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Biological and Nanocomposite Control of *Fusarium W*ilt of Potato Caused by *Fusarium Oxysporum* f. sp. *Tuberosi*

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Abstract

The present study indicated the antagonists activity of different biocontrol agents against a soil – born *pathogen Fusarium oxysporum*, that causes wilt disease of potato (Hermis and Spunta cvs.). *In vitro*, the effect of antagonists by the modified montmorillonite particles (Mod- MMT) as a fungicide with concentration (1,3 and 5%) , and some strains of fungus *Trichoderma* and *Pseudomonas* revealed the presence of clear antagonistic action against *Fusarium oxysporum* f. sp. *tuberosi* . The highest mean inhibition values were 93.33% and 90% with Mod-MMT 5% and Mod-MMT 3% combined with *Trichoderma harzianum* respectively ,while the lowest effect was investigated with *Pseudomonas fluorescens* only 51.11%. Under green house conditions the results proved that soil treatments was more effective than tuber treatments against pre and post wilting disease. The highest protection against the pathogen cleared with Mod-MMT 5% and Mod-MMT 3% combined with *Trichoderma harzianum* respectively as soil treatment . This treatment was superior to Mod-MMT 5% and Mod-MMT 3% combined with *Pseudomonas fluorescens* in reducing the disease in comparison to infested control. All biological treatments also significantly decreased the incidence of disease specially Mod-MMT 5% and Mod-MMT 3% combined with *Trichoderma*. On the other hand the all bio- control agents reduced the population of *Fusarium oxysporum* gradually up to 8weeks after sowing. Also all biocontrol agents increased plant hight(cm./plant), fresh and dry weight(gm./ plant) , number of tubers/ plant and weight of tubers (gm./ plant).

Key words: biological control –nanocomposite- potato - *Fusarium* wilt- *Trichoderma- Pseudomonas*

Introduction

Potato (*Solanum tuberosum* L.) is a crop of major economic importance worldwide (Tsegaw, 2005; FAO, 2008).It is the world's fourth-largest food crop, following rice, wheat and maize (Harris, 1992; FAO, 2008). It widely cultivated, and could contribute to reducing worldwide food shortages , So it is a useful crop that provide high yields of carbohydrate and protein from their tubers so it has an important role in human nutrition (OrzoLek *et al*.2010). The potato tubers could be easily invaded by pests and pathogens such as *Fusarium oxysporum* (Agrios,1997).

Fusarium wilt is a worldwide important disease of potato and can be transmitted by seed cuts (Powelson and Rowe, 1993). This disease caused by *F. oxysporum tuberosi* which is a soil-borne hyphomycete and causes vascular wilts of flowering plants (Domsch *et al*. 1980; Nelson *et al*. 1983) . It considers one of the most destructive and economically damaging diseases of potato that lead to yield Losses (Stevenson *et al* .,2001 and Haggag,2008).This disease, characterized by generally symptoms such as wilting, chlorosis, necrosis, premature leaf drop, browning of the vascular system, stunting, and damping-off. The most important of these is vascular wilt., frequently results in the death of infected plants (Jones *et al*.,1991).

In view of difficulties and problems associated with chemical control of soil borne plant pathogens and environmental pollution, employment of biocontrol agents for plant disease management is considered as a good alternative (Blond 1990).

Using *Trichoderma* species as biocontrol agents was recognized for the first time by (Weindling, 1932). During recent decades, attention has been paid to this group of fungi and subsequently they have been applied successfully as biocontrol agents against several plant diseases in commercial agriculture (Howell, 2003 and Farkhondeh *et al* .,2013). *Trichoderma* spp. is an unique ability to control the plant pathogens by using the mechanism of mycoparasitism, antibiosis, competition, siderophore production, induction of systemic resistance etc (Chet, 1987; Dennis and Webster 1971, Upadhyay and Mukhopadhyay, 1986; Howell, 2003). Many studies cleared that *Trichoderma* spp.have antagonistic and biologically control potential against a diversity of soil borne pathogens.(Grondona *et al*., 1997; Hanson and Howell, 2004; Bajwa *et al* ., 2004). Antagonistic ability of different isolates of *Trichoderma* on different formae specials of *Fusarium oxysporum* is well documented (Kerkeni *et al*.,2007; Dohroo, 1995; Orole and Adejumo, 2009 ; Sivan and Chet, 1989 ; Pawan and Vijay 2011).

Pseudomonas spp. are suitable for application as agricultural bio-control agents since they can use many exudates compounds as a nutrient source. It has been widely used as antagonists against fusarium wilt of several agriculture important crops (Alabouvette and Steinberg, 1995; Aeshah *et al* ., 2011) .Certain members of the *P. fluorescens* have been shown to be potential agents for the bio-control which suppress plant diseases by protecting the seeds and roots from fungal infection. They are known to enhance plant growth promotion and reduce severity of many fungal diseases (Hoffland *et al*. 1996, Wei *et al*. 1996). This effect is the result of the production of a number of secondary metabolites including antibiotics, siderophores and hydrogen cyanide (O'Sullivan and O'Gara 1992). Hass and Defago (2005) reviewed the mechanisms by which *P. fluorescens* control pathogenic microorganisms in detail. Competitive exclusion of pathogens as the result of rapid colonization of the rhizosphere by *P. fluorescens* may also be an important factor in disease control (Girija and Manoj 2005).

Montmorillonite (MMT), a bioinert clay mineral with fine grain and large inter-planner spacing in the (001) plane, has superior capability to intercalate large molecules into the interlayer space of the (001) plane. The structure of MMT is an octahedral laminated sheet, sandwiched between tetrahedral silicate layers (Bremner , 1982 and Boyd et al., 1988). The expandable layered aluminosilicate structure of clay minerals, consisting of stacks of plate-like layers of ~1 nm thickness, separated by an interlayer distance of ~1 nm. The platelets with an aspect ratio in the range of 20-100 nm have an extremely large surface area of ~750 m2 g-1. The layers are not stacked regularly enough to form true crystal but are grouped in small numbers not exceeding dozen-forming stacks, inside which the only degree of organization is a parallelism sufficient to result in selective d001 reflection. Stacks of parallel layers form primary particle and number of primary particles form aggregate and microaggregate (0.1 -10 µm) (Mering, 1946). It has some excellent properties such as good water absorption, swelling, absorbability, cation exchange and drug carrying capability (Macter et al., 1990), that are considered beneficial from the viewpoint of synthesis of pharmaceutical products as both inactive and active substances (Ricka et al. 1984 and Hirokawa et al.,1984). However, in spite of the beneficial effects of clay minerals, there are some inherent drawbacks associated with their use for drug delivery. Under physiological conditions clay dispersions are unstable and tend to flocculate and precipitate in ion containing solution, because of high salt concentration and the presence of polyelectrolytes such as proteins. Stability of dispersion is an important requirement for drug carriers because it plays a determining role with regard to adsorption and bioavailability. Furthermore, the ability of clay particles to adsorb negatively charged or neutral drugs is low, restricting their application as carriers of negatively charged or neutral drugs (Aguzzi et al., 2007). MMT is a natural biodegradable copolymer of N-acetylglucosamine and D-glucosamine that is usually obtained from exoskeletons of shellfish and insects. With regard to its unique properties such as biocompatibility, biodegradability, non-toxicity and non allergenic with antimicrobial effect, it is widely used in fields like biotechnology, biomedical, pharmaceutics, cosmetics, textile and agriculture (Mazzarelli, 1973 and Darder et al., 2003).

The present study was undertaken to find out environmental friendly methods of controlling *Fusarium oxysporum* f. sp. *Tuberosi*, using modified montmorillonite nanocomposite with different ratio (Mod-MMT), *Trichoderma harzianum* and *Pseudomonas fluorescens* individually or combination.

Materials and Methods

Isolation and identification of *F.oxysporum*

Potato plants showing wilt symptoms were collected from different locations at Kafr El-Sheikh and Minufiya governorates, Egypt at flowering stage. Small pieces of diseased specimens (stem vascular tissue) were grown on Potato Dextrose Agar plates (PDA) , then kept in incubator at 25˚C for 3days .After purification, isolates were identified according to their morphological ,microscopical and pathological characteristics with the help of standard key (Gilman1957; Burnett and Hunter 1972; Nelson *et al*., 1983).

Pathogenicity tests

For pathogenicity tests ten isolates of *F. oxysporum* from thirty two isolates were selected. Pathogenicity of the isolates toward potato plants (Hermis and Spunta cvs.) were estimated on potted potato plants according to Khalifa (1991). Nine days old *F. oxysporum* isolates grown on potato dextrose agar (PDA) at 25 °C were transferred to 500 ml Erlenmayer flasks containing 250 ml PDB (potato dextrose liquid broth) and the flasks were incubated in shaker at 25 °C for 4 days. Cultures were passed through cheesecloth to separate the mycelia from the spores and the final concentration of the spore suspension was adjusted to10^8 spores/ml. Twenty ml of these suspensions was added to each pot in which potato tubers were grown. Three pots were used for each isolate and plants were observed for appearance of disease symptoms Pathogens were re-isolated from randomly selected diseased plants, the Koch's postulates were followed in order to confirm the pathogenicity of *F. oxysporum* isolates .

Determine the index of leaf damage

A scale of 0-4 according to (Beye and Lafay ,1985) was used to determine the disease severity every weak 0: none symptoms on leaf , 1: leaf wilted , 2:leaf with yellowing , 3:leaf with necrosis and 4: dead leaf .

ILD= $\sum$ notes / Max

ILD : Index of leaf damage

$\sum$ notes : Total notes

Max : 4 times of developed leaf number

Isolation and identification of antagonists:

From soil rhizosphere samples (20 cm deep) of healthy potato plants antagonists were isolated from different fields at Kafr El - Sheikh and Minufiya governorates, Egypt. The used bioagents *Pseudomonas fluorescens* and *Trichoderma harzianum* were isolated on selected media according to the methods recommended by (Burgess *et al* .1994) ; King *et al* . 1954 ; Davet 1979 and Turner *et al*. 1998) five isolates from each antagonist were used. After proper growth they were incubated at 25 °C., isolates were purified and identified on the basis of their morphological characteristics(Buchanan and Gibbons, 1974; and Rifai, 1969).

Praperatiom of modified MMT

The clay mineral used in this study was sodium MMT (Colloid BP) from Southern Clay Products Inc (Gonzales, Texas, USA) with cation exchange capacity (CEC) of 114.8 meq/100g. Chitosan (CS) with the deacetylation degree of 70 % and the molecular weight (Mw) of 400.000 was purchased from Aldrich chemicals. Triphenyl phosphine and chloroacetylchloride were purchased from Aldrich chemicals and were used as received without further purification. The structure of MMT is shown in Fig. 1.

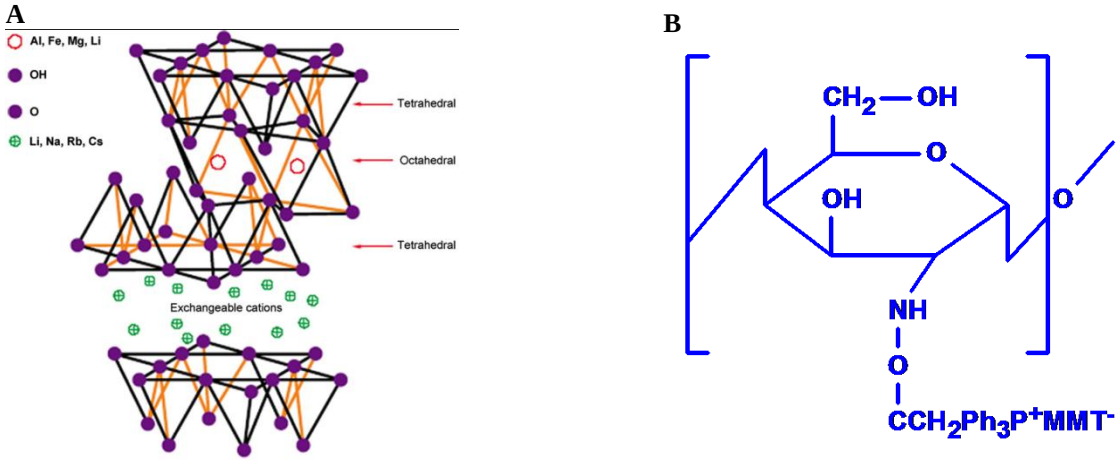


Fig 1: (A) Structure of MMT and (B) Structure of Mod-MMT

Chloroacetylation of chitosan.

Under magnetic stirring drops of pyridine (75.2 ml, 99.6 mmol) were added to 1 g chitosan. The mixture was cooled in an ice-salt bath and chloroacetyl chloride (7.965 ml, 99.6 mmol) was added drop wise with stirring. The reaction mixture was stirred overnight at 40 ºC for three days. The chloroacetylated chitosan was precipitated by addition of dilute HCl (1N), filtered off and washed with distilled water several times and the product was dried to give 0.95 g (N. Salahuddin,2013).

Synthesis of Triphenyl-chloro acetylated chitosan phosphonium salt (mod-CS).

2 g of chloro acetylatyed chitosan was dissolved in 20 ml dry DMF followed by addition of 5.33 g (20 mmol) triphenyl phosphine. The reaction mixture was stirred for four days at 80 °C in an oil bath. The product was filtered off, washed with DMF to give 1.5 g (N. Salahuddin,2013).

Preparation of Triphenyl- (chloro acetylated chitosan) phosphonium salt-montmorillonite intercalates (Mod/MMT).

1 g of Na-MMT was swelled in 30 ml water by stirring several hours at 40°C. A solution of 100 g mod-CS dissolved in 100 ml DMF was added and stirred for 24 hr. The product (Mod-MMT 1%) was filtered, washed several times with water and collected by a filter press and dried at 80 °C in vacuum oven for 10 hr. The same procedure was done using 3 g and 5 g of mod-CS to prepare (Mod-/MMT 3%) and (Mod-MMT 5%) respectively **(Table 1).**

Table 1. Composition data of mod-MMT with different concentrations.

Antagonistic effect *in vitro:*

Organo/clay Support	clay (wt)	Condition of reaction	Mod-Cs(wt)	Yield %
Mod-MMT (1%)	(1)	**Solvent method** at room temp.	(100 g)	75
Mod-MMT (3%)	(3g)	**Solvent method** at room temp.	(100 g)	82
Mod-MMT (5%)	(5g)	**Solvent method** at room temp.	(100 g)	89

Antagonism of *pseudomonas flourescens*

Discs (5mm) of 7days old mycelium of *Fusarium oxysporum f. sp. tuberose.*

Were placed in the one side of Petri plates with PDA. The streaking colony of *pseudomonas flourescens* were placed equidistant on the other side from plates. After 7days of inoculation at 25°C the percentages of antagonistic colonies of bacteria inhibit growth of *Fusarium oxysporum f. sp. tuberosi* mycelium in the whole population was assessed .

Antagonism of *Trichoderma harzianum*

The antagonistic effect of *Trichoderma harzianum* against *Fusarium oxysporum f. sp. tuberosi in vitro* was examined on Petri plates (9 cm. in diameter) containing potato dextrose agar (PDA) medium . Discs (5mm) of 7days old mycelium of *Fusarium oxysporum f. sp. tuberosi* was transferred to one side of Petri plates and the other side was inoculated with mycelia disc from *Trichoderma harzianum.* Five plates were inoculated with the pathogen only as a control . Plates were incubated at 25°C for seven days .

Antagonism of Montmorillonite particles

(Mod-MMT) solutions of 10, 30 and 50 ppm were prepared . 5 ml of each solution was spread aseptically on the surface of an inoculated PDA Petri dishes with mycelium of *Fusarium oxysporum f. sp. tuberosi*. Five plates were inoculated with the pathogen only as a control . All Petri dishes were incubated at 25°C for one week in the dark (due to the photocatalytic nature of nanocomposite). Five plates were inoculated with the pathogen only as a control .

The radial growth inhibited percentage of the pathogen was calculated using the equation (Frolich, 1979) of all bio-control agents.

I.P % = C – T / C × 100 where

I.P= inhibition %

C = Radial growth of control
T= Radial growth of treatment

The experiment included the following treatments in laboratory:

1- Control
2- *Trichoderma harzianum*
3- *Pseudomonas fluorescens*
4- MMT1%
5- MMT3%
6- MMT5%
7- MMT1% + *Trichoderma harzianum*
8- MMT1% + *Pseudomonas fluorescens*
9- MMT3% + *Trichoderma harzianum*
10- MMT3% + *Pseudomonas fluorescens*
11- MMT5% + *Trichoderma harzianum*
12- MMT5% + *Pseudomonas fluorescens*
13- MMT1% + *Trichoderma harzianum* + *Pseudomonas fluorescens*
14- MMT3% + *Trichoderma harzianum* + *Pseudomonas fluorescens*
15- MMT5% + *Trichoderma harzianum* + *Pseudomonas fluorescens*

Green house experiment

Plant materials

We selected a commercial potato cultivars (Hermis and Spunta cvs.) , which are susceptible to *Fusarium* potato wilt. Tubers were surface sterilized with 1% sodium hypochlorite (NaOCl) for 2 min, then triple rinsed with sterilized distilled water.

Pot experiment:

A pot experiment was designed under greenhouse conditions using plastic pots (15 cm, diameter) each pot containing 8 kg. of sterilized sandy clay soil. Soil was infested with *Fusarium oxysporum f. sp. tuberosi* grown on barely grain at the rate of 5g/kg soil before sowing. Infested pots were irrigated for 5 days before sowing. Three potato tubers (Hermis and Spunta cvs.) were sown in each pot, six replicate pots were specified for each treatment in completely randomized experimental design. The experiment included the treatments as laboratory.

The bio- control agents were applied in two methods

First :Tuber treatment

Spores with along mycelia fragments of *Trichoderma harzianum* and colonies of *Pseudomonas fluorescens* were collected from the surface of agar cultures after 7 days of inoculation by adding 10 ml of sterile distilled water . After collecting by brush, blended the mycelia was filtrated and centrifuged at 3000 rpm for 10 minutes. The spore suspension was adjusted to 5×10^9 conidia / ml using haemocytometer for *Trichoderma harzianum* and (10^8 cfu/mL) for *Pseudomonas fluorescens* . potato tubers were wounded and put in each suspensions of previous treatments for half an hour and sown in pathogen inoculated soil. Tubers with no treatments as control.

Second : Soil treatments

Suspensions inoculum of the antagonists was mixed with the soil at the rat of 10 ml / pot at planting time. The percentages of pre and post – emergence damping –off were recorded after 20 and 40 days from planting ,respectively, fresh weight of plant**,** plant height**,** weight of tubers/ plant and number of tuber / plant.

Population of *Fusarium oxysporum* f. sp. *tuberosi*

To determine the Population of *Fusarium oxysporum* f. sp. *tuberosi* pots and tubers infested with all bio- control agents individually or combine and compare the effect of them on the Population of *Fusarium*. Dilution plate technique were used and the population was expressed as colony forming units (cfu)/gm soil

Statistical analysis:

The obtained data were statistically analysed according to the method of Gomez and Gomez (1984).

Results and Discussion

Isolation and identification of *Fusarium oxysporum f. sp. tuberosi*

The isolated organism were including (10) pathogen isolates belonging to the genus *Fusarium* as cleared by preliminary microscopic examination. The isolated *Fusarium* were identified as *Fusarium oxysporum f. sp. tuberosi* according to (Gilman1957; Burnett and Hunter 1972; Nelson *et al*., 1983).

Pathogenicity tests of *Fusarium oxysporum f. sp. tuberosi*

All isolates of *Fusarium oxysporum f. sp. tuberosi* were pathogenic to all potato cultivars (Hermis and Spunta cvs.) and typical symptoms of *Fusarium* wilt disease were appeared . The symptoms appeared for 25 days after inoculation at 22-25°C. Inoculated potato plants showed yellow color of lower leaves, browning in the vascular stem , ascending wilt symptoms and inoculated plants completely wilted eighty days after planting (Fig 2) ,this results were similar to (Fakher *et al*., 2006).

A B

Fig (2) **Pathogenicity tests of *Fusarium oxysporum f. sp. tuberosi* under greenhouse conditions (A: healthy potato plant and B: inoculated plant by pathogen at 60 days after planting) .**

Determine the index of leaf damage (ILD)

There were significant reduced in index of leaf damage of potato plants(Hermis c.v) that treated with all biocontrol agents compared with control *(F. oxysporum f. sp. tuberosi*).The index of leaf damage of potato plants treated with Mod-MMT 5% combine with *T. harzianum* decreased significantly the severity of *Fusarium* wilt disease and it was 0.15, Mod-MMT 3% combine with *T. harzianum* 0.36 , Mod-MMT 1% combine with *T. harzianum* 0.46 , Mod-MMT 5% combine with *P. fluorescens* 0.67, MMT3% combine with *P. fluorescens* 0.91 , Mod-MMT1% combine with *P. fluorescens* 0.96 . In the other hand the index of leaf damage of potato plants treated with all biocontrol agents individually were also effective but its effects were lowest than the combined one . The ILD of potato treated with Mod-MMT5 %, Mod-MMT3% , Mod-MMT1% , *T. harzianum* and *P. fluorescens* were 1, 1.02,1.03,1.06 and 1.08 respectively compared with control (*F. oxysporum f. sp. tuberosi*) 3.2. Asimilar results were obtained with Spunta c.v.

Evaluation antagonistic effect *in vitro:*

The antagonistic activities of all biocontrol agents fungal , bacteria strains and nanocomposites were evaluated on Petri dishes containing PDA medium against *F. oxysporum f. sp. tuberosi in vitro* . Data in table (2) show that all concentration of nanocomposite (Mod-MMT) and bioagent strains succeeded in reducing the radial growth of *F. oxysporum f. sp. tuberosi* . Mod-MMT with concentration 5% was more active than Mod-MMT with concentration 3, 1% for reducing the radial growth of *F. oxysporum f. sp. tuberosi* (0.6 , 0.9 and 1 cm respectively) and these value equal to 88.88, 81.88 and 76.33 inhibition percentage respectively .Moreover, *T. harzianum* inhibited the over growth of *F. oxysporum f. sp. tuberosi*, by 56.33% comparing with *P. fluorescens* 51.11% . Both of nano particles and bioagents strains reduced growth percentage comparing with control of *F. oxysporum f. sp. tuberosi*. These results are agreement with (Howell , 2003)

Table (2) Effect of composite (MMT) and bioagents strains on the radial growth of *F. oxysporum f. sp. tuberosi in vitro.*

Bio- agents	Radial growth (cm)	Inhibition (%)
T. harzianum	3.93	56.33
P.fluorescens	4.40	51.11
MMT1%	3.0	66.66
MMT3%	2.13	76.33
MMT5%	2.0	77.77
MMT1%+ *T. harzianum*	1.00	88.88
MMT1% + *P.fluorescens*	1.9	78.88
MMT3%+ *T. harzianum*	0.9	90
MMT3% + *P.fluorescens*	1.8	80
MMT5%+ *T. harzianum*	0.6	93.33
MMT5%+ *P.fluorescens*	1.8	80
MMT1% + *T.harzianum* + *P.fluorescens*	3.79	57.88
MMT1% + *T.harzianum* + *P.fluorescens*	3.42 3.21	62 64.33
MMT1% + *T.harzianum* + *P.fluorescens*	9.00	00.00
Control		

LSD = 0.5017 at alpha 0.05

T.= Trichoderma *P.= Pseudomonas* MMT = montmorillonite particles

Green house experiment

Effect of bio control agents on *Fusarium* wilt disease

From table (3) we can indicate that, in tuber treatments adding tubers in suspensions of all concentrations of MMT combine with *T. harzianum* in both two cultivars Hermis and Spunta gave the lowest percentage of pre and post emergence damping off specially with concentration 5% (5.14,1.83, 6.72 and 3.55%) followed by concentration 3% (5.59, 3.19 , 7.12 and 5.44) respectively . Also all concentrations of Mod-MMT combine with *P. fluorescens* reduced the pre and post emergence damping off specially with concentration 5% (10.38 ,6.24 ,12.71 ,and 8.12) followed by concentration 3% (14.31 ,7.31 ,17.48 and 9.25) compared to control (*F. oxysporum f. sp. tuberosi*). Whereas *P. fluorescens* individually gave the highest percentage of damping off in either Hermis and Spunta cultivars (27.01 ,18.57

,31.22 and 19.1 % respectively) compared to all biocontrol agents. Data in table (4) showed that soil treatments with all tested biocontrol agents gave higher protection against *Fusarium* wilt disease than tuber treatments in both Hermis and Spunta cultivars and significantly reduced the percentage of pre and post emergence damping off from 33.17 and 20.89 to 1.66 and 0 % in Hermis cultivar and from 37.27 and 23.97 to 3.36 and 0% in Spunta cultivar. Also all biocontrol agents increased the percentage of the survivals .The highest percentage of survival was 98.34% and 96.64 % with treatment MMT 5% combine with *T. harzianum* but the lowest one was 64.04 and 58.65 % with treatment *P. fluorescens* individually in Hermis and Spunta cultivars respectively .

Table (3) Effect of tuber treatments with all tested antagonists on *Fusarium* wilt disease caused by *F. oxysporum f. sp. tuberosi* under greenhouse conditions.

Antagonists \ **Tuber treatments**	**Hermis cultivar**			**Spunta cultivar**		
	Pre-emerg.	**Post-emerg.**	**Survival**	**Pre-emerg.**	**Post-emerg**	**Survival**
T. harzianum	23.64 c*	16.45 c	59.91 h	27.52 c	16.8 c	55.68 g
P.fluorescens	27.01 b	18.57 b	54.42 h	31.22 b	19.1 b	49.68 h
Mod-MMT1%	22.43 cd	16 c	61.57 f	23.68 de	16.24 cd	60.08 f
Mod-MMT3%	23.27 c	15.5 c	61.23 f	23.08 e	15.8 de	61.12 f
Mod-MMT5%	23.03 cd	15.43 c	61.54 f	22.87 e	15.11 e	62.02 f
Mod-MMT1%+ *T. harzianum*	8.27 fg	5.04 g	86.69 b	10.53 h	7.39 h	82.08 b
Mod-MMT1%+ *P.fluorescens*	15.52 e	10.04 e	74.44 e	17.51 f	12.52 f	69.97 e
MMT3%+ *T. harzianum*	5.59 gh	3.19 h	91.22 a	7.12 i	5.44 i	87.44 a
MMT3%+ *P.fluorescens*	14.31 e	7.31 f	78.37 d	17.48 f	9.25 g	73.27 d
MMT5%+ *T. harzianum*	5.14 h	1.83 h	93.03 a	6.72 i	3.55 j	89.73 a
MMT5%+ *P.fluorescens*	10.38 f	6.24 fg	83.37 c	12.71 g	8.12 h	79.17 c
MMT1%+ *T. harzianum* + *P.fluorescens*	21.66 cd	15.56 c	62.78 f	23.2 e	13.21 f	63.59 f
MMT3%+ *T. harzianum* + *P.fluorescens*	21.31 cd	12.48 d	66.21 g	24.63 d	13.35 f	62.02 f
MMT5%+ *T. harzianum* + *P.fluorescens*	20.34 d	12.07 d	67.59 g	23.11 e	13.08 f	63.81 f
Control	40.17 a	26.07 a	33.76 i	44.45 a	28.76 a	26.79 i

***: There are significant differences between carrying different litters.**

Table (4) Effect of soil treatments with all tested antagonists on *Fusarium* wilt disease caused by *F. oxysporum f. sp. tuberosi* under greenhouse conditions

Antagonists \ Soil treatments	Hermis cultivar			Spunta cultivar		
	Pre-emerg.	Post-emerg.	Survival	Pre-emerg.	Post-emerg	Survival
T. harzianum	17.44 d*	10.59 cd	71.97 de	22.22 e	15.56 b	62.22 f
P.fluorescens	21.74 b	14.22 b	64.04 e	25.09 b	16.26 b	58.65 g
Mod-MMT1%	19.14 c	8.56 de	73.3 d	20.14 f	11.67 cd	68.19 d
Mod-MMT3%	17.82 d	7.66 e	74.52 d	18.68 g	9.39 e	71.93 d
Mod-MMT5%	15.28 f	5.32 fg	79.4 c	18.49 g	6.23 f	75.28 bc
Mod-MMT1%+ *T. harzianum*	4.51 j	0 h	95.49 a	6.16 j	5.25 fg	88.59 b
Mod-MMT1%+ *P.fluorescens*	12.42 g	5.13 fg	82.45 bc	16.47 h	6.35 f	77.18 c
Mod-MMT3%+ *T. harzianum*	3.39 j	0 h	96.61 a	4.11 k	0 h	95.89 a
Mod-MMT1%+ *P.fluorescens*	9.36 h	3.45 g	87.19 b	14.92 i	5.57 fg	79.51 c
Mod-MMT5%+ *T. harzianum*	1.66 k	0 h	98.34 a	3.36 l	0h g	96.64 a
Mod-MMT5%+	7.73 i	3.41 g	88.86 b	14.5 i	4.74 c	80.76 c

P.fluorescens						
Mod-MMT1%+ *T. harzianum* + *P.fluorescens*	15.2 f	11.49 c	73.31 d	23.79 c	12.83 d	63.38 f
Mod-MMT3%+ *T. harzianum* + *P.fluorescens*	16.03 ef	11.11 c	72.86 d	23.05 d	10.85 e	66.1 e
Mod-MMT5%+ *T. harzianum* + *P.fluorescens*	16.63 de	6.8 ef	76.57 c	22.15 e	8.16 a	69.69 d
Control	33.17 a	20.89 a	45.94 f	37.27 a	23.97	38.76 h

***: There are significant differences between carrying different litters.**

Growth parameters:

Effect of antagonists on fresh , dry weight and plant height of potato plants

The effect of antagonists on plant growth that presented in Table (5 and 6) reveal that, soil treatments with the antagonists increased the fresh , dry weight and plant height of potato plants more than tuber treatment of both Hermis and Spunta cultivars .

Data showed in Table (5) indicated low values of growth parameters, (fresh , dry weight of plants and plant height,) with the control treatment (6.45 , 8.36 , 1.89, 1.95 , 21.86 and 26.78) in both two cultivars Hermis and Spunta in comparison with other treatment. The growth parameters of potato plants were significantly increased with the dual inoculation of treatment MMT combine with either *T. harzianum* and *P. fluorescens* compared with the individual one. Data in table (6) revealed that treating the soil with all concentration of MMT5 combine with *T. harzianum* followed by the same treatment with *P. fluorescens* were the most effective in improving fresh , dry weight and plant height from 32.83 to 29.77 , 25.41 , 24.68 , 22.3 and 19.73 gm/ plant of fresh weight respectively in Hermis cultivar ; from 43.89 to 40.67 , 36.57, 31.58 , 31.48 and 27.68 gm/ plant of fresh weight respectively in Spunta cultivar ; from 11.36 , 10.23 ,10.11 , 9.36 , 9.11 and 8.38 gm/ plant of dry weight respectively in Hermis cultivar ; from 11.39 to 11.06 ,10.93 , 8.67 ,7.98 and 7.88 gm/ plant of dry weight respectively in Spunta cultivar ; from 62.78 , 95.24 , 54.14 , 55.71 , 50.32 and 44.98 cm of plant height in Hermis cultivar and from 83.18 , 79.16 , 78.63 , 72.94 , 68.48 and 64.25 cm of plant height in Spunta cultivar . Treatment of *P. fluorescens* individually was the least effective in this respect ,while the other antagonists fall in between .

Table (5): Effect of antagonists on fresh ,dry weight/ plant (gm) and plant height (cm) in tuber treatments experiment under greenhouse conditions.

Antagonists	Mean of fresh weight/plant(gm)		Mean of dry weight/ plant(gm)		Mean of plant height (cm)	
	Hermis	Spunta	Hermis	Spunta	Hermis	Spunta
T. harzianum	11.36 f*	14.26 g	2.81 c	3.71 d	30.25 f	40.85 f
P.fluorescens	8.67 h	9.64 j	2.22 c	2.81 d	27.64 h	35.56 h
Mod-MMT1%	11.26 f	14.0 g	2.87 c	3.88 d	28.24 g	39.25 g
Mod-MMT3%	13.37 e	14.21 g	3.21 c	3.88 d	29.14 g	42.17 f
Mod-MMT5%	13.36 e	15.36 f	3.16 c	3.87 d	31.25 f	42.36 f
Mod-MMT1%+ *T. harzianum*	18.66 c	24.37 c	5.11 a	6.87 c	43.58 c	52.36 c
Mod-MMT1%+ *P.fluorescens*	15.22 d	17.21 e	3.64 b	4.74 b	37.22 e	46.12 e
Mod-MMT3%+ *T. harzianum*	21.86 b	27.98 b	5.28 a	7.22 a	47.36 b	57.69 b
Mod-MMT3%+ *P.fluorescens*	15.58 d	17.68 e	3.88 b	5.01 b	41.18 d	46.58 e
Mod-MMT5%+ *T. harzianum*	23.95 a	31.29 a	6.39 a	8.38 a	51.23 a	59.59 a
Mod-MMT5%+ *P.fluorescens*	17.36 c	20.69 d	4.65 b	5.63 b	41.21 d	50.34 d
Mod-MMT1%+ *T. harzianum* + *P.fluorescens*	10.36 g	13.98 g	2.01 d	3.55 d	27.56 h	38.16 g
Mod-MMT3%+ *T. harzianum* + *P.fluorescens*	10.89 g	13.57 gh	2.66 c	3.57 d	29.48 g	38.15 g
Mod-MMT5%+ *T. harzianum* + *P.fluorescens*	11.06 f	14.59 g	2.66 c	3.88 d	29.56 g	40.81 f
Control	6.45 i	8.36 i	1.89 e	1.95 e	21.86 i	26.78 i

***: There are significant differences between carrying different litters.**

Table (6): Effect of antagonists on fresh ,dry weight/ plant (gm) and plant height (cm) in soil treatments experiment under greenhouse conditions.

Antagonists	Mean of fresh weight/plant(gm)		Mean of dry weight/ plant(gm)		Mean of plant height (cm)	
	Hermis	Spunta	Hermis	Spunta	Hermis	Spunta
T. harzianum	18.42 f*	22.02 f	7.23 bc	6.32 c	39.54 g	57.36 g
P.fluorescens	14.26 h	16.32 h	4.51 d	3.82 d	30.34 j	51.34 j
Mod-MMT1%	16.31 g	19.36 g	6.0 c	5.72 c	33.86 i	55.31 h
Mod-MMT3%	17.15 de	21.56 f	6.11 c	5.88 c	36.71 h	57.34 g
Mod-MMT5%	18.14 f	22.18 f	7.25 b	5.62 c	41.82 f	59.57 f
Mod-MMT1%+ *T. harzianum*	25.41 c	36.57 c	10.11 a	10.93 a	54.14 c	78.63 b
Mod-MMT1%+ *P.fluorescens*	19.73 e	27.68 e	8.38 b	7.88 bc	44.98. e	64.25 e
Mod-MMT3%+ *T. harzianum*	29.77 b	40.67 b	10.23 a	11.06 a	59.24 b	79.16 b
Mod-MMT3%+ *P.fluorescens*	22.38 d	31.48 d	9.11 b	7.98 b	50. 32 d	68.45 d
Mod-MMT5%+ *T. harzianum*	32.83 a	43.89 a	11.36 a	11.39 a	62.78 a	83.18 a
Mod-MMT5%+ *P.fluorescens*	24.86 c	31.58 d	9.36 b	8.67 b	55.71 c	72.94 c
Mod-MMT1%+ *T. harzianum* + *P.fluorescens*	17.85 d	19.46 g	5.68 cd	4.95 d	34.24 i	53.48 i
Mod-MMT3%+ *T. harzianum* + *P.fluorescens*	16.56 g	19.12 g	6.83 c	4.68 d	34.52 i	55.14 h
Mod-MMT5%+ *T. harzianum* + *P.fluorescens*	18.06 f	21.15 f	6.35 c	5.25 cd	38.82 g	56.57 g
Control	5.43 i	10.36 i	2.12 e	2.97 e	20.14 k	31.54 k

***: There are significant differences between carrying different litters.**

Effect of antagonists on number and weight of potato plants

Data in table (7 and 8) demonstrate that the application of all antagonists to soil gave the maximum yield as average number and weight of tubers compared with tuber treatments in both Hermis and Spunta cultivars. Treating soil withMod- MMT5% + *T. harzianum* increased the yield as average number of tubers / plant to 13.68 and 6.32 compared with control 1.79 and 1.04 tubers /plant in Hermis and Spunta, respectively and average weight of tubers to 63.85 and 68.34 gm/plant in Hermis and Spunta, respectively. In this respect *P.fluorescens* gave the minimum yield , whereas other antagonists fall in between.

Table (7) Effect of antagonists on average number of tubers /plant(gm) and average weight of tubers/ plant(gm) in tuber treatments experiment under greenhouse conditions.

Antagonists	Ave. number of tubers /plant(gm)		Ave. weight of tubers/ plant(gm)	
	Hermis	Spunta	Hermis	Spunta
T. harzianum	2.34 g*	2.23 g	27.31 de	32.26 de
P.fluorescens	1.4 h	1.167 h	14.39 g	18.57 g
Mod-MMT1%	2.43 g	2.05 g	21.58 f	25.28 f
Mod-MMT3%	3.01 fg	2.29 g	23.15 e	26.27 c
Mod-MMT5%	3.01 fg	2.77 ef	25.39 d	30.25c e
Mod-MMT1%+ *T. harzianum*	5.51 c	5.11 c	35.36 b	40.97 b
Mod-MMT1%+ *P.fluorescens*	3.54 ef	3.05 de	28.38 d	33.18 d
Mod-MMT3%+ *T. harzianum*	6.43 b	5.12 b	36.15 b	42.29 a
Mod-MMT3%+ *P.fluorescens*	4.04 de	3.23 de	31.19 c	37.28 c
Mod-MMT5%+ *T. harzianum*	8.2 a	5.12 a	39.26 a	42.36 a
Mod-MMT5%+ *P.fluorescens*	4.79 cd	3.33 d	31.26 c	39.26 b
Mod-MMT1%+ *T. harzianum* + *P.fluorescens*	2.69 g	2.37 fg	24.39 d	26.38 c
Mod-MMT3%+ *T. harzianum* + *P.fluorescens*	2.42 g	2.37 fg	24.26 d	30.56 e
Mod-MMT5%+ *T. harzianum* + *P.fluorescens*	2.81 fg	2.06 g	23.15 e	32.58 de
Control	1.43 h	1.06 h	8.26 h	9.35 h

***: There are significant differences between carrying different litters.**

Table (8) Effect of antagonists on average number of tubers /plant(gm) and average weight of tubers/ plant(gm) in soil treatments experiment under greenhouse conditions.

Antagonists	Ave. number of tubers /plant(gm)		Ave. weight of tubers/ plant(gm)	
	Hermis	Spunta	Hermis	Spunta
T. harzianum	6.58 d*	3.89 b	44.39 ef	50.37 f
P.fluorescens	3.66 f	2.167 c	32.69 i	36.57 i
Mod-MMT1%	5.28 e	3.08 b	36.16 h	40.98 h
Mod-MMT3%	6.54 d	3.19 b	40.86 g	45.39 g
Mod-MMT5%	6.38 d	3.26 b	45.19 f	49.37 f
Mod-MMT1%+ *T. harzianum*	10.26 b	5.01 a	56.37 c	59.36 c
Mod-MMT1%+ *P.fluorescens*	7.26 d	4.0 a	50.39 e	52.39 e
Mod-MMT3%+ *T. harzianum*	11.68 b	5.01 a	60.28 b	62.38 b
Mod-MMT3%+ *P.fluorescens*	8.28 c	4.0 a	51.13 d	53.39 e
Mod-MMT5%+ *T. harzianum*	13.68 a	6.32 a	63.85 a	68.34 a
Mod-MMT5%+ *P.fluorescens*	9.34 c	4.16 a	51.37 d	55.12 d
Mod-MMT1%+ *T. harzianum* + *P.fluorescens*	4.99 e	3.0 b	39.25 ef	41.86 h
Mod-MMT3%+ *T. harzianum* + *P.fluorescens*	5.12 e	3.01 b	41.29 g	47.38 g
Mod-MMT5%+ *T. harzianum* + *P.fluorescens*	5.68 e	3.16 b	44.35 ef	49.38 f
Control	1.79 g	1.04 d	9.68 j	10.28 j

***: There are significant differences between carrying different litters.**

Effect of tuber and soil treatment with some antagonists on the soil population of *Fusarium oxysporum* f. sp. *tuberosi*

Data in Figures (3 to 6) showed that, treating the tubers and soil with all bio control agents significantly decreased the population of *Fusarium oxysporum* f. sp. *tuberosi* by time compared with control (*Fusarium oxysporum* f. sp. *tuberosi*) in both Hermis and Spunta cultivars. Reduction in *Fusarium oxysporum* f. sp. *tuberosi* was investigated when MMT5%+ *T. harzianum* and MMT3%+ *P.fluorescens* were added to the soil. These bioagents reduced the population of *Fusarium oxysporum* f. sp. *tuberosi* from 31.25 and 35.25 to1.15 and 0.5 (cfu)× 10^6 gm/soil after 8 weeks of planting in Hermis and Spunta cultivars respectively .On the other hand the highest values of population was with *P.fluorescens* only compared with other bioagents . While the population was increased by time in the untreated control from 27.25 and 23.25 to 41.15 and 36.15 (cfu)× 10^6 gm/soil after 8 weeks of planting in Hermis and Spunta cultivars respectively

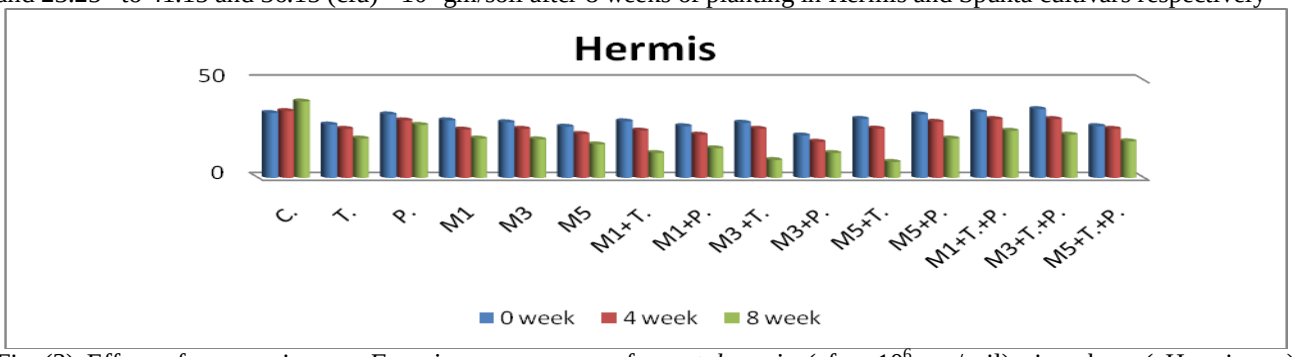


Fig (3) Effect of antagonists on *Fusarium oxysporum* f. sp. *tuberosi* (cfu× 10^6 gm/soil) in tubers (Hermis c.v) treatments at different intervals week

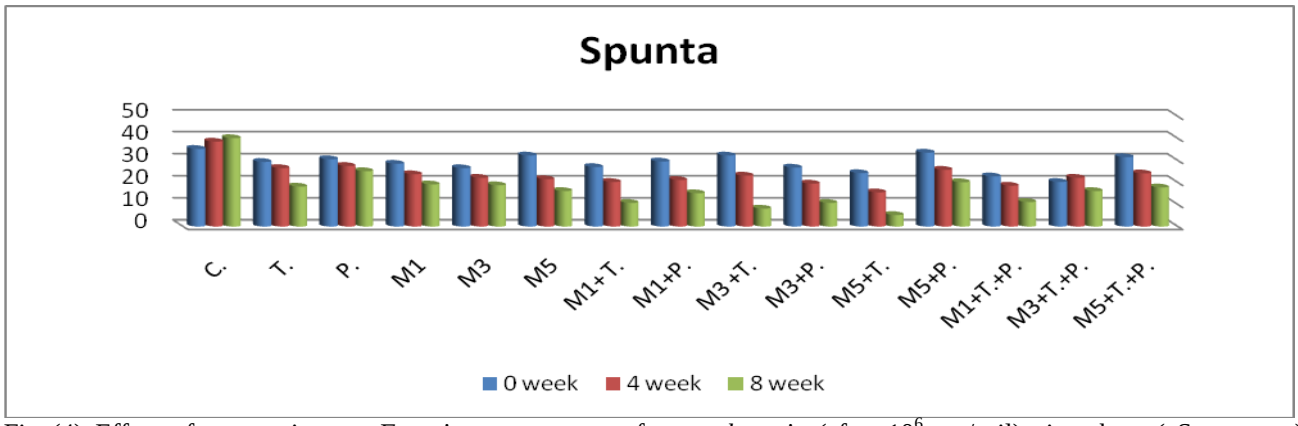


Fig (4) Effect of antagonists on *Fusarium oxysporum* f. sp. *tuberosi* (cfu× 10^6 gm/soil) in tubers (Spunta c.v) treatments at different intervals week

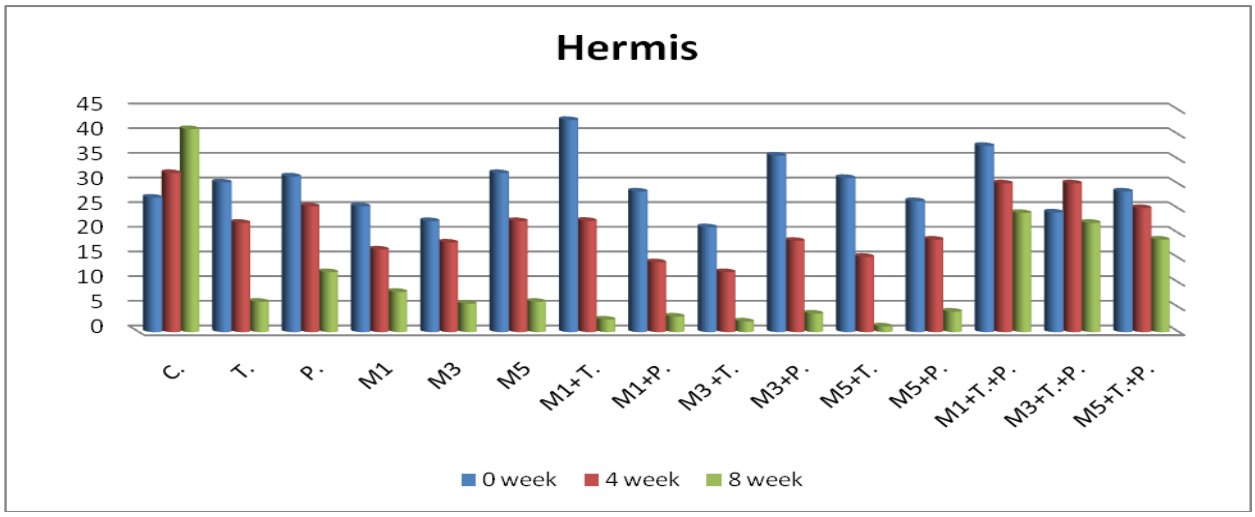
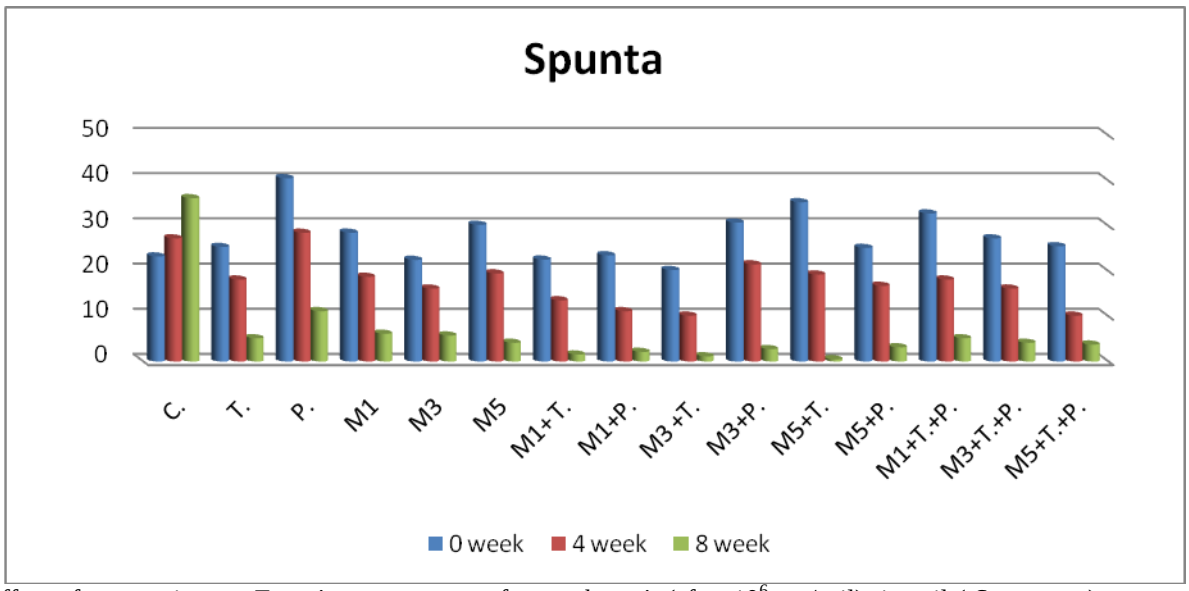


Fig (5) Effect of antagonists on *Fusarium oxysporum* f. sp. *tuberosi* (cfu× 10^6 gm/soil) in soil(Spunta c.v) treatments at different intervals week.



(6) Effect of antagonists on *Fusarium oxysporum* f. sp. *tuberosi* (cfu× 10^6 gm/soil) in soil (Spunta c.v) treatments at different intervals week

C=Control
T. =*Trichoderma harzianum*
P. =*Pseudomonas fluorescens*
M1= Mod-MMT1%
M3 = Mod-MMT3%
M5 = Mod-MMT5%
M1+T. = Mod-MMT1% + *Trichoderma harzianum*
M1 + P. = Mod-MMT1% + *Pseudomonas fluorescens*
M3+T. = Mod-MMT3% + *Trichoderma harzianum*
M3 + P. = Mod-MMT3% + *Pseudomonas fluorescens*
M5+T. = Mod-MMT5% + *Trichoderma harzianum*
M5 + P. = Mod-MMT5% + *Pseudomonas fluorescens*
M1+T.+P. = Mod-MMT1% + *Trichoderma harzianum* + *Pseudomonas fluorescens*
M3+T.+P. = Mod-MMT3% + *Trichoderma harzianum* + *Pseudomonas fluorescens*
M5+T.+P. = Mod-MMT5% + *Trichoderma harzianum* + *Pseudomonas fluorescens*

Discussion

Bio-agents have remarkable capacity of multiplication, thus, when applied; they multiply in exponential ratio and even can overcome stress condition by forming thick walled spores. Hence, bioagents are the solution for safer environmental issues and needs proper attention for seed treatment (Bharath *et al*., 2005). There are three strategies in considering biological control with introduced biocontrol agents : (a) to reduce the population of the pathogens , (b) to prevent the pathogen to infect the plant , and (c) to limit the disease development after infection (Cook, 1993). *Trichoderma* spp. are among the most - promising bio control agents that can be used against many fungal plant pathogens. *T. harzianum* has multiple mechanisms of action (Benitez *et al*., 2004), including antibiotics, competition, co-parasitism via production of chitinases, β-1-3 glucanases and β- - 4 glucanases, , solubilisation of inorganic plant nutrients, induced resistance and inactivation of the pathogen's enzymes involved in the infection process (Sivan and

Chet, 1993; Altomare *et al*., 1999; Elad and Kapat, 1999; Morsy, 2005 and Harman, 2006 .Our results are agreement with those recorded by(Ercole *et al* ., 1993 and Karunanithi and Usman, 1999). They suggested that the dual culture in Petri-dishes may be useful for detecting the micro-organism as biocontrol agent. The antagonistic effect of *Trichoderma spp*. may be due to faster mycelia growth than pathogenic fungi (Wei *et al.*, 1999). In addition to its produced the non-volatile compounds of ethylene and formic aldehyde (Karunanithi & Usman, 1999).

Also, our results indicat that *P. fluorescens* has great potential to be used as biocontrol agents for the management of the fusarium wilt of potato. *P. fluorescens* is consider from one of PGPR bacteria that have great potential in both phytostimulation and the biocontrol of plant pathogens (Akhtar and Sidiqui, 2009). The results from both soil and tubers treatments that were treated with *P. fluorescens* strains showed varying degrees of bioprotection that of fusarium wilt of potato (Kloepper *et al*., 1980; Maurhofer *et al*., 1995; Raaijmakers and Weller, 1998). PGPR antagonize plant pathogenic fungi, mainly by the production of antimicrobial metabolites but also by the competition for iron or rhizosphere niches (Keel et al., 1992) and the stimulation of the host defenses (induced systemic resistance) (Van Loon *et al*., 1998). Other mechanisms are involved directly in the promotion of plant growth and modulate the biocontrol activity of the bacteria (Lemanceau and Alabouvette, 1993; De Werra *et al*., 2009).

In this study, MMT was modified by modifies chitosan with different ratio producing nanocomposites with positive charge. The positive charges of these nanocomposites enhance their ability to adsorb bacteria through electrostatic interactions. In addition, there may be hydrophobic interaction between mod-chitosan and lipophilic components of the bacterial cell walls such as lipoproteins, liposaccarides and phospholipids. Therefore, microorganisms in this system can be adsorbed and immobilized on the surface of the nanocomposites and then biocide exerts its effect. And this agree with our result give the highest mean inhibition values (N. Salahuddin,2013)..

The obtained results of this study suggest that the tested microorganisms proved to be an effective bioagents in controlling the tested potato pathogenic fungi and it can reduce the environmental pollution in controlling plant diseases.

Conclusion

From the results of present study it is concluded that, although all bio control agents applied individually reduced disease incidence, under laboratory and greenhouse conditions. Species *Trichoderma.harzianum* and *Pseudomonas fluorescens* combine with nanocomposites Mod-MMT5% and Mod-MMT3% have great potential to control *Fusarium* wilt disease caused by *Fusarium oxysporum* f. sp. *tuberosi*.

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