa* + *P. fluorescens*	6.1 f	81.3	2.5 b	57.0	91.4 ab	48.6
Rizolex–T	5.8 f	82.3	2.3 b	60.3	91.9 a	49.4
Untreated control	32.7 a	0.0	5.8 a	0.0	61.5 f	0.0
(B): Etai Elbaroud Res. Stat.						
Mycorrhizeen	9.5 d	72.1	2.9 b	53.2	87.6 bc	46.7
P. polymyxa	15.3 b	55.1	1.7 b	72.6	83.0 d	39.0
P. fluorescens	15.6 b	54.3	1.5 b	75.8	82.9 d	38.9
P. polymyxa + *P. fluorescens*	11.8 c	65.4	2.5 b	59.7	85.7 cd	43.6
Mycorrhizeen + *P. polymyxa*	11.7 c	65.7	1.5 b	75.8	86.8 bc	45.4
Mycorrhizeen + *P. fluorescens*	10.3 cd	69.8	2.5 b	59.7	87.2 bc	46.0
Mycorrhizeen +*P. polymyxa* + *P. fluorescens*	5.5 e	83.9	2.8 b	54.8	91.7 ab	53.6
Rizolex–T	5.9 e	82.7	2.1 b	66.1	92.0 a	54.1
Untreated control	34.1 a	0.0	6.2 a	0.0	59.7 e	0.0

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

The weight of one hundred seed:

In the two locations, all treatments significantly increased the weight of one hundred seed as compared with untreated control. The maximum increase was recorded with the triple combination and Rizolex-T treatments. Also, there is no significant difference among all the dual combination treatments at two locations. However, the lowest figure was observed with *P. fluorescens*.

Seed yield:

At Giza Res. Stat., the maximum increase in seed yield was recorded with the triple combination followed by Rizolex-T and the dual combination (Mycorrhizeen plus *P. fluorescens*) treatments, respectively. While in the Etai-El-Baroud Res. Stat. the maximum increase was noticed with Rizolex-T treatment followed by the triple combination treatment.

Table 5. Effect of the tested bio-agents and Rizolex-T as seed treatments on some cropparameters of soybean plants under field conditions (natural infection) at Giza Agric. Res. Stat. (A) and Etai El-Baroud Agric. Res. Stat. (B) during the summer growing season 2017

Treatment	Plant height (cm)	No. of branches/ plant	No. of pods/ plant	Seed weight/ plant(g)	100 seed weight (g)	Seed yield (kg/fed)
(A): Giza Res. Stat.						
Mycorrhizeen	90.0 bc	3.7 bc	74.3 abcd	30.1 bc	18.2 bc	1510 bc
P. polymyxa	87.0 cd	3.3 c	71.8 cd	28.7 bc	18.2 bc	1430 d
P. fluorescens	85.7 d	3.4 c	67.8 d	27.9 c	17.6 c	1272 e
P. polymyxa + *P. fluorescens*	88.7 bcd	4.0 ab	77.4 abc	29.9 bc	18.3 bc	1408 cd
Mycorrhizeen +*P. polymyxa*	90.3 bc	3.9 b	72.5 bcd	30.5 b	18.3 bc	1384 de
Mycorrhizeen +*P. fluorescens*	91.7 b	4.1 ab	77.8 abc	30.8 b	18.6 b	1582 ab
Mycorrhizeen +*P. polymyxa* + *P. fluorescens*	97.9 a	4.5 a	81.0 a	34.0 a	19.1 a	1643 a
Rizolex –T	95.9 a	4.0 ab	79.8 ab	33.2 a	18.7 ab	1630 a
Untreated control	69.4 e	2.8 d	38.0 e	23.7 d	15.6 d	968 f
(B): Etai Elbaroud Res. Stat.						
Mycorrhizeen	94.8 ab	3.8 bc	73.0 bc	30.9 b	18.4 ab	1483 c
P. polymyxa	89.7 bc	3.5 cd	65.8 de	28.7 cd	17.8 bc	1342 de
P. fluorescens	86.8 c	3.4 d	63.0 e	27.5 d	17.4 cd	1281 e
P. polymyxa + *P. fluorescens*	92.5 bc	3.9 bc	68.2 cde	30.3 bc	17.7 bc	1395 d
Mycorrhizeen +*P. polymyxa*	94.1 b	4.2 ab	69.0 cd	30.8 bc	18.2 abc	1361 de
Mycorrhizeen +*P. fluorescens*	91.4 bc	4.2 ab	74.6 ab	32.0 b	18.2 abc	1517 bc
Mycorrhizeen +*P. polymyxa* + *P. fluorescens*	98.7 a	4.3 a	78.5 a	34.7 a	18.6 a	1603 ab
Rizolex–T	96.9 a	4.0 ab	76.5 ab	34.1 a	18.4 ab	1625 a
Untreated control	67.5 d	2.8 e	36.3 f	23.9 e	14.5 e	1005 f

Means in each column followed by the same letters are not significantly different using Duncan's multiple range test, (p = 0.05)

From the results, it could be noticed that no significant differences between the triple combination treatment and Rizolex-T treatment in all growth parameters under study at two locations. However, there were no significant differences between the two dual combination treatments (*P. polymyxa* plus *P. fluorescens*), and (Mycorrhizeen plus *P. polymyxa*) in all crop parameters under study at two locations. Meanwhile, no significant differences were found between the two sole treatments with *P. polymyxa* or *P. fluorescens* in all crop parameters under study at two locations with exception of seed yield (kg/feddan) at Giza Res. Stat.

4- Effect of the tested bioagents as seed treatments on the activity of oxidative enzymes and content of phenolic compounds:
a. Activity of oxidative enzymes:

Data in Table 6 showed increases of peroxidase (PO) and polyphenol oxidase (PPO) in treated soybean plants with different bioagent treatments over the untreated control plants sown in infested soil with *R. solani*. The maximum increase was recorded with the triple combination treatment and this was true for both enzymes. However, the lowest increase was observed with *P. fluorescens* as a sole treatment for the two enzymes.

Table 6. Effect of the tested bio-agents as seed treatments on the activity of peroxidase and polyphenol oxidase in soybean plants grown in artificially infested soil by *R. solani* under greenhouse condition

Treatment	Peroxidase activity (absorbance at 430 nm) (Enzyme unit /mg protein/min)		Polyphenol oxidase activity (absorbance at 495 nm) (Enzyme unit /mg protein/min)	
	Activity	% increasing over control	Activity	% increasing over control
Mycorrhizeen	2.052	21.3	0.215	58.1
P. polymyxa	2.131	25.9	0.221	62.5
P. fluorescens	1.997	18.0	0.193	41.9
P. polymyxa + *P. fluorescens*	2.216	30.9	0.232	70.6
Mycorrhizeen +*P. polymyxa*	2.672	57.9	0.245	80.1
Mycorrhizeen +*P. fluorescens*	2.531	49.6	0.253	86.0
Mycorrhizeen +*P. polymyxa* + *P. fluorescens*	2.736	61.7	0.321	136.0
Untreated control (infested soil)	1.692	0.0	0.136	0.0
Untreated control (non-infested soil)	1.256		0.129	

b. Phenol content:

Data in Table 7 indicated that all treatments enhanced the total phenol content in soybean plants compared with untreated control. The maximum increase was recorded with the triple combination treatment followed by the two dual treatments of the Mycorrhizeen. However, the lowest increase was noted with *P. fluorescens* and *P. polymyxa* as a sole treatment. The same results were observed for the contents of free phenol. Meanwhile, the fewer figure for total phenol, free phenol, and conjugated phenol was observed with *P. fluorescens* treatments.

Table 7. Effect of the tested bio-control agents as seed treatments on the levels of phenolic compounds in soybean plants grown in artificially infested soil by *R. solani* under greenhouse condition

Treatment	Phenolic contents (catechol equivalents mg/g fresh weight)					
	Total phenols	% Increase over control	Free phenols	% Increase over control	Conjugated phenols	% Increase over control
Mycorrhizeen	3.325	48.3	1.693	16.5	1.632	105.8
P. polymyxa	3.123	39.3	1.532	5.4	1.591	101.6
P. fluorescens	2.857	27.4	1.493	2.7	1.364	72.9
P. polymyxa + *P. fluorescens*	3.456	54.1	1.792	23.3	1.664	110.9
Mycorrhizeen +*P. polymyxa*	3.543	58.0	1.893	30.2	1.65	109.1
Mycorrhizeen +*P. fluorescens*	3.573	59.4	1.935	33.2	1.638	107.6
Mycorrhizeen +*P. polymyxa* + *P. fluorescens*	3.828	70.7	2.135	46.9	1.693	114.6
Untreated control (infested soil)	2.242	0.0	1.453	0.0	0.789	0.0
Untreated control (non-infested soil)	1.523		1.259		0.323	

D i s c u s s i o n

The wide host range exhibited by *R. solani* complicates management schemas because there are no commercial soybean cultivars resistant to this pathogen (Bradley *et al.,* 2001). However, the Intensive use of agrochemicals has been a subject of public concern because of harmful effects on the natural resources such as water and potential carcinogenicity of some chemicals (Carvalho, 2017). So, recent research priorities preferred disease control programs that are safe and compatible with sustainable agriculture. The present study demonstrates that not only the seed treatment with Rizolex-T decreased the disease incidence significantly but also treated seeds with *P. polymyxa*, *P. fluorescens* and Mycorrhizeen formulation (mainly *Glomus* sp.) in combination as dual or triple inoculation decreased significantly the severity of damping-off disease of soybean plants under greenhouse and field conditions. In this respect, *P. fluorescens* isolates which non-pathogenic saprophytes that colonize soil and plant surface environments are effective for the management of soil-borne disease (Bakker *et al.,* 2007 & Susilowati *et al.,* 2011).

This action believed to be related to: (1) competition for nutrients (Sharma, 2006); (2) produce a wide variety of antimicrobial metabolites such as the antibiotic 2,4-diacetyl phloroglucinol and pyrrolnitrin (Zhang *et al*., 2016); cyclic lipopeptides (Michelsen *et al*., 2015); (3) iron-chelating siderophores (Gupta and Gopal, 2008); (4) hydrolytic enzymes from bacteria such as chitinase, *β*-1,3-glucanase, and protease (Solanki *et al*., 2014) and (5) its induction of systemic resistance (Bakker *et al*., 2007).

However, *P. polymyxa* is known for its ability to produce antimicrobial compounds that suppress plant pathogens in the soil such as fusaricidins (Beatty & Jensen, 2002); polymyxins and lantibiotics (He *et al.,* 2007). Meanwhile, hydroxamate-type siderophores have been detected in cultures of *P. polymyxa* SQR-21, in the late log phase (Raza and Shen, 2010). Also, *P. polymyxa* can help to control phytopathogens by triggering a hypersensitive defensive response of the plant, known as induced systemic resistance (Atwa *et al.,* 2014).

Additionally, biocontrol effects of AM fungi on root pathogens have been previously reported (Borowicz, 2001), the AMF play an important role in the management of plant pathogens, such as *Rhizoctonia solani* (Yao *et al.,* 2002) and *Macrophomina phaseolina* (Akhtar and Siddiqui, 2010 a). This action may be due to several mechanisms, for example, competition for space and nutrients with soil-borne pathogens within the mycorrhizosphere and the host roots. (Azcón-Aguilar and Barea, 1996); activation of plant defense mechanisms against soil-borne pathogens (Bagyaraj, 2006) by modification of cell wall, production of antimicrobial compounds and altered rhizosphere microflora (Sampangi and Bagyaraj 1989; Azcón-Aguilar and Barea, 1996). The antagonistic interaction between AMF with various soilborne plant pathogens is the reason for their use as biocontrol agents. However, a synergistic or additive control of pathogen impact on plant growth by dual inoculation of AM fungi with rhizobacteria showing biocontrol properties (Barea *et al.,* 2005).

A relational trend for the effect of bioagents on suppression of soybean damping off, that was the increase in activities of peroxidase (PO) and polyphenol oxidase (PPO) enzymes in samples of treated plants. Here again, combining bio-treatments resulted in the highest enzymatic activity which was coincided with suppression of *R. solani* pathogenesis to soybean seedlings in soil. Wang *et al.,* (2000) stated that the increase in oxidases activity in plants was related to increased plant resistance against infection by many diseases. Among the proteins induced during plant defense and playing a key role in several metabolic responses, plant peroxidases are well known. It is widely known that peroxidases play a central role in host plant defences against necrotrophic or biotrophic pathogens (Van Loon *et al.,* 2006). Moreover, peroxidases are involved in phytoalexin synthesis, and the production of reactive oxygen species (ROS) that have antifungal properties (Almagro *et al.,* 2009).

Meantime, polyphenol oxidases or tyrosinases (PPO) are able to oxidize the ortho-diphenolic compounds to o-quinones (antimicrobial compounds). Quinones are effective inhibitors of SH group of enzymes which may inhibit the pathogens (Goodman *et al.,* 1967). Also, several studies report a positive correlation between

PPO expression and resistance/tolerance to biotic stresses (Taranto *et al.,* 2017). In fact, Polyphenol oxidases may act through: (1) direct toxicity of quinones; (2) reduced bioavailability and alkylation of cellular proteins to the pathogen; (3) cross-linking of quinones with protein or other phenolic compounds to form physical barriers; and (4) the production of reactive oxygen species (ROS) (Yoruk and Marshall, 2003).

Accordingly, the total phenolic compounds content of soybean treated plants with the tested bioagents and sown in infested soil with *R. solani* was remarkably increased over untreated control plants. Such data are related to the recorded increase in oxidases in the same treatments. The highest increase in phenolic compounds was recorded with the triple combined bioagents. In this regard, the role of phenolic compounds in disease resistance was postulated by Nicholson and Hammerschmidt (1992). They indicated that phenols are oxidized to quinones or semi-quinones which are more toxic and play a great role as antimicrobial substances on the fungal pathogens (Farkas and Kiraly, 1962).

As for the impact of the tested bioagents on the nodulation of soybean plants compared with untreated control plants grown in *R. solani* infested soil, different bioagent treatments resulted in a significant increase in nodules number and nodules dry weight when co-inoculated with *B. japonicum*. Bai *et al.* (2003) stated that co-inoculation of *B. japonicum* with *Bacillus* strains resulted in the greatest increase in the number of nodules, nodules weight, shoot and root weight and grain yield in soybean plants. Silva *et al.* (2007) found nodulation stimulus in *Vigna unguiculata* co-inoculated with *Bradyrhizobium* sp. and *P. polymyxa*. Nishijima *et al.* (1988) reported that *P. fluorescens* can increase the nodulation ability of *B. japonicum*, preincubation of *B. japonicum* and *P. fluorescens* before inoculation further enhanced the level of nodulation of soybean. Sayeed and Siddiqui (2008) reported that AM fungus can coexist along with root nodule bacterium without exhibiting adverse effects on each other, and it could be utilized as a biocontrol agent to control most of the soil-borne disease. Generally, legumes such as soybean can form symbiotic associations with both rhizobia and AM fungi (Lisette *et al.,* 2003). The encouragement of shoots and roots dry weight caused by the bioagents treatments have come back on seeds yield per feddan in the field, so a clear increase was obtained with all bioagents and Rizolex-T over the untreated control. In this respect, the possible explanations for growth promotion by *P. polymyxa* that it produces many plant growth stimulators, including auxin as indole 3-acetic acid (da Mota *et al.,* 2008); cytokinin and increased P-uptake (Timmusk *et al.,* 1999). Also, *Paenibacillus* may be capable of stimulating mycorrhizal colonization as in sorghum (Budi *et al.,* 1999). On the other hand, the increase in P content in plants co-inoculated with *P. fluorescens* and *G. mosseae* was achieved (Gamalero *et al.,* 2004). Previously, phosphate solubilizing *Pseudomonas* sp. was observed from the rhizoplane of rice (Islam *et al.,* 2007). Bapiri *et al.* (2012) notified zinc solubilization in *P. aeruginosa* and *P. fluorescens.* Meanwhile, the most frequently documented response to AM colonization is an increase in phosphorus nutrition of the host plant, which would consequently be more vigorous and more resistant to pathogen invasion (St-Arnaud and Elsen, 2005 and Toussaint *et al.,* 2008).

There was an increase in NPK contents due to the treatments over the untreated control in the greenhouse experiment. High levels of N and K were found in single AM fungi inoculated plants, and this is in line with previous studies (Toussaint *et al.,* 2004 and Mortimer *et al.,* 2009) by enhancing biological N fixation. Also, AM symbiosis resulted in more vigorous growth of plants and plants may be more resistant or tolerant to pathogen attacks (Linderman, 1994). Also, Jäderlund *et al.* (2008) reported that dual inoculation of *Paenibacillus* with AM fungi stimulated growth and exerted strong stimulatory effects on AM colonization and promoted mycorrhizal root colonization. The AM symbiosis enhances the feeding of mineral nutrients to the plant, particularly the ionic forms nutrient that has poor mobility or those existing in low concentrations in the soil solution. This mainly applies to phosphate, ammonium, zinc, and copper. The AM association also upgrades plant soundness through increased defense against soil-borne microbial pathogens in the bioremediation of polluted soils (Barea *et al.,* 2005). Based on the fact that each biocontrol agent may have a different mechanism of action to fight the pathogen, so the use of a combination of biocontrol agents expects to accomplish better results. The combined use of *Bacillus pumilus, Pseudomonas alcaligenes* and *Glomus intraradices* increases the availability of N and P which may have an adverse effect on pathogens, improves root growth than individual application (Akhtar and Siddiqui, 2008). So, Dwivedi *et al.* (2009) found that antifungal compounds like phenazine and di-acetyl-phloroglucinol (DAPG) produced by *P. fluorescens* has a positive influence on mycorrhizal colonization as well as soybean growth parameters. Xavier and Germida (2003) reported that PGPR are known to enhance AM fungal growth and root colonization. Also, high NPK concentrations were observed when inoculated plants with PGPR, Mycorrhizae which allow for increased shoot growth as well as greater above-ground biomass (Singh *et al.,* 2012). Moreover, Souchie *et al.* (2003) reported the dual application of AMF and PGPR inhibited the pathogen more efficiently and reduced infections.

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الجمع بين عوامل المكافحة الحيوية لمكافحة مرض
موت البادرات في فول الصويا المتسبب عن فطر
Rhizoctonia solani

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يهدف هذا البحث إلى تأثير معاملة فول الصويا صنف جيزة ٢٢ بعوامل المكافحة الحيوية *Paenibacillus polymyxa* ، *Pseudomonas fluorescens* والمركب الحيوي ميكوريزين (يحتوي بشكل رئيسي علي الجنس *Glomus*) إما بشكل فردي أو في توليفات مختلفة ضد الإصابة بفطر *Rhizoctonia solani* تحت ظروف الصوبة والحقل. كما تم استخدام المبيد الفطري رايزولكس – تي (٥٠%) للمقارنة مع النتائج المتحصل عليها. وقد أدت جميع المعاملات تحت ظروف الصوبة إلي اختزال معنوي في نسبة موت البادرات قبل وبعد الظهور فوق سطح التربة مقارنة بالبذور غير المعاملة المنزرعة في التربة المعداة بالفطر، وقد تحققت أعلى نسبة للنباتات الباقية علي قيد الحياة (٩٢%) من المعاملة الثلاثية لعوامل المكافحة الحيوية (مركب الميكوريزين مضاف اليه *P. polymyxa* و *P. fluorescens*) وكذلك المعاملة بالمبيد رايزولكس – تي (بمعدل ٣ جم /كجم تقاوي) مقارنة بالنباتات الناتجة من البذور غير المعاملة (٤٨%). وقد سُجلت زيادة معنوية في أطوال النباتات والوزن الجاف لكل من المجموع الخضري والمجموع الجذري لنباتات فول الصويا فى حالة المعاملة الثلاثية ويليها المعاملة بالمبيد. كما أدت كل المعاملات إلى زيادة معنوية في عدد العقد الجذرية / نبات وكذلك الوزن الجاف للعقد الجذرية علي النبات مقارنة بالنباتات الناتجة من البذور غير المعاملة والنامية في التربة المعداة بالفطر *R. solani*، وقد لوحظت أعلي قيم للزيادة بالنباتات فى حالة المعاملة الثلاثية يليها المعاملة المزدوجة (مركب الميكوريزين مضاف اليه *P. fluorescens*) ثم المعاملة بمركب الميكوريزين على التوالي. من ناحية أخرى، أظهرت النتائج أن التوليفات المختلفة لعوامل المكافحة الحيوية مع مركب الميكوريزين أدت لزيادة النسبة المئوية لإستعمار الميكوريزا في الجذور عن المعاملة الفردية للميكوريزين والتى زادت بنسبة ١٨٫٧ % فى حالة المعاملة الثلاثية. تحت ظروف الحقل في محطة البحوث الزراعية بالجيزة ومحطة البحوث الزراعية بايتاي البارود خلال موسم صيف ٢٠١٧، أدت المعاملة الثلاثية إلى ارتفاع قيم المكافحة للأمراض بالقرب من تلك القيم التي تم الحصول عليها باستخدام معاملة المبيد ريزوليكس- تي. كان هذا التأثير واضحا في اختزال نسبة الموت للبادرات قبل وبعد الظهور فوق سطح التربة وكذلك زيادة النسبة المئوية للنباتات الباقية على قيد الحياة. لقد أظهرت جميع المعاملات بعوامل المكافحة الحيوية تأثيرًا تحفيزيًا بشكل معنوى لمقاييس النمو المدروسة لنباتات فول الصويا مثل طول الساق، عدد الأفرع/نبات، عدد القرون/نبات ووزن البذور/نبات وكذلك المحصول (كجم/فدان) بالمقارنة بالنباتات الناتجة من البذور غير المعاملة في كلا الموقعين. تم تقدير نشاط الإنزيمات المؤكسدة المختزلة ومحتوى الفينولات في نباتات فول الصويا المعاملة بعوامل المكافحة الحيوية المختلفة سواء فردية أو في توليفات مختلفة. أدت كل المعاملات إلى زيادة فى نشاط إنزيمى البيروكسيديز، البولي فينول اكسيديز ومحتوى الفينولات في النباتات المعاملة بعوامل المكافحة الحيوية المختلفة مقارنة بالنباتات الناتجة من البذور غير المعاملة والنامية في التربة المعداة بالفطر *R. solani* وقد سجلت أعلى زيادة في حالة المعاملة الثلاثية وذلك بالنسبة لكلا الانزيمين. على الجانب الآخر سجلت الزيادة القصوى في المحتوى الكلي للفينولات في المعاملة الثلاثية يليها المعاملات المزدوجة مع مركب الميكوريزين.