Middle East Journal of Applied Sciences **Volume : 10 | Issue :04 |Oct.-Dec.| 2020**
EISSN: 2706 -7947 ISSN: 2077- 4613 **Pages: 638-648**
DOI: 10.36632/mejas/2020.10.4.54

Integration between Antagonistic Fungi and Bacteria for Controlling of Peanut Pod Rot Incidence and Occurrence of Aflatoxigenic fungi

Zeinab N. Hussien[1] and Ahmed M. Gomaa[2]

[1]Plant Pathol. Res. Inst., Agric. Res. Center (ARC), Giza., Egypt
[2]Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Foods, Ministry of Agriculture and Land Reclamation, Giza, Egypt.

Received: 15 August 2020 / Accepted 20 Oct. 2020 / Publication date: 25 Oct. 2020

ABSTRACT
Peanuts are infected with many diseases affecting the productivity, especially pod rot diseases caused by many fungi as well as aflatoxigenic fungi (*Aspergillus flavus* and *Aspergillus parasiticus*) which conceder a major health and food safety problem in the worldwide. In this study, *Trichoderma harzianum*, *Trichoderma viride*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Bacillus subtilis* were tested alone and in combinations for their effect as biocontrol against peanut pod rot pathogenes (*Fusarium moniliforme*, *Fusarium solani, Macrophomina phaseolina*, *Rhizoctonia solani* and *Sclerotium rolfsii*) and aflatoxigenic fungi (*Aspergillus flavus* and *Aspergillus parasiticus*). The fungal and bacterial isolates which have not to reverse effect between them were used in this study. *In vitro* studies, the highest antagonistic effect against the tested pathogenic fungi was shown by *P. fluorescens* followed by *T. viride* (Tv 5) and *B. subtilis*. In greenhouse and field experiments the isolets of selscted biocontrol againt beside standard consisting of fungicide (Rizolex-T) were evaluated for peanut pod rots as well as incidence of *A. flavus, A. parasiticus* and peanut aflatoxin contaminations. In this respect, all tested biocontrol agents and their mixture had a significant effect in reducing peanut pod rots incidence compared to the control. *Pseudomonas fluorescens* alone was superior followed by *T. viride* (Tv 5) and *B. subtilis* in reducing of peanut pod rots incidence. While the mixture of *P. fluorescens* and *T. viride* (Tv 5) gives the best effect in reducing of peanut pod rots incidence compared to other treatments and their effect was the nearest one to Rizolex-T effect in reduction of diseases incidence. Regard to aflatoxigenic fungi and aflatoxin contamination, isolates of *A. flavus* were more invasive to peanut pod than *A. parasiticus*. The most effective treatment was the mixture of *P. fluorescens* and *T. viride* (Tv 5) which gave the least incidence of *A. flavus* and *A. parasiticus* and reduced preharvest aflatoxins contamination compared to other treatments including fungicide. The highest pod yield obtained with the mixture of *T. viride* (Tv 5) and *B. subtills*, followed by *T. viride* (Tv 5) and *P. fluorescens* compared with other biocontrol agents while Rizolex-T gave the highest pod yield at all in the two seasons 2017 and 2018. This study conclusion that, the application of more than one antagonists of diverse origin consider a reliable means of reducing diseases and increasing the reliability of biological control.

***Keywords*:** Bioagents, Biocontrol agent, *Trichoderma harzianum*, *Trichoderma viride*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Basillus subtilis*, *Aspergillus flavus* and *Aspergillus parasiticus* Aflatoxin and Crop safety

Introduction

Peanut, (*Arachis hypogaea* L.) is one of the most export and locally consumed crops in Egypt. Pod rots disease considered among the most destructive disease attacking peanuts and causing quantitative and qualitative losses of yield in Egypt. Meanwhile, aflatoxin contamination caused by the ubiquitous *Aspergillus* group of fungi is a major production constraint and is responsible for huge economic losses to the farming community and trade that makes it is one of the most challengers facing the peanut producers (Felicia *et al.,* 2008 and Kifle *et al.,* 2016). *Aspergillus flavus* and *A. parasiticus* were the predominant fungi infected peanut before harvest (Mahmoud and Gomaa, 2015). Aflatoxins are toxic secondary metabolites produced by some species of the *Aspergillus* fungus (Calvo and Cary, 2015). *Aspergillus flavus* and *A. parasiticus* are the most common species associated with aflatoxin

Corresponding Author: Ahmed M. Gomaa, Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Foods, Ministry of Agriculture and Land Reclamation, Giza, Egypt.
E-mail: ahmedmamdouh1@gmail.com

contamination of peanut crops (Mahmoud, 2004). Aflatoxin B1 is the most toxic and potent carcinogen and the exposure to aflatoxin B1 leads to liver cancer and strongly associated with stunting and immune suppression in children (Mossanda, 2015).

Due to the environment need to more regulations and increasing of organic agriculture besides the danger of chemical control which resulted from farmers apply increasing amounts of fungicides and pesticides to avoid devastating harvest losses. Which poses risks to environmental and human health, and contributes to the increasing incidence of fungal resistance to chemical fungicides. It is therefore imperative that we find alternative approaches to crop protection. One popular suggestion is to increase the efficacy of biocontrol, which is the use of living organisms to control plant disease vectors (Price *et al.,* 2015, Mahmoud *et al.,* 2016, Hawkins *et al.,* 2018 and Ng *et al.,* 2019)

Bacillus and *Pseudomonas* were considered as an important genus of plant growth-promoting rhizobacteria (PGPR) (Mishra *et al.,* 2013, Bhimeshwari *et al.,* 2018, Muhammad *et al.,* 2018 and Wang *et al.,* 2018). *Pseudomonas fluorescens* is considered as an important antagonistic bacteria, the maximum of their antagonistic effect produced *in vitro* by HCN, salicylic acid siderophores such as pyoverdine (pseudo action) pyochelin, pyrrolnitrin (antibiotic) and Beta–1,3 gluconase (Bhimeshwari *et al.,* 2018). Also, *B. subtilis* can induce resistance by stimulation of phytoalexins production and increasing the activity of lytic enzymes (Wang *et al.,* 2018). While, in general, fungal antagonists depend mainly on physical contact with their pathogen besides their ability to secretion analyses enzymes (Muhammad *et al.,* 2018).

Biological control is a recognized method of controlling several plant diseases. Most of the studies on biological control of plant pathogens deal with single biocontrol agents as the antagonist to a single pathogen. While single biocontrol agent is not likely to be active in all soil environments or against all pathogens that attack the host plant and control of a wide spectrum of pathogens under a wide range of environmental conditions. This could be overcome by developing strain mixtures with superior biocontrol activity (Mishra *et al.,* 2011). Consequently, the application of a mixture of introduced biocontrol agents would more closely mimic the natural situation and might broaden the spectrum of biocontrol activity, enhance the efficacy and reliability of biological control (Mishra *et al.,* 2011 and 2013). Previous studies on combinations of biocontrol agents for plant diseases have included mixtures of fungi (Datnoff *et al.,* 1995), mixtures of fungi and bacteria (Mishra *et al.,* 2013 and Mahmoud *et al.,* 2016), and mixtures of bacteria (Raupach and Kloepper, 1998).

With the hypothesis that the combination of two entirely different antagonists would enhance the level of disease management, this study was conducted to evaluate the effect of mixed compatible efficient antagonists of fungi (*Trichoderma*) and bacteria (*Pseudomonas* and *Bacillus*) and testing their efficacy against peanut pod rot incidence, aflatoxigenic fungi, and peanut aflatoxin contaminations under artificial inoculation in greenhouse and field conditions.

Materials and Methods

1. Source of pathogenic fungi:

The fungal isolates used throughout this study were previously isolated by Mahmoud and Gomaa, (2015) from diseased peanut pods and their pathogenic capabilities were also confirmed.

2. Preparation of fungal inoculum:

(A): Inocula of *Fusarium moniliforme*, *Fusarium solani, Macrophomina phaseolina*, *Rhizoctonia solani* and *Sclerotium rolfsii* were prepared using sorghum - coarse sand - water (2:1:2 v/v) medium. The ingredients were mixed, bottled and autoclaved at 15 pounds/ sq. inch for 2h. Each bottle of the sterilized medium was inoculated with a 5mm fungal growth disc obtained from the periphery of the 5-day-old culture of each fungus. The infested media were incubated at 28°C for 15 days before used for soil infestation.

(B): Inocula of aflatoxigenic fungi, *i.e. Aspergillus flavus* and *A. parasiticus* were prepared, as described by Mahmoud and Gomaa, (2015) by growing each isolate on potato dextrose agar (PDA) medium for 7 days at 27°C. Fungal spores were released from culture surface using hair camel brush and sterilized water containing 0.1% agar to prepare spore suspension of $4 \times l0^6$ spores/ml for artificial infestation of soil.

3. Soil Infestation:

Two different methods were used for soil infestation with the tested pathogens throughout this study:

(A): The inoculum of *F. moniliforme*, *F. solani, M. phaseolina*, *R. solani* and *S. rolfsii*, were mixed thoroughly with soil surface of each pot, at the rate of 2% w/w, and were covered with a thin layer of sterilized soil for studying pod rots complex diseases. Pots containing infested soil were irrigated and kept for 10 days until sowing.

(B): Infestation with the conidial suspension at the rate of 10 ml ($4x10^4$ spore/ml) per kg of soil from a mixture of *A. flavus* and *A. parasiticus*. The infestation was carried out, 30 days after sowing, to study the effect of aflatoxigenic fungi and aflatoxin contaminations (Mahmoud, 2004).

4. Disease assessment

(A) At harvest, the percentage of pod rot was recorded. Four categories for apparent symptoms of pod rot beside the healthy pods were adopted according to Mahmoud (2004): a) Rhizoctonia rot, pods with a dry brown lesion, b) Fusarium rot, pods with pink discoloration and c) complex rot pod with general breakdown resulting from many fungi.

$$Pod\ rot\ categories\ (\%) = \frac{Number\ of\ rotted\ pods}{Number\ of\ total\ pods} \times 100$$

(B) Aflatoxigenic fungi, which associated with the four categories, were isolated after harvesting according to Mahmoud (2004). Seeds fruits were shelled and the seed was surface-disinfested for three minutes in 1% sodium hypochlorite and plated on potato dextrose agar (PDA) medium (4 plates in 4 replicates, 5 seeds per dish). Plates were examined after 7 days from incubated at 27 °C. The identification of the isolates was carried out based on taxonomic criteria for these fungi as described by Maren and Johan (1988).

5. Determination of aflatoxins:

The extraction and determination of aflatoxins was carried out according to Malone *et al.* (2000) with accordance to VICAM manul guidance for using immuinoaffinaty colum together with Fluorometer Series-4 (VICAM 1. P, USA), figure no. 1 sumerizes the sample preparation procedure.

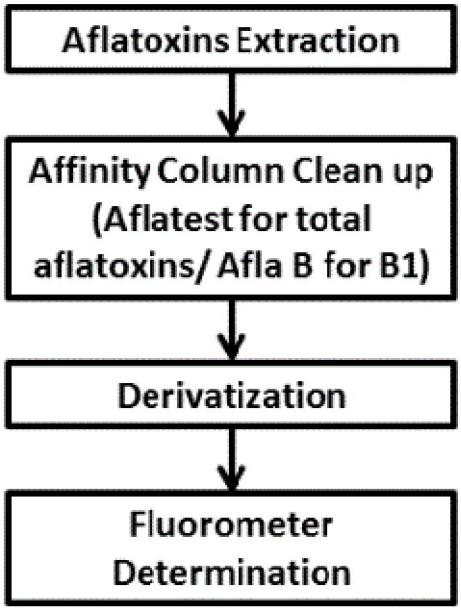

Fig. 1: Aflatoxin sample preparation procedure

5. The sources of biocontrol agents:

Five known isolates of *P. fluorescens* (Pf5), *P. putida*, *B. subtilis* (Bs1), *Brevibacterium carei* and *Bacillus amyloliquefcciens* were obtained from Mahmoud, (2014). However, the tested bioagents included antagonistic fungal isolates *i.e. Trichoderma viride* and *Trichoderma harzianum*, were obtained from Onion, Garlic and Oil Crops Diseases Res. Dept., Plant Pathology Res. Inst., Agric., Res. Center Giza, Egypt.

6. Testing of compatibility of fungal and bacterial biocontrol agents:

The method described by Mahmoud *et al.* (2016) was used for *in-vitro* testing. Five mm size sterilized paper (Whatman paper No. 1) discs impregnated with a bacterial isolate suspension containing 10^6 CFU/ ml (prepared in 0.1 M Mg SO_4) of individual isolates were placed at 5 mm apart from one side of a Petri plate filled with the growth media. The bacterial isolates were allowed to grow for 24 hr at 26±2°C. Five mm diameter plug from a 5- day-old culture of *Trichoderma* isolate was placed on the opposite side of the plate. After 5 days incubation at 26±2°C the zone of inhibition was estimated. Three replications were considered for each treatment.

7. Evaluation of biocontrol agents *in vitro*:

7.1. Effect of antagonistic fungi:

Two discs (5 mm diam.) of plain agar culture of both antagonistic fungi and pathogenic fungi (*F. moniliforme, F. solani, M. phaseolina*, *R. solani* and *Sclerotium rolfsii*) were inoculated (7-day-old) opposite to each other 1 cm apart from the dish edge (9 cm diameter) containing 10 ml PDA medium. The dishes were inoculated with one disc of mycelial growth from the same fungi as the control treatment. Four replicates were used for each particular treatment and then incubated at 26°C ±2 for 5-7 days. Percentage of the fungal growth reduction (X) was calculated using the following formula:

$$X = [(A- B) / A] \times 100$$

Where: A: Diameter of pathogenic fungi growth without biocontrol agents (control).
B: Diameter of pathogenic fungi growth with biocontrol agents.

7.2. Effect of antagonistic bacteria:

Bacillus subtilis and *P. fluorescens* antagonists were tested in this study. Plats of PDA medium were streaked 1 cm apart at one side of the dish edge with a given antagonistic bacteria and incubated for 24 hrs at 26°C ±2. Then, the same plate was inoculated at the opposite side, 1 cm apart from the dish edge, with a disc (5 mm diam.) from *F. moniliforme, M. phaseolina, R. solani* and *Sclerotium rolfsii* of 4-day-old plain agar culture. Plates control inoculated with one disc of mycelial growth of *Fusarium moniliforme, Macrophomina phaseolina, Rhizoctonia solani and Sclerotium rolfsii* in the absence of bacteria were prepared as a control. The percentage of the fungal growth reduction was calculated as mentioned before.

8. Preparation of biocontrol agents:

Bacterial suspensions (1 x 10^6 CFU / ml) were prepared by dilution plate assay as described by Mahmoud, (2014). The tested antagonistic fungal isolates were prepared as an adjusted suspension with approx. 5 X 10^8 conidia/ml as described by Mahmoud, (2014).

9. Methods of application:

The both kind of biocontrol agents (bacteria and fungi) have applied either alone or mixed as a foliar spray after 20 days and soil drench after 40 days. While fungicide Rhizolex-T 50% (Tolclofos-methyl 20% + thiram 30%) were applied as soil treatment at the rate of 3kg/fed after 30 and 60 days.

10. Evaluation of biocontrol agents under greenhouse conditions:

Pot experiments were carried out in order to study the effect of biocontrol agents in controlling peanut pod rots incidence (%), the frequency of *A. flavus, A. parasiticus* and peanut seed contaminations with aflatoxin. The experiment was carried out at Agric. Res. Center, Giza. Peanut seeds were sown in 50 cm-diameter pots containing sterilized soil previously infested with a mix of *F. moniliforme, M. phaseolina, R. solani and S. rolfsii* (2% w/w). Ten seeds were sown per pot, five replicate (pots) were used for each treatment. The biocontrol agents and fungicides were applied as

mentioned before. Disease assessment was recorded as a percentage of pod rots incidence.

11. Evaluation of biocontrol agents under field conditions:

The field experiments were performed at Nubaria, during 2017 and 2018 seasons to study the effect biocontrol agents in controlling peanut pod rots incidence, the frequency of *A. flavus, A. parasiticus* and peanut seed contaminations with aflatoxin. The selected fields considered were known to have a natural infestation with pod rots pathogens. The biocontrol agents were applied as mentioned before. Seeds were sown on the first week of April with 10 cm spacing between hills. Cultural practices and fertilization for the peanut crop were applied as recommended. The fungicide Rhizolex-T50% was applied as previously mentioned under the experimental unit area was 21 m^2 (1/200 fed.).The treatments were arranged in a completely randomized block design with four replicates. Disease assessment was recorded as mentioned before.

Statistical analysis:

The data were statistically analyzed by analysis of variance (ANOVA) using the Statistical Analysis software "COStat 6.4" (CoStat, 2005). Means were separated by the least significant difference (LSD) test at $P \leq 0.05$ levels.

Results

1. Compatibility of fungal and bacterial biocontrol agents:

Table (l) shows that the compatibility of *Trichoderma* isolates with bacterial biocontrol agents varied greatly according to the isolates. In this respect, Th 1, Th 2, Th 6**,** Tv 1, Tv 3, Tv 4 and Tv 5, were compatible with *P. fluorescens* (Pf5) and exhibited no antagonistic interaction against it. Moreover, Th1, Th 5, Th 6**,** Tv 3, Tv 4 and Tv 5, showed a similar trend with *B. subtilis* (Bs1). While Th 1, Th 3, Th 6**,** Tv 1, Tv 3, Tv 4 and Tv 5, were compatible with *P. putida* (PP). only both of Th 6**,** Tv 5 were compatible with *B. carei* (Bc). Based on these results, Trichoderma (Th 1, Th 6**,** Tv 3, Tv 4 and Tv 5), *P. fluorescens* (Pf5), *B. subtilis* (Bs1) and *P. putida* (PP) were evaluated *in vitro* for their antagonistic potential against tested pathogenic fungi (Table 2).

Table 1: Test of compatibility of biocontrol agents.

Biocontrol agents	*P. fluorescens* (Pf5)	*P. putida* (PP)	*B. carei* (Bc)	*B. amyloliquefcciens* (Ba)	*B. subtilis* (Bs1)
***T. harzianum* (Th 1)**	+	+	-	+	+
***T. harzianum* (Th 2)**	+	-	-	-	-
***T. harzianum* (Th 3)**	-	+	-	-	-
***T. harzianum* (Th 4)**	-	-	-	-	-
***T. harzianum* (Th 5)**	-	-	-	-	+
***T. harzianum* (Th 6)**	+	+	+	-	+
***T. viride* (Tv 1)**	+	+	-	-	-
***T. viride* (Tv 2)**	-	-	-	+	-
***T. viride* (Tv 3)**	+	+	-	+	+
***T. viride* (Tv 4)**	+	+	-	-	+
***T. viride* (Tv 5)**	+	+	+	-	+
***T.a viride* (Tv 6)**	-	-	-	+	-

Compatible = inhibition zone < 1 mm (+) Non-compatible= inhibition zone > 1 mm (-).

2. Screening of biocontrol control potential *in vitro*:

Five isolates of Trichoderma (Th 1, Th 6, Tv 3, Tv 4 and Tv 5) in addition to three bacteria (*P. fluorescens, P. putida* and *B. subtilis*) were evaluated *in vitro* for their antagonistic effect against tested pathogenic fungi (Table 2).

P. fluorescens gave the high significant antagonistic effect against the tested pathogenic fungi followed by *T. viride* (Tv 5) and *B. subtilis*. However, *T. viride* (Tv 3), *T. harzianum* (Th 6) and *P. putida* give a moderate effect against tested pathogenic fungi. While both of *T. viride* (Tv 4) and *T. harzianum* (Th 1) had a little effect.

Table 2: Antagonistic effect of biocontrol agents on the percentage of liner growth reduction (%).

Biocontrol agents	*R. solani*	*F. moniliforme*	*F. solani*	*M. phaseolina*	*S. rolfsii*	*A. flavus*	*A. parasiticus*
***T. harzianum* (Th 1)**	13.33	17.78	17.78	22.22	26.67	18.16	19.33
***T. harzianum* (Th 6)**	23.44	30.11	29.00	29.56	31.56	30.77	31.61
***T. viride* (Tv 3)**	22.33	27.22	26.44	25.00	26.11	27.82	29.91
***T. viride* (Tv 4)**	11.11	20.00	22.22	27.78	27.78	20.43	24.15
***T. viride* (Tv 5)**	26.67	33.90	33.33	31.11	33.33	35.04	36.23
P. fluorescens	28.89	40.00	38.89	32.22	36.67	40.85	42.27
P. putida	22.44	29.44	28.33	26.78	30.11	30.11	30.97
B. subtilis	24.60	33.67	30.56	30.00	32.44	34.39	33.22
L.S.D. 5%	1.02	0.53	1.03	1.02	1.05	0.86	1.03

3. Evaluation of biocontrol agents under greenhouse conditions:

3.1. On peanut pod rots incidence:

Two selected fungal isolates and three bacterial isolates beside standard consisting of Rizolex-T (fungicide) were evaluated for peanut pod rots control under greenhouse conditions (Table 3).

Table (3) shows that all tested biocontrol agents and their mixture had a significant effect in reducing peanut pod rots incidence compared to the control. *Pseudomonas fluorescens* alone was superior followed by *T. viride* (Tv 5) and *B. subtilis* in reducing peanut pod rots incidence.

The present data indicated that when supply compatible mixing of fungi and bacteria give more activity in control of peanut pod rots. In this respect, the mixture of *P. fluorescens* and *T. viride* (Tv 5) gives the best effect in reducing of peanut pod rots incidence compared to other treatments except for fungicide treatment.

Data also showed that the mixture of *T. viride* (Tv 5) and *P. fluorescens* was the nearest one to the Rizolex-T effect in the reduction of disease incidence (Table 3).

Table 3: Effect of biocontrol agents on peanut pod rots incidence under greenhouse conditions.

Biocontrol agents	Disease incidence (%)			Apparent healthy (%)
	Dry brown lesion	Pink discoloration	General breakdown	
T.harzianum* (Th 6)+ *P. fluorescens	10.14	0.80	13.87	75.19
T.harzianum* (Th 6)+ *P. putida	12.82	1.30	15.82	70.06
T.harzianum* (Th 6)+ *B. subtilis	10.64	1.13	14.92	73.31
***T. harzianum* (Th 6)**	11.62	0.57	14.85	72.96
T. viride* (Tv 5)+*P. fluorescens	6.67	0.44	10.18	82.71
T. viride* (Tv 5)+*P. putida	10.78	0.74	15.15	73.33
T. viride* (Tv 5)+*B. subtilis	8.92	0.54	13.00	77.54
***T. viride* (Tv 5)**	9.95	0.62	13.22	76.21
P. fluorescens	9.54	0.35	12.55	77.56
P. putida	12.54	1.78	16.04	69.64
B. subtilis	11.13	0.96	13.21	74.70
Rizolex-T 50%	6.00	0.28	9.01	84.71
Control	15.07	2.06	17.79	65.08
L.S.D. 5%	2.11	0.08	2.03	2. 25

3.2. On the frequency of *A. flavus, A. parasiticus* and peanut seed contaminations with aflatoxin:

Two selected fungal isolate and three bacterial isolates beside of Rizolex-T (fungicide) were evaluated for the occurrence of *A. flavus, A. parasiticus* and aflatoxin content in peanut pods under greenhouse conditions (Table 4).

Generally, all tested biocontrol agents and their mixture had an effect in reducing the frequency of aflatoxigenic fungi. The occurrence of *A. flavus* was higher than that of *A. parasiticus* in all treatments whether any of them was infested separately or in the mixture (Table 4). In respect to separate treatment, *P. fluorescens* followed by *T. viride* (Tv 5) and *B. subtilis* give the highest reduction of the frequency of aflatoxigenic fungi as well as peanut seed contaminations with aflatoxin. While in general, the compatibility between fungi and bacteria led to more effective in reducing the frequency of aflatoxigenic fungi and seed content of aflatoxin whether total aflatoxin or B_1. Regard to this point, the

mixture of *P. fluorescens* and *T. viride* (Tv 5) gives the best effect in reducing the frequency of aflatoxigenic fungi and peanut seed contamination wih aflatoxin.

Table 4: Effect of biocontrol agents on the frequency of *A. flavus, A. parasiticus* and aflatoxin contaminations in peanut seed under greenhouse conditions.

Biocontrol agents	*A. flavus*			*A. parasiticus*			*A. flavus* + *A. parasiticus*			
	*A.f %	Aflatoxin (ppb)		**A.p %	Aflatoxin (ppb)		A.f %	A.p %	Aflatoxin (ppb)	
		B_1	Total AFT		B_1	Total AFT			B_1	Total AFT
T.harzianum* (Th 6)+ *P. fluorescens	25	50	57	15	0	0	20	15	90	99
T.harzianum* (Th 6)+ *P. putida	40	100	112	25	80	89	50	30	200	219
T.harzianum* (Th 6)+ *B. subtilis	35	90	99	25	40	45	40	30	170	188
***T. harzianum* (Th 6)**	45	170	185	35	140	156	50	35	210	233
T. viride* (Tv 5)+*P. fluorescens	20	30	34	5	0	0	15	10	70	77
T. viride* (Tv 5)+*P. putida	25	80	85	15	0	0	25	20	100	110
T. viride* (Tv 5)+*B. subtilis	30	80	88	20	70	79	30	25	100	110
T. viride (Tv 5)	30	70	77	20	30	35	30	10	150	168
Pseudomonas fluorescens	30	60	66	20	0	0	20	15	70	79
Pseudomona. putida	40	90	99	30	100	110	40	30	200	220
Basillus subtilis	35	85	100	20	60	66	30	25	180	189
Rizolex-T 50%	30	80	89	20	50	55	30	20	110	121
Control	50	200	220	35	200	230	60	40	350	400

***A.f** = *A. flavus* ** **AFT** = aflatoxin

Data in Table (4) also indicate that the effect of Rizolex-T was not the best in reducing the frequency of aflatoxigenic fungi and peanut seed contaminations with aflatoxin but the mixture of *P. fluorescens* and *T. viride* (Tv 5) gives the best effect.

4. Evaluation of biocontrol agents under field conditions:

4.1. On peanut pod rots incidence:

Data in Tables (5 and 6) indicate that all tested biocontrol agents and their mixtures had a significant effect in reducing peanut pod rots incidence during the two successive seasons, 2017 and 2018.

In general *P. fluorescens* showed greater influence in the reduction of all types of pod rot and increased apparent healthy either as single or as mixed application during the two successive seasons, 2017 and 2018.

Moreover, *P. fluorescens* when mixed with *T. viride* (Tv 5) recorded the highest effect in reducing pod rots disease compared with that being mixed with *T. harzianum* (Th 6) along with increasing the percentage of apparent healthy peanut pods during the two seasons, 2017 and 2018. The mixture of *T. viride* (Tv 5) also with *B. subtilis* or *P. putida* gave more effective in reducing peanut pod rots during 2017 and 2018 while, the mixed of *T. harzianum* (Th 6) with other bacterial species gave weak effect in reducing of peanut pod rot Tables(5 and 6).

4.2. On the frequency of *A. flavus, A. parasiticus* and peanut seed contaminations with aflatoxin:

In general the frequency of *A. flavus* was higher than *A. parasiticus* in all treatments during the two growing seasons 2017 and 2018 (Table 7). Data also indicate that the compatible between fungi and bacteria led to more effective in reducing the frequency of aflatoxigenic fungi and seed content of aflatoxin whether total aflatoxin or B_1. In this respect compatible with *P. fluorescens* and fungi gives the highest effect on reducing of the frequency of aflatoxigenic fungi as well as peanut seed contamination with aflatoxin followed by *B. subtilis* while the mixture with *P. putida* gives the lowest effect.

Table 5: Effect of biocontrol agents on peanut pod rots incidence under field conditions during season 2017

Biocontrol agents	Disease incidence (%)			Apparent healthy (%)
	Dry brown lesion	Pink discoloration	General breakdown	
T.harzianum* (Th 6)+ *P. fluorescens	7.72	0.50	11.93	79.85
T.harzianum* (Th 6)+ *P. putida	11.03	1.01	14.60	73.36
T.harzianum* (Th 6)+ *B. subtilis	9.15	0.77	13.83	76.25
***T. harzianum* (Th 6)**	10.00	0.42	13.77	75.81
T. viride* (Tv 5)+*P. fluorescens	6.74	0.12	8.75	84.39
T. viride* (Tv 5)+*P. putida	9.27	0.24	13.03	77.46
T. viride* (Tv 5)+*B. subtilis	7.67	0.30	11.18	80.85
***T. viride* (Tv 5)**	8.56	0.33	11.37	79.74
Pseudomonas fluorescens	8.21	0.30	10.79	80.70
Pseudomona. putida	11.79	1.13	14.79	72.29
Basillus subtilis	10.57	0.83	12.36	76.24
Rizolex-T 50%	5.16	0.19	7.75	86.90
Control	12.96	1.89	15.30	69.85
L.S.D. 5%	1.26	0.06	1.25	1.55

Table 6: Effect of biocontrol agents on peanut pod rots diseases under field conditions during season 2018

Biocontrol agents	Disease incidence (%)			Apparent healthy (%)
	Dry brown lesion	Pink discoloration	General breakdown	
T.harzianum* (Th 6)+ *P. fluorescens	8.09	0.46	11.06	80.39
T.harzianum* (Th 6)+ *P. putida	10.23	0.94	12.61	76.22
T.harzianum* (Th 6)+ *B. subtilis	8.49	0.71	11.90	78.90
***T. harzianum* (Th 6)**	9.27	0.39	11.84	78.50
T. viride* (Tv 5)+*P. fluorescens	6.25	0.11	8.11	85.53
T. viride* (Tv 5)+*P. putida	8.60	0.22	12.08	79.10
T. viride* (Tv 5)+*B. subtilis	7.11	0.28	10.37	82.24
***T. viride* (Tv 5)**	7.94	0.31	10.54	81.21
Pseudomonas fluorescens	7.61	0.28	10.01	82.10
Pseudomona. putida	10.01	1.05	12.79	76.15
Basillus subtilis	8.87	0.77	11.53	78.83
Rizolex-T 50%	4.79	0.18	7.19	87.84
Control	12.00	1.73	15.19	71.08
L.S.D. 5%	1.21	0.05	1.23	1.49

Table 7: Effect of biocontrol agents on the frequency of *A. flavus, A. parasiticus* and aflatoxin contaminations in peanut seed under field conditions during the two growing seasons 2017 and 2018.

Seasons	2017				2018			
Biocontrol agents	A. f. (%)	A. p. (%)	Aflatoxin (ppb)		A. f. (%)	A. p. (%)	Aflatoxin (ppb)	
			B_1	Total AFT			B_1	Total AFT
T.harzianum* (Th 6)+ *P. fluorescens	15	0	80	88	10	0	0	0
T.harzianum* (Th 6)+ *P. putida	25	20	180	218	20	10	120	132
T.harzianum* (Th 6)+ *B. subtilis	20	15	160	178	15	10	70	77
***T. harzianum* (Th 6)**	20	20	100	110	20	15	60	67
T. viride* (Tv 5)+*P. fluorescens	10	0	0	0	0	0	90	99
T. viride* (Tv 5)+*P. putida	20	15	150	165	20	15	100	112
T. viride* (Tv 5)+*B. subtilis	15	10	80	88	10	5	0	0
***T. viride* (Tv 5)**	25	20	90	100	15	10	50	56
Pseudomonas fluorescens	15	5	0	0	5	0	0	0
Pseudomona. putida	30	20	150	160	20	20	140	150
Basillus subtilis	25	20	110	120	20	15	80	88
Rizolex-T 50%	20	15	50	55	15	15	0	0
Control	40	30	270	300	30	25	200	223

***A.f** = *A. flavus* ** **AFT** = Aflatoxin

In the case of separate treatment, *P. fluorescens* followed by *T. viride* (Tv 5) and *B. subtilis* give the highest reduction of the frequency of aflatoxigenic fungi as well as peanut seed content of aflatoxin. Data also indicate that the effect of Rizolex-T was not the best in reducing the frequency of aflatoxigenic fungi and peanut seed contaminations but the mixture of *P. fluorescens* and *T. viride* (Tv 5) gives the best effect during the seasons 2017 and 2018.

5. Effect of a biocontrol agent on peanut pod yield under field conditions:

Data presented in Table (8) demonstrate that all tested biocontrol agents either single or in combination caused a significant increase in total peanut pod yield. Percentage of increases however reached (10.94-22.48) and (10.17-29.25) in the first and second seasons respectively.

The highest pod yield in the two seasons obtained with mixed of *T. viride* (Tv 5) and *B. subtills*, followed by *T. viride* (Tv 5) and *P. fluorescens* compared with other biocontrol agents. While Rizolex-T gave the highest pod yield at all as well as their effect on increase yield in the two successive seasons 2017 and 2018 compared with other treatments. On the other hand, the mixture of *Trichoderma viride* and *B. subtilis* was the nearest biocontrol agent to fungicides (Rizolex-T) effect in increase of pod yield in the two seasons 2017 and 2018 compared with other biocontrol agents while *T. harzianum* (Th 6) gives the lowest effect in increase of pod yield compared with other biocontrol agents in the two seasons (Table 8).

Table 8: Effect of biocontrol agents on peanut yield and increase of yield under field conditions during the two growing seasons 2017 and 2018.

Seasons	2017		2018	
Biocontrol agents	**Yield (Ton)**	***Increases (%)**	**Yield (Ton)**	***Increases (%)**
T.harzianum* (Th 6)+ *P. fluorescens	1.338	15.25	1.395	16.25
T.harzianum* (Th 6)+ *P. putida	1.302	12.14	1.358	13.17
T.harzianum* (Th 6)+ *B. subtilis	1.355	16.71	1.402	16.83
***T. harzianum* (Th 6)**	1.288	10.94	1.322	10.17
T. viride* (Tv 5)+*P. fluorescens	1.366	17.66	1.432	19.33
T. viride* (Tv 5)+*P. putida	1.300	11.97	1.392	16.00
T. viride* (Tv 5)+*B. subtilis	1.396	20.24	1.485	23.75
***T. viride* (Tv 5)**	1.305	12.40	1.365	13.75
Pseudomonas fluorescens	1.315	13.26	1.378	14.83
Pseudomona. putida	1.299	11.89	1.353	12.75
Basillus subtilis	1.319	13.61	1.371	14.25
Rizolex-T 50%	1.422	22.48	1.551	29.25
Control	1.161	--	1.200	--
L.S.D. 5%	0.053		0.049	

*Increases related to the control

Discussion

Biological control of plant pathogens by microorganisms decreases the use effect of fungicides hazardous to humans and the environment (Cook, 1993).

The present study results indicated that the tested biocontrol agents significantly reduced peanut pod rot disease, occurrences of aflatoxigenic fungi (*A. flavus* and *A. parasiticus*) and seed content of aflatoxin whether under artificial infection or natural infection and showed antagonistic effect against the tested pathogeneses fungi *in vitro*. Regarded to this, *Pseudomonas fluorescens* (Pf5) followed by *Trichoderma viride* (Tv5) and *Bacillus subtilis* (Bs1) gave the highest significant antagonistic effect against the tested pathogen growth. These results are in harmony with those reported Mishra *et al.,* (2011&2013), Mahmoud *et al.,* (2016), Kifle *et al.,* (2016), Bhimeshwari *et al.,* 2018 and Wang *et al.,* (2018).

Who's reported that *B. subtilis* and *P. fluorescens*, as well as *Trichoderma viride*, were found to be the most effective biocontrol agent against various soilborne diseases caused by *F. moniliforme, F. solani R. solani, M. phaseolina, S. rolfsii, A. flavus,* and *A. parasiticus,* and other. On the other hand, Torres *et al*. (2014) confirmed the use of biocontrol agents to reduce *Aspergillus* contamination of crops pre-harvest. While, Mahmoud, (2004), reported that, Under field condition, *Bacillus* spp. (Sp2)

followed by *Bacillus* spp. (Ss2) and *Pseudomonas fluorescens* (Pf 5) had a significant effect on reduced the pod rot incidence and recorded the highest effect on reduced the occurrence of aflatoxigenic fungi and the aflatoxin contaminations. And in 2014 he stated that, in greenhouse and field experiments, the most effective isolates in reducing peanut damping-off, root and pod rot diseases were *P. fluorescens* (Pf.5) followed by *B. subtilis* (Bs1) and *Brevibacterium carei* (S.5). Moreover, Kifle *et al.* (2016) found that *Trichoderma harzianum* strain kd can reduce infection of the groundnut seeds by *Aspergillus flavus*, and hence it may reduce the contamination of the seed by aflatoxin, especially under drought stress condition.

Many studies in this respect showed that certain *P. fluorescens* and *B. subtilis* isolates were effective rhizobacteria for suppression of soil-borne fungi, by production of certain substance such as enzymes, phenazines, pyrrole type antibiotics, pyo-compounds, indole derivatives peptide antibiotic, moenomycins, difficidins, bacillomycins and bacillaenes (Bhimeshwari *et al.,* 2018 and Wang *et al.,*2018). Moreover, certain strains of Pseudomonas can produce several siderophores such as pyoverdine (pseudobactin) pyochelin, pyrollnitrin (antibiotic), salicylic acid, HCN and lytic enzymes (Bhimeshwari *et al.,* 2018). Also, *B. subtilis* can induce resistance by stimulation of phytoalexins production and increasing the activity of lytic enzymes (Wang *et al.,* 2018). While, Trichoderma isolates can be antagonistic on other fungi through lyses to host hyphae by the action of hydrolytic enzymes such as chitinases, beta–glucanases and proteases alone or in combination with secondary metabolites also, it competition for nutrients by produce siderophores basically concentrates on carbon, nitrogen and essential micronutrients such as iron and manganese (Muhammad *et al.,* 2018).

The present study is in agreement with the studies conducted by different workers, where they have reported that increased biocontrol activity might be achieved by combining different isolates of biocontrol agents (Duffy *et al.,* l996; Raupach and Kloepper, l998). Further, Duffy *et al.,* (1996) indicated that *P. fluorescent* species and *T. koningii* are compatible when applied to wheat simultaneously. The performance of all bacterial treatments was greatly enhanced by combination with *T. koningii*. While, Mishra *et al.,* (2013) reported that, *Trichoderma harzianum* and *Pseudomonas fluorescens* when tested alone or combined under glass-house and field conditions against many soil-borne plant pathogens viz., *Rhizoctonia solani, Sclerotium rolfsii* and *Macrophomina phaseolina* responsible for root and stem rot disease of soybean give a greater suppression and enhanced consistency against the pathogens by mixed of them. However, Mahmoud *et al.,* (2016) stated that the mixed with *T. viride* either with *B. subtilis* or *P. fluorescens* revealed greater effect in control of sunflower charcoal rot compared to the single application of any of them, especially the mixed with *B. subtilis* treatment.

These results strengthen the opinion that control with fungicides can be partially replaced by biological control because it's effectively protected the peanut pod from aflatoxigenic fungi and aflatoxin contamination. The results clearly showed that Rizolex-T was not the best in reducing the frequency of aflatoxigenic fungi and peanut seed contaminations but the mixture of *P. fluorescens* and *T. viride* (Tv 5) record the best effect during the seasons 2017 and 2018.

References

Bhimeshwari S., S. Jahaar, S. Girija and A. Pradhan, 2018. *Pseudomonas fluorescens* PGPR bacteria as well as biocontrol agent: A review. International Journal of Chemical Studies 6(2): 01-07.

Calvo, A.M. and J.W. Cary, 2015. Association of fungal secondary metabolism and sclerotial biology. Frontiers in Microbiology 6.

Cook, R.J., 1993. Making greater use of introduced microorganisms for biological control of plant pathogens Annu. Rev. Phytopathol., 31: 53-80.

CoStat, 2005. CoStat program, version 6.4. CoHort software, Monterey, CA, USA.

Datnoff, L.E., S. Nemec and K. Pernezny, 1995. Biological control of Fusarium crown and root rot of tomato in Florida using Trichoderma harzianum and Glomus intraradices. Biol. Control, 5:427-431.

Duffy, B.K., A. Simon and D.M. Weller, 1996. Combinations of Trichoderma koningii and fluorescent pseudomonad for control of take-all of wheat. Phytopathology, 86:188-194.

Felicia, W., Yan, L., and B. Deepak, 2008. Cost-effectiveness of aflatoxin control methods: Economic incentives. Toxin Reviews, 27(3&4):203-225.

Hawkins, N.J., C. Bass, A. Dixon, and P. Neve, 2018. The evolutionary origins of pesticide resistance. Biol. Rev. Camb. Philos. Soc. 94, 135–155. doi: 10.1111/ brv.12440

Kifle, M.H., K.S. Yobo and. M.D. Laing, 2016. Biocontrol of Aspergillus flavus in groundnut using Trichoderma harzianum stain kd Journal of Plant Diseases and Protection -New Series- · December 2016 DOI: 10.1007/s41348-016-0066-4.

Mahmoud, E.Y., 2004. Integrated control of pod rot diseases of peanut. Ph.D. Thesis, Fac. Of Agric, Ain – Shams Univ.154 pp

Mahmoud, E.Y., 2014. Performance of some antagonistic bacteria in minimizing occurrence of peanut damping - off, root and pod rot diseases. Egypt j. Phytopathol. 42 (1):205-220

Mahmoud, E.Y., and M.G. Ahmed, 2015. Impact of some essential plant oils for controlling of peanut pod rots diseases and aflatoxin. J. Biol. Chem. & Envirom. Sci., 10 (1): 261-280.

Mahmoud, E.Y., M.M. Ibrahim, Wagida A.M. Saleh and M.I.M. Ahmed, 2016. Compatibility between antagonistic fungi and bacteria and their influence in controlling sunflower charcoal rot. Egypt J. Phytopathol., 43(1-2): 53-65

Malone, B.R., C.W. Humphrey, T.R. Romer, and J.L. Richard, 2000. Determination of Aflatoxins in Grains and Raw Peanuts by a Rapid Procedure with Fluorometric Analysis. Journal of aoac International, 83: 95-98.

Maren, A.K. and I.P. Johan, 1988. A laboratory guide to the common Aspergillus Species and Their Teleomorph. Commonwealth Scientific and Industrial Res. Org. Division of Food Processing., 116 pp.

Mishra, D.S., A.K. Gupta, C.R. Prajapati and U.S. Singh, 2011. Combination of fungal and bacterial antagonists for management of root and stem rot disease of soybean Pak. J. Bot., 43, 2569-2574.

Mishra, D.S., A. Kumar, C.R. Prajapati, A.K. Singh and S.D. Sharma, 2013. Identification of compatible bacterial and fungal isolates and their effectiveness against plant disease. J. of Environmental Biology, 34:183-139.

Mossanda, K.S., 2015. Hepatocellular carcinoma: Putative interactive mechanism between aflatoxins and hepatitis viral infections implicating oxidative stress during the onset and progression of cancer. Hypothesis 1: 18.

Muhammad, U., M.R. Ghazanfar, R. Waqas, and I.Q. Misbah 2018. Trichoderma as potential biocontrol agent, its exploitation in agriculture: A review. Plant Protection, 02 (03):109-135

Ng, A., D. Weerakoon, E. Lim, and L.P. Padhye, 2019. Fate of environmental pollutants. Water Environ. Res. 91, 1294–1325. doi: 10.1002/wer.1225

Price, C.L., J.E. Parker, A.G. Warrilow, D.E. Kelly, and S.L. Kelly, 2015. Azole fungicides - understanding resistance mechanisms in agricultural fungal pathogens. Pest Manag. Sci. 71, 1054–1058. doi: 10.1002/ps.4029

Raupach, G.S. and J.W. Kloepper, 1998. Mixtures of plant growth promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology, 88:1158-1164.

Torres, A., G. Barros, S. Palacios, S. Chulze and P. Battilani, 2014. Review on pre-and post-harvest management of peanuts to minimize aflatoxin contamination. Food Research International 62: 11-19.

Wang, X.Q., D.L. Zhao, L.L. Shen, C.L. Jing, and C.S. Zhang, 2018. Application and mechanisms of *Bacillus subtilis* in biological control of plant disease„" in Role of Rhizospheric Microbes in Soil: Volume 1: Stress Management and Agricultural Sustainability, ed. V. S. Meena (Singapore: Springer Singapore), 225–250.