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POTENTIAL OF *Trichoderma viride* AND *Rhizobium leguminosarum* IN COMBINATION WITH TOPSIN M70 FUNGICIDE FOR MANAGEMENT DAMPING-OFF DISEASE OF PEA PLANTS CAUSED BY *Rhizoctonia solani*

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ABSTRACT: Efficacies of *Trichoderma viride*, *Rhizobium leguminosarum* and the fungicide Topsin M70% individually and/or their mixtures were tested *in vitro* and greenhouse conditions to control damping-off and root rot diseases of pea plants (*Pisum sativum* L., cv. Master P) caused by *Rhizoctonia solani.* The ability of tested *Rhizobium leguminosarum* and *Trichodermia viride* to exhibit plant growth promoting rhizobacteria (PGPR)-properties including ability to solubilize-P and production of IAA, as well as production of siderophores, hydrocyanic acid (HCN) and secretion of cell-wall degrading enzymes (chitinase and protease) were investigated. Also, under *in vitro* conditions the effect of Topsin M70% on growth of *R. solani* and *T. viride*, *R. leguminosarum* and their mixtures was determined. The fungicide effective concentration was found to range from 10 to 50 ppm for *R. solani* and *T. viride* mycelial growth being 83.30, 100, 76.08 and 100% at 40 and 50 ppm of Topsin M70%. The same trend was obtained with *R. leguminosarum* that showed maximum tolerance at 40 and 50 ppm of Topsin M70% with the average of 52.19 and 59.85% inhibition over control, respectively. Additionally, greenhouse conditions were conducted on sandy clay soil at Etay El Baroud Agricultural Research Station, Beheria Government, Egypt to study the singly effect of seed soaking in *T. viride*, *R. leguminosarum* or their mixtures with the soil drench fungicide in pots after sowing of pea cv. Master P in concern. Significant decrease of damping-off and root rot of pea was obtained. Numbers of survived plants, shoot length as well as, fresh and dry weight were recorded. The combined treatments *R. leguminosarum* + Topsin M70%, *T. viride* + Topsin M70% and *R. leguminosarum* + *T. viride* + Topsin M70% were the most effective ones resulting the least percentage of total damping-off and the highest percentage of healthy plants being 96.67 and100.00%, respectively. Seed treatment with *T. viride*, *R. leguminosarum* and drenched fungicide individually or in mixture improved plant growth as indicated by the increased growth parameters and the physiological activities (phytosynthetic pigments, peroxidase and polyphenol oxidase), especially in combined treatments. Peroxidase and polyphenol oxidase activities were increased in the different treatments- even, the fungicide drench treatment. Reduction in total damping-off was positively correlated with both peroxidase ($R^2 = 96.70$, $P < 0.005$) and polyphenol oxidase ($R^2 = 89.60$, $P < 0.005$) activities.

Key words: Pea, damping-off, *T. viride*, *R. leguminosarum*, PGPR- properties, peroxidase, polyphenol oxidase.

INTRODUCTION

Pea (*Pisum sativum* L.) is one of the most important leguminous plant. It is considered as most important inexpensive source of protein in Egypt. However, soil-borne fungal pathogens limited its growth and productivity in Egypt and around the world. *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk.) attacks a wide range of plant species

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causing seed decay, damping- off and root rot (**Ogoshi, 1976; Abawi *et al.*, 1985**). The present research discusses the need of biological ways than dependence on chemical ones. Biological agents may take some time to establish in soil before their significant effect are seen on disease control and crop yield. Several application schedules may be required to obtain desired effect keeping in consideration human and animal health. Consequently searching for alternative control approaches were considered (**Pal and Gardener, 2006**).

Rhizobia, which have been extensively studied as plant growth promoting rizobacteria (PGPR), have also been found to be an important biocontrol agent for the inhibition of several soil - borne plant pathogens.

PGPR isolated from plant rhizosphere have been shown to inhibit plant pathogens either directly through competition for nutrients, competition for iron by siderophores, antibiosis or lytic enzymes as well as inducing systemic resistance (ISR), or indirectly as a biocontrol agent inhibiting the growth of the pathogen (**Deshwal *et al.*, 2003; Lugtenberg and Kamilova, 2009**). Also, other reports (**Estevez *el al.*, 2002; Baraka *et al.*, 2009**) revealed that *Rhizobium* spp. was effective to control of pathogen legume crops, such as *Rhizoctonia solani*., *Fusarium* spp., *Sclerotium rolfsii*, *Macrophomina phaseolina* and *Pythium* spp. In this connection, **Papavizas (1985) Dubey *et al.* (2007) and Bastakoti *et al.* (2017)** reported that *Trichoderma viride* and *Trichoderma harzianum* were naturally existing biological agents against *R. solani, Fusarium oxysporium, Sclerotium* spp. and *Pythium* spp. Antagonistic activity of *Trichoderma* strains is attributable to one or more complex mechanisms, including mycoparasites, nutrient competition, antibiosis, the activity of cell wall-lytic enzymes, induction of systemic resistance and increased plant nutrient availability (**Naseby *et al.*, 2000**). Combined inoculation of *Rhizobium* sp. and a biocontrol fungus *Trichoderma* sp. decreased damping –off and root rot disease; increase growth, nutrient uptake and yield of several legume crops under glasshouse and field conditions (**Shaban and El-Bramawy, 2011**). Hence, combining these two organisms together may have a great potential value in organic agriculture to avoid the side effects of chemical fertilizers and pesticide. **Mahmood *et al.* (2015)** evaluated the combination of bio-control microorganism and fungicide in glasshouse assay; they concluded that combination of fungicide and biocontrol agents proved to be more effective than individual one. Also, **Ainmisha and Zacharia (2011)** found that, minimize of wilt of chickpea caused by *Fusarium oxysporum* could be useful application by carbendazim and *Trichoderma viride.*

Nowadays, there are strict regulations on chemical fungicides used in disease management. Consequently, effort on developing alternative inputs controlling disease and management of chemical fungicides usage were done by **Asghar and Mohammad (2010)**. Combination fungicide at reduced rates with biocontrol agents has significantly enhanced disease control, compared to biocontrol agent alone (**Frances *et al.*, 2002; Buck, 2004**). Integrated use of biocontrol agent with reduced dose of fungicide was effective against Fusarium crown and root rot of tomato (**Omar *et al.*, 2006**), late leaf spot of groundnut (**Kishore *et al.*, 2005**), Rhizoctonia root rot and take-all disease of spring wheat (**Duffy, 2000**).

The objectives of the present study were to evaluate potential of *T. viride*, *R. leguminosarum* bioagents to control *R. solani* the causal agent of pea damping-off disease and to enhance growth parameters under greenhouse conditions. Also, identify the associated biochemical changes in plants and the ability of *T. viride* and *R. leguminosarum* for their PGP induction.

MATERIALS AND METHODS

In vitro Experiments

The ability of the tested bioagents

The tested *Rhizobium leguminosarum* was obtained from the Biofertilizes Production Unit, Agric. Microbiology. Dept., Soils, Water and Environ. Res. Inst. (SWERI), Agric. Res. Center (ARC), Giza, Egypt. *Trichoderma viride* was also kindly obtained from Mycology Research and Plant Diseases Survey Department, Plant Pathology Res. Inst., ARC, Giza, Egypt.

Bacteria culture was grown on tryptone yeast agar (**Beringer, 1974**) in petri dishes for 48 hr., at room temperature (25±2°C). The resulting colonies in each dish were suspended in 5ml of 1% methyl cellulose (Sigma-Aldrich, Milwauka, USA) in sterile distilled water, and scraped gently with a spatula to obtain bacterial slurries.

T. viride was grown on Molasses M medium in Erlenmeyer flasks (500 ml.) containing 200ml medium (**Abd El Moity, 1985**).

Assay of PGP-related properties of tested bioagents

Ability of the *Rhizobium leguminosarum* and *Trichoderma viride* to exhibit PGP-properties was evaluated *in vitro* conditions, through the determination of their efficiency to solubilize phosphate on dichloropropionic acid (DCP) media **Pikovskaya (1948)** while production of indol acetic acid (IAA) was also evaluated according to **Gordon and Waber (1951)**. Production of siderophores was estimated according to **Alexander and Zuberer (1991)**. Meanwhile their ability to produce hydrocyanic acid (HCN) was checked as described by **Bakker and Schippers (1987)**, as well as chitinase and protease production were checked according to **Lingappa and Lockwood (1962) and Dunne *et al*. (1997)**.

Effect of a fungicide on bicontrol agents and *R. solani*

The effect of the fungicide Topsin M70% (Table 1) on *R. solani* and *T. viride, R. leguominosarum* strains was evaluated *in vitro* through the ability of these bioagents growth on media saturated with different concentrations of Topsin M70% according to the methods described by **Mohiddin and Khan (2013)**. Different concentrations of the fungicide were prepared (10, 20, 30, 40 and 50 ppm) in distilled water and used in the experiment. PDA plates without a fungicide but inoculated with the fungi and bacteria served as a control. Each treatment was replicated three times. The inoculated plates were incubated at 25±2 ˚C or 5 days. The radial growth of the colony in each treatment was measured and the percent inhibition of growth was calculated by the formula:

$$I = \frac{C - T}{C} \times 100$$

Where:

I = Growth inhibition percent, C = Radial growth in control (cm), T = Radial growth in treated plates (cm).

Isolation and identification of *Rhizoctonia solani*

Roots of pea, showing typical symptoms of root rot was collected from infected field in Etay-El Baroud Research Station. Surface sterilized, transferred to Petri dishes containing PDA medium and incubated at 25±2˚C. Finally growth fungus was purified using hyphal tip technique (**Domsch *et al*., 1980**). The isolated fungal pathogen was identified by the aid of Department of Mycology, Plant Pathology Institute, Agricultural Research Center Giza, Egypt.

Determination of chlorophyll content

Total chlorophyll, Ch. a and Ch. b contents were determined following the method of **Mackinney (1941)** at 25-30 days old pea plants and at harvest. The final volume of extract was made to 40 ml. The absorbance of the extract was read at 645 and 663 nm in spectrophotometer (Elico Sl. 159) and for blank 95 percent acetone was used expressed in mg/g fresh weight.

$$\text{Chlorophyll a}'' = (12.7 \times A663) - (2.69 \times A645) \times \frac{V}{1000 \times W \times a}$$

$$\text{Chlorophyll b}'' = (22.9 \times A645) - (4.68 \times A663) \times \frac{V}{1000 \times W \times a}$$

A663 = Absorbance of the extract at 663 nm

A645 = Absorbance of the extract at 645 nmg

W = Fresh weight of the sample (0.25)

V = Volume of the extract (25 ml)

a = Path length of light (1 cm)

Total chlorophyll = Ch. a + Ch.b

The amount of chlorophyll content a and b in the sample was calculated by using the previously maintained formulae.

Determination of oxidative enzymes

The activity of the oxidative enzymes, peroxidase (PO) and polyphenol oxidase (PPO) were determined in the 30 – day old pea plants grown under greenhouse conditions.

Table 1. Chemical name, common name, recommended dose and active ingredient of the investigated fungicide

Fungicide	Chemical name	Trade name	Recommended dose	Active ingredient
Topsin M70%	dimethyl 4,4- (o-phenylene) bis (3-thioallophanate)	Topsin M70%	70g/100 l water	Thiophanate methyl

Preparation of enzymatic filtrate

The enzymatic filtrate was prepared according to **Maxwell and Baterman (1967)**. The resulted supernatant fluid was used for enzyme assays.

Peroxidase activity

Peroxidase activity was determined according to the method described by **Allam and Hollis (1972)** by the oxidation of pyrogallal to pyrogallin in the presence of H_2O_2 at 425 nm. The rate of peroxidase activity was expressed as the change in absorbance at 425 nm/gram fresh/min.

Polyphenol oxidase

The activity of polyphenol oxidase was measured as described by **Matta and Dimond (1963)**. The rate of polyphenol oxidase was expressed as the change in absorbency at 490 nm/gram fresh/min.

Greenhouse Experiments

Pathogenicity of *Rhizoctonia solani* isolates

Experiments were carried out in artificially infested sandy clay soil at the greenhouse of Etay El Baroud Agricultural Experiments Station, in Beheria Government, Egypt. *R. solani* inoculum was prepared by growing the fungus on autoclaved maize-sand medium in glass bottles for 15 days at 25°C. Soil infestation was achieved by mixing inoculum of *R. solani* with the soil at the rate 3% (*W/W*) in sterlized sandy clay pots (25× 25× 30 cm) and watered regularly for one week before planting. The same amount of fungus free autoclaved maize sand medium was added to the soil to serve as a control. Each pot was sown by ten seeds of pea (Master P) and watered when needed in a complete randomized design with four replicates. The percentage of seed germination was estimated after one week of seedling emergence. The higher pathogenic isolate was used in the next experiments.

Combined evaluation of bio-control microorganisms and a fungicide in greenhouse assay

Seeds of pea (Master P) were first surface sterilized with sodium hypochlorite 1% and soaked in prepared bacterial suspension for 20 min, spread on screen cloth with paper towel to absorb the excess slurry, and air- dried overnight (**Rudresh *et al*., 2005**). *T. viride* was applied as seed soaking in spore suspension of 5×10^6 conidia/ml at 30 min (**Shaban and El-Bramawy, 2011**). Infested soil with the pathogen was prepared as described above in pathogenicity. In each pot ten seeds were sown and four replicates were used for each treatment in (Table 2). Percentage of pre and post – emergence damping-off and survival were calculated at 15, 30 and 45 days after planting, respectively. The pots were arranged in a randomized complete block design (RCBD) with four replicates. The Fungicide Topsin M70% proved to be recommend at rate (0.7 g/l) was drenched in pots as described above in Table 1 after two days from seeding in pots.

Statistical Analysis

Results were expressed as mean ± standard error (SE). the data were analyzed by using Two-Way ANOVA followed by LSD test through SPSS 16 (version 4). The treatment means were compared using least significant difference (LSD) test at level of probability 5% as described by **Gomez and Gomez (1984)**.

Table 2. Randomized design of experimental setup

Treatment set no.	Abbreviation	Treatment
1	Rh	Rhizobium
2	Tv	*Trichoderma viride*
3	Fu	Fungicide Topsin M 70% WP (70g/100 l water)
4	Rh+Tv	Rhizobium + *Trichoderma viride*
5	Rh+Fu	Rhizobium + Fungicide
6	Tv+Fu	*Trichoderma viride* + Fungicide
7	Rh+Tv+Fu	Rhizobium + *Trichoderma viride* +fungicide
8	Control (CI) Infected	Infected soil with *Rhizoctonia solani*
9	Control (CH) Healthy	Healthy plants (Non infected)

All comparison were made with treatment 8 and 9.

RESULTS AND DISCUSSION

In vitro Ability of the Tested Bioagents for PGP Induction

Results in Table 3 show that, both of the tested microorganisms were apparently able to trigger PGP *in vitro* conditions.

The result of the qualitative and quantitative IAA reflected the ability of the two tested bioagents to produce indole compounds as they exhibited a pink to red colours with little variation in intensity. However, the highest value of auxin production was obtained by *T. viride* followed by the *R. leguominosarum* as they produced with the averages of 105.61 and 82.84 µg/ml, respectively. Several investigators reported that indole secretion, consider as a vital mechanism, to clarify plant promotion to stimulate root growth that helps plants to tolerate fungal plant pathogens (**Zahir *et al*., 2004; Abdel-Wahab *et al*., 2008**).

Hydrocyanie, a secondary metabolite produced by several microorganisms, has deleterious effect on the growth of some microbes (**Knowles, 1996**). Meanwhile, *T. viride* showed a reddish brown colour in the *in vitro* test which an indication to its ability to produce cyanide. However, *R. leguominosarum* was considered a moderately cyanogen as appeared as yellow to light brown colour. Some rhizospheric microorganisms have been known to protect their host plants through the inhibition of pathogen growth by HCN production (**Deshwal *et al.,* 2003**).

The ability of the tested PGP bioagents for solubilizing calcium phosphate was examined and the results are presented in Table 3 and Fig. 1. It was apparent that ability to dissolve phosphate was markedly differed among the tested PGP bioagent, which reflected by the variation of clearing zone extend for each tested bioagent. Diameters of the developed clear zone values were 2.8 and 2.1 for *T. viride* and *R. leguominosarum*, respectively revealing that bioagents were strong phosphate solubilizers as the amounts of available phosphorus measured were 111.25ppm and 106.89 ppm for *T. viride* and *R. leguominosarum*, respectively. In this concern, **Altomare *et al*. (1999) and Neumann and Laing (2006)** reported the capability of the plant-growth-promoting and biocontrol agents to solubilize *in vitro* some insoluble minerals *via* three possible mechanisms of acidification of the medium, production of chelating metabolites, and redox activity which import plant growth against plant pathogens.

Results presented also in Table 3 and Fig. 1 elicited the qualitative screening of the tested bioagents for excreting siderophores compounds. It is clear that both tested bioagents are able to produce siderophores. *T. viride* was superior for

Table 3. *In vitro* ability of the tested bioagents induction

PGP-properties		*R. leguominosarum*	*Trichoderma viride*
IAA-production	Colour intensity	++	+++
	Quantity (µg/ml)	82.84	105.61
Hydrocyanic (HCN)	Colour intensity	++	+++
P-solubilization	Zone diameter (cm)	2.8	2.1
	Quantity (ppm)	106.89	111.25
Siderophores production	Colour intensity	+ve	++ve
Protease production	Clear zone	++	+
	Zone diameter (cm)	2.5	1.2
Chitinase production	Clear zone	+	++
	Zone diameter (cm)	1.9	3.2

- Negative result; +, low; ++, moderate; +++, high.

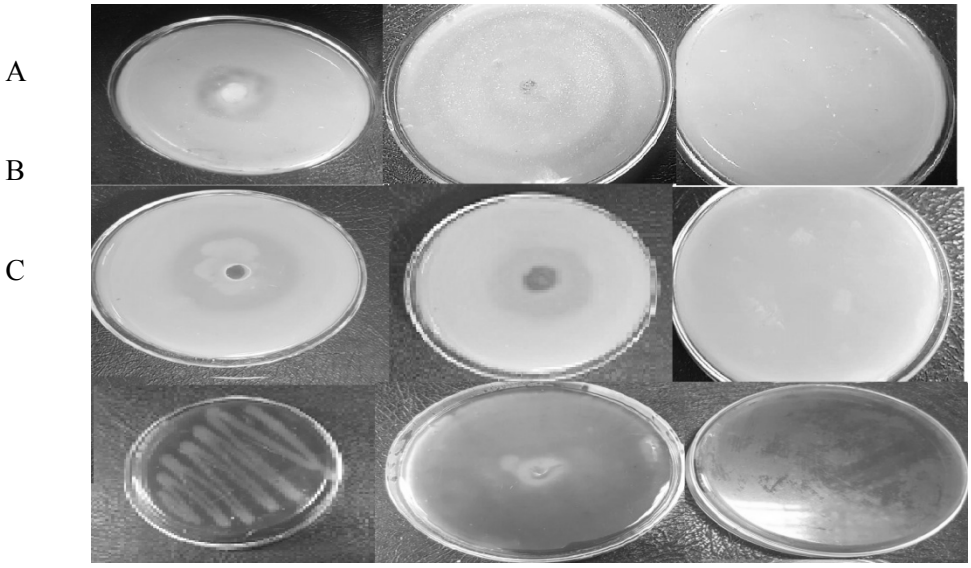

Fig. 1. The *in vitro* ability of tested bioagents to induce PGP A: Phosphate solubilization B: Protease production C: Siderophores production

siderophores excretion, while *R. leguominosarum* was recorded as moderately producers. In fact, under iron-limiting conditions, some microorganisms produce a range of low molecular weight compounds, namely siderophores which are able to acquire ferric iron. These iron chelators are thought to sequest the limited supply of iron available in the rhizosphere, thereby depriving pathogenic fungi of this essential element and consequently restricting their growth, as well as contribute to iron uptake and transport in the plant. Thus, cyanide and siderophores have an important role in the biocontrol activity against soil borne phytopathogens, beside the essential function of siderophores in the improvement of iron nutrition. These results are in harmony with those obtained by **Press *et al.* (2001), Zahir *et al*. (2004) and Neumann and Laing (2006)**.

Results of the same Table and Figure showed the chitinolytic and protiolytic activities indicating the ability of tested bioagents to form clearing zone around their growth colonies. *T. viride* and *R. leguominosarum* exhibited chitinolytic activity being of clear zones of 3.2 and 1.9 cm in diameter, respectively. However, *R. leguominosarum* showed higher protiolytic activity as exerted 2.5 cm diameter of inhibition, while, *T. viride* a clear zone of (1.2 cm). Several reports have demonstrated positive relationship between the production of chitinase and protease and the ability to control plant disease. Enzymes induction by bioagents during the parasite interaction could inhibit the growth of several fungal plant pathogens by degrading cell walls (**Melo and Faull, 2000; Harman *et al*., 2004**). The destructive parasitizing of lyses of the pathogen by extracellular, degrative enzymes such as chitinas considered an effective mechanism implicated in biological control against soil borne pathogenic fungi (**Chet *et al.*, 1990; Bastakoti *et al*., 2017**). Microbes with these enzymatic activities positively induce the nodulation when used in combined with rhizobia to inoculate legumes seeds (**Sindhu and Dadarwal, 2001**).

Consequently, as *T. viride* and *R. leguominosarum* induce PGP- properties *in vitro* testes, they were selected to apply as seed soaking for peas singly or in mixtures grown in soil infested with *R. solani.*

Effect of a Topsin M70 on biocontrol agents and *R. solani*

Results in Fig. 2 illustrate the growth of *R. solani*, *T. viride* and *R. leguoeminasarum* in medium saturated with different conc. of Topsin M70%. Obtained results showed that the highest inhibitory effect of *R. solani* was noticed with Topsin M70% at rate 40 and 50 ppm with the averages of 83.48 and 100%, respectively. Increase in conc. of Topsin M70% was directly proportion to increase inhibition growth percent of bioagents. *T. viride* has more mycelial growth inhibition (76.08%) at 40 ppm of fungicide, while at 10 ppm the growth inhibition percent of *T. viride* was 51.27%, which showed resistance of *T.viride* to different fungicidal conc. as proved in results obtained. This finding was in harmony with those of **Singh and Singh (2007)** who evaluated five pesticides (carbendazin, captan, vitavax, monocrotophos and thiram) against seven biocontrol agents, namely, *T. viride*-1*, T. viride*-2*, T. harzianum*-1, *T. harzianum*-2, *T. harzianum*-3, *Glioclodium virens* and T-35 (*T. harzianum*) and they found that all the tested pesticides inhibited growth of all these biocontrol agents to varying extends. The same trend was obtained with *R. leguoeminasarum*, in which, the growth inhibition percents of Rhizobium at different fungicide conc. 10, 20, 30, 40 and 50 ppm were (34.66, 41.08, 46. 66, 52.19 and 59.85%). In addition, *T. viride* when mixed with *R. leguoeminasarum* in medium saturated with different conc. of Topsin M70%, obtained results was illustrated reading visible in Fig. 3 appeared the growth of *T. viride* limit at 50 ppm. On the other hand, *R. leguoeminasarum* showed that the safe tolerance limit were 40 and 50 ppm. Similar results have been obtained by other workers **Mohiddin and Khan (2013)** found that the biocontrol bacteria *viz.*, *Pseudomonas fluorescens* and *Bacillus subtilis* were found more tolerant to fungicidal action than fungi. This may be due to that, some bacteria can use pesticides as nutrients and hence can tolerate higher concentrations of chemicals (**Kishore and Jacob, 1987; Aislabie and Jones, 1995**).

Moreover, the ability tolerance fungicide broadened the use at these bioagents in conjugation with fungicides can be selected to apply as seed pea soaking singly or mixture in soil infested with *R. solani* in the integrated disease management.

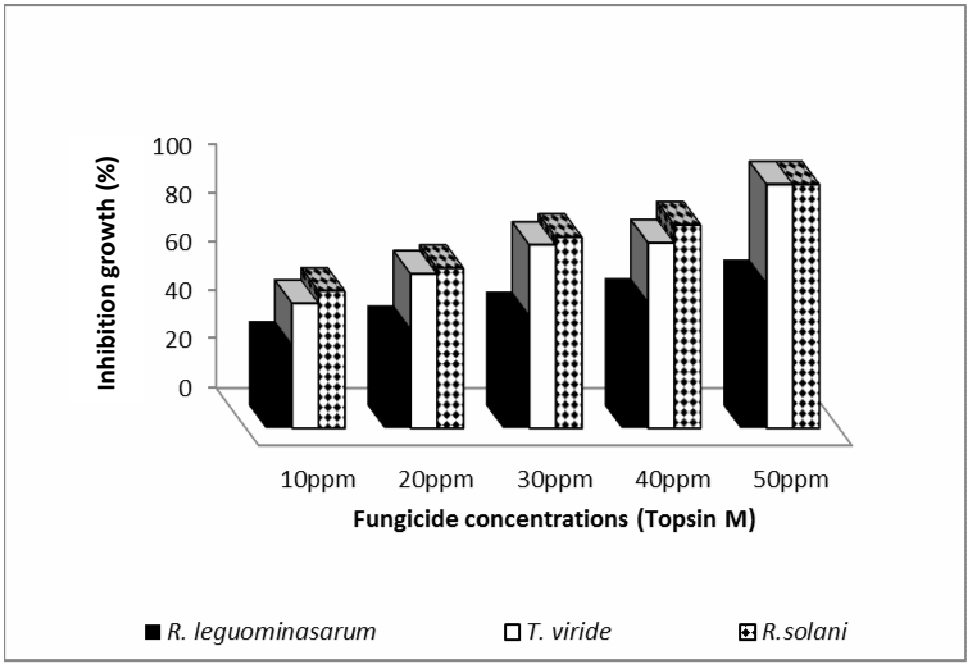

Fig. 2. Effect of various concentrations of Topsin M70% on inhibition growth percentage of *R. leguoeminasarum*, *T.viride* and *R. solani in vitro* assay

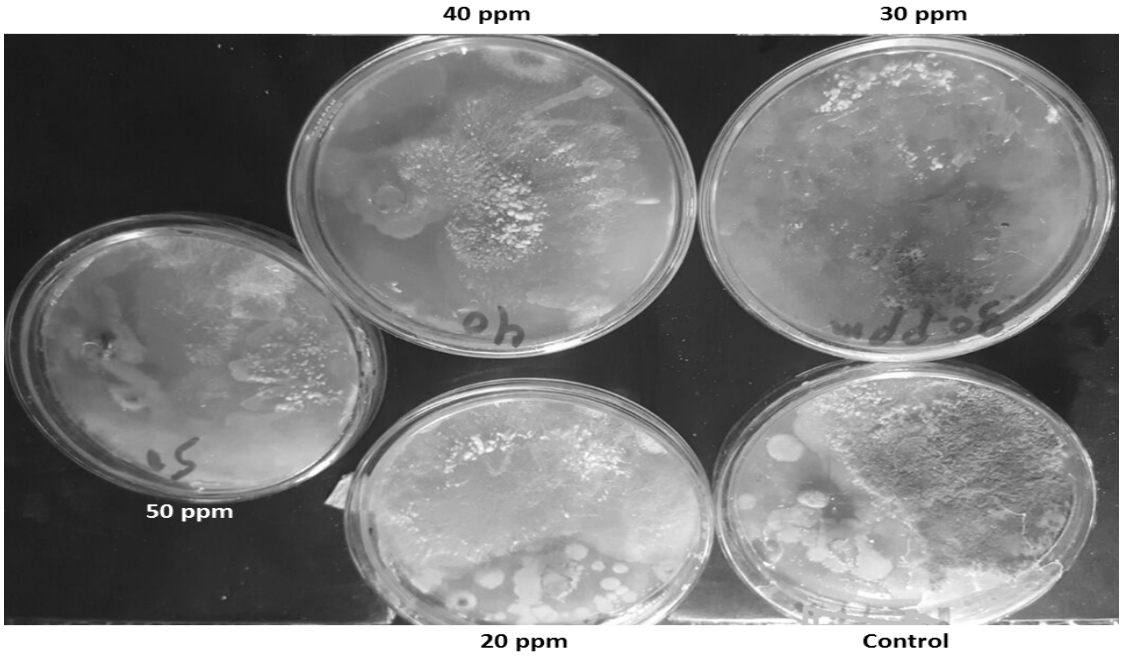

Fig. 3. Effect of various concentrations of Topsin M70% on mixture growth of *R. leguoeminasarum* and *T.viride in vitro* assay

Pre and Post Emergence Damping-off as Effected by Combined Bioagents and Topsin M70% under Greenhouse Conditions

Results in Table 4 indicate significant differences for pre and post emergence and total damping off among all treatments at level probability of 5 or 1%. Individual seed soaking with *R. leguominosarum* (Rh), *T. viride* (Tv) and fungicide (Fu) recorded the lowest percentages of pre emergence damping-off with averages of 53.33, 60.00 and 3.33%, respectively compared with control (66.67). The highest percentage of infection with *R. solani* (CI) being 20.00, 10.00 and 95.00%, respectively. The obtained results are in harmony with those reported by **Bardin *et al.* (2004)** who found that the seeds treated by *R. leguominosarum* bv. *viceae* was effective in decrease damping-off in pea. Soil application with *T. hamatum*, *T. harzianum* or *T. viride* are active colonizers in soil (**Akramin *et al*., 2009**) and produce antibiotics like trichodermin, gliotoxins, viridian, cell wall- degrading enzymes (**Bruckner and Przbylski, 1984**) and certain biologically active heat stable metabolites like ethyl acetate. These substances may inhibit the activity of soil-borne pathogens (**Chet and Baker, 1981; Khan *et al*., 2004 and 2011**). Reduction of damping-off may be due to antagonistic effects *T. viride* and *R. leguominosarum* as shown in Table 4. Furthermore combined application of Topsin M70 with *R. leguominosarum* and or *T. viride* were more effective compared with individual one, especially Fu+Tv+Rh, Rh+Fu and Tv+Fu that reveal the least percentages of total damping-off (0.00 and 3.33%) compared with control (CI) 73.33%. In consequence that highest survival plants were of averages 100 and 96.67%, respectively. Combined treatments also increased percentage efficiency over infected control (100 and 95.45%). These results are in agreement with **Mahmood *et al.* (2015)** who treated chickpea seeds with *T. harzianum* then drenched the infected soil with Carbendazim, that proved to be very effective against chickpea wilt disease incidence. **Gupta (2006)** also found that presence of rhizobia in the rhizosphere may also protect chickpea seedling roots from damage caused by pathogens when, integrated Vitavax 200 with biocontrol agents. Moreover, **Podder *et al.* (2004)** found effective management of chickpea diseases, through combined fungicides with bi-control agents. **Wang *et al.* (2005)** found that fludioxonil inhibited Fusarium strongly but showed little effect on Trichoderma. Thus, an integrated pest management (IPM) schedule using a balanced application of biological, chemical and physical methods is necessary.

However, the combined treatment of Rh+Th also reduced the percentage of total damping-off (46.66%), and increased survived plants (53.34%). On the other hand, increased percentage over control (CI) the analogous value was 36.37%. These results are confirmed by the findings of the benefits on chickpea, when treated by combined inoculation of *R. leguominosarum* and *Trichoderma* spp. where, seems likely as cumulative effect on processes due to supply of N and P to the crop considered. In addition to the growth promoting substances, which in turn produced by these organisms and the biological control of soil-borne fungal pathogens (**Windham *et al*., 1986; Alagawadi and Gaur, 1988**). These reduction of damping-off may be clear and illustrated back to the *T. viride* and *R. leguominosarum* in Table 4. The combined effect of *T. harizanium* and *R. leguminosarum* showed a maximum effect in controlling damping-off and root rot for chickpea and lupine plants (**Shaban and El-Bramawy, 2011**).

Generally, from the previous results it could be clear that Topsin M70% was the best in controlling and reducing damping off in pea plants if it is used in individual or mixed with Rh or Tv.

Plant Growth Parameters as Affected by Combined Bioagents and Topsin M70 under Greenhouse Conditions

Results in Table 5 indicate significant differences for the plants growth parameters among all treatments compared with two controls (CH or CI).

The highest values of plant length were obtained in seed socked in Tv combined with Rh solution and drenched fungicide where the analogous values were (25.00 cm). However, Fu revealed similar values when seeds were soak in

Table 4. Pre and post-emergence damping-off as effected by combined bioagents and Topsin M70% under greenhouse conditions

Treatment	Pre emergence damping-off		Post emergence damping-off		Total damping-off		Survival
	Mean	Over CI (%)	Mean	Over CI (%)	Mean	Over CI (%)	
Rh	53.33	20.00	0.00	100.00	53.33	27.27	46.67
Tv	60.00	10.00	0.00	100.00	60.00	18.18	40.00
Fu	3.33	95.00	0.00	100.00	3.33	95.45	96.67
Rh+Tv	43.33	35.00	3.33	50.00	46.66	36.37	53.34
Rh+Fu	3.33	95.00	0.00	100.00	3.33	95.45	96.67
Tv+Fu	0.00	100.00	3.33	50.00	3.33	95.45	96.67
Fu +Tv+Rh	0.00	100.00	0.00	100.00	0.00	100.00	100.00
Cont. (Healthy)	0.00		0.00		0.00		100.00
Cont. (Infected)	66.67		6.67		73.33		26.67
LSD 5%	8.178		4.383		6.434		
LSD 1%	12.098		6.485		9.519		

Rh = *R. leguominosarum*, Tv = *T. virid* , Fu = Topsin M70 Fungicide
Over CI (%) = (CI-T)/CI)*100 Where : CI= Control infected T= Treatment

Table 5. Plant growth parameters of infected pea plants as affected by combined bioagents and Topsin M70 under greenhouse conditions

Treatment	Plant length (cm)			Fresh weight (g)			Dry weight (g)		
	Mean	Over CH (%)	Over CI (%)	Mean	Over CH (%)	Over CI (%)	Mean	Over CH (%)	Over CI (%)
Rh	16.25	-19.75 *	30.00 *	4.46	10.59 ns	81.22 **	0.77	12.04 ns	99.35 *
Tv	18.50	-8.64 ns	48.00 **	5.16	27.82 *	109.44 **	0.95	38.32 ns	146.10 **
Fu	22.00	8.64 ns	76.00 **	7.06	74.97 **	186.70 **	0.98	43.80 ns	155.84 **
Rh+Tv	18.25	-9.88 ns	46.00 **	5.43	34.57*	120.51**	0.73	6.93 ns	90.26**
Rh+Fu	23.00	13.58 ns	84.00 **	5.60	38.66 **	127.21 **	0.90	30.66 ns	132.47 **
Tv+Fu	23.00	13.58 ns	84.00 **	7.34	81.78 **	197.87 **	1.41	106.57 **	267.53 **
Fu +Tv+Rh	25.00	23.46 *	100.00 **	10.99	172.42 **	346.39 **	2.14	212.41 **	455.84 **
Cont. (Healthy)	20.25			4.04			0.69		
Cont. (Infected)	12.50			2.46			0.38		
LSD 5%	3.53			1.10			0.30		
LSD 1%	4.80			1.49			0.41		

Rh = *R. leguominosarum*, Tv = *T. virid*, Fu = Topsin M70 Fungicide
Over CH (%) = (CH-T)/CH)*100 Over CI (%) = (CI-T)/CI)*100
Where : CH= Control Healthy CI= Control infected T= Treatment

Rh and/or Tv with an average of 23.00 cm for both. An increase percentage of plant length over control healthy (CH) was obtained with Rh+Fu (13.58), Tv+Fu (13.58) and Fu+Tv+Rh (23.46), respectively. These results are in harmony with the findings of **Shaban and El-Bramawy (2011)** who showed that the combination of *T. harzianum* with *R. leguminosarum* improved more effective growth of legume plants than all of each individually.

Concerning the fresh weight/plant treatments of Fu+Tv+Rh, Tv+Fu and Fu gave the highest values of fresh weight/plant with averages of 10.99, 7.34 and 7.06 gm/plant, respectively compared with the control that infected by *R. solani* (CI) (2.46 g). The obtained results are confirmed by findings of **Huang and Erickison (2007)** who reported that seed treatment with *R. leguminosarum* was effective in controlling damping-off in pea and also improved plant growth. These improvements may be due to the combined action of both *R. leguminosarum* that stimulated plant growth through production of growth promoter substances (Table 3) and also might be due to that *T. viride* have been, recently reported as plant growth promoter fungus (**Adams *et al*., 2007**). Also, growth promotions induced by rhizobia may be directly affect through nitrogen fixation and production of plant growth regulators. Several researchers reported that rhizobia produced plant growth regulators such as indole acetic acid, auxins, cytokinins, gibberellins-like substance and rhizopine that stimulated and enhanced plant growth (**Noel *et al*., 1996; Boddey and Hungeria 1997; Deshwal *et al*., 2003; Sharif *et al*., 2003**). It was also reported that rhizobia increase P- availability to plants. (**De Freitas *et al.*, 1997**). The lowest values investigation of the present were obtained from individual Rh and or Tv treatments with the averages of 4.46 and 5.16 g, respectively.

Dry weight of pea plants was the highest mean values obtained from three treatments Fu+Tv+Rh (2.14), Tv+Fu (1.41) and Fu (0.98) g/plant, respectively.

Combined inoculation of *Rhizobium* sp. with a biocontrol fungus *Trichoderma* spp. increased growth, nutrient uptake and yield of chickpea under greenhouse and field conditions (**Rudresh *et al*., 2005**). In addition, the promotion of plant growth may indirectly suppress soil-borne pathogens by rhizobia metabolites. Several previous studies reported that rhizobia increased significantly seed germination and improve plant growth and yield through a reduction of soil-borne pathogens (**Sheikh *et al*., 2006; Mazen *et al*., 2008**).

Some Physiological Activities as Effected By Combined Bioagents and Topsin M70 of Pea Plants (cv. Master P)

Chlorophyll contents of tested pea plants

Results recorded in Table 6 indicate that seed soaking singly in Rh, Tv and Fu or their mixture caused significant increase in the photosynthetic pigments contents (chrophyll a, b and a + b) in plant leaves collected after 30 days from sowing revealing that seed soaking with Fu + Tv + Rh (1.73), Rh + Tv (1.58) and Tv + Fu (1.53) mg/g fresh weight, respectively gave the highest values of chlorophyll a with percentage increase over healthy control (CH) where the analogous values were 17.93, 7.43 and 4.09%, respectively. The same trend being obvious in case of chlorophyll b where Fu + Tv + Rh (0.77) revealed the highest values followed by Rh + Fu (0.66) mg/g fresh weight, respectively. Thus, seed soaking with Fu + Tv + Rh, Rh + Tv and Tv + Fu were more effective revealing total chlorophyll with averages of 2.49, 2.22 and 2.17 mg/g fresh weight, respectively. The present findings are harmony with those obtained by **Saber *et al.* (2009)** who reported that Chl. b and total Chl. significantly increased in plants developed from faba bean treated with *R. leguminosarum +T.vride*. On the other hand, the present treatments recorded the least percentage over infected control (CI) with single treatments of Rh, Tv and/or Fu with the averages of -4.68, -7.64 and -5.15%, respectively.

Changes of polyphenol oxidase (PPO) and peroxidase (PO) activities

Results presented in Table 7 clear that seed soaking with Rh, Tv and Fu individually or in combinations significantly increased the activity of PPO and PO compared the control treatment. Polyphenol oxidase was markedly increased after treatment with Tv+Rh+Fu (0.110) and Rh + Fu (0.101) compared with control (CI) 0.023

Table 6. Chlorophyll contents of treated infected pea plants as effected by combination of bioagents and Topsin M70%

Treatment	Chl. (a)			Chl. (b)			Chl. (a+b)		
	Mean	Over CH (%)	Over CI (%)	Mean	Over CH (%)	Over CI (%)	Mean	Over CH (%)	Over CI (%)
Rh	1.48	0.68 ns	5.42 **	0.61	-4.98 **	-4.68 **	2.09	-1.00 *	2.30 **
Tv	1.40	-4.29 **	0.21 ns	0.59	-7.93 **	-7.64 **	2.00	-5.40 **	-2.25 **
Fu	1.44	-1.98 **	2.64 **	0.61	-5.44 **	- .5.15 **	2.05	-3.03 **	0.20 ns
Rh+Tv	1.58	7.43 **	12.49 **	0.65	0.57 ns	0.88 ns	2.22	5.34 **	8.85 **
Rh+Fu	1.48	0.68 ns	5.42 **	0.66	3.27 **	3.59 **	2.14	1.47 **	4.85 **
Tv+Fu	1.53	4.09 **	8.99 **	0.64	0.16 ns	0.47 ns	2.17	2.89 **	6.32 **
Fu +Tv+Rh	1.73	17.93 **	23.48 **	0.77	18.97 **	19.34 **	2.49	8.25 **	22.18 **
Cont. (Healthy)	1.47			0.64			2.11		
Cont. (Infected)	1.40			0.64			2.04		
LSD 5%	0.013			0.009			0.0196		
LSD 1%	0.018			0.012			0.0271		

Rh = *R. leguominosarum*, Tv = *T. virid* , Fu = Topsin M70 Fungicide
Over CH (%) = (CH-T)/CH)×100 Over CI (%) = (CI-T)/CI)×100
Where : CH= Control healthy CI= Control infected T= Treatment

Table 7. Polyphenol oxidase and peroxidase activities treated infected pea plants as effected by combination of bioagents and Topsin M70%

Treatment	Poly phenol oxidase* (PPO)			Peroxidase activity** (PO)		
	Mean	Over CH (%)	Over CI (%)	Mean	Over CH (%)	Over CI (%)
Rh	0.048	42.34**	-107.25	0.106	66.22	-1118.39**
Tv	0.054	34.27**	-136.23	0.117	62.71	-1244.83**
Fu	0.096	-16.53ns	-318.84	0.240	23.51	-2658.62**
Rh+Tv	0.030	63.99**	-29.42	0.104	66.85	-1095.40**
Rh+Fu	0.101	-22.18*	-339.13	0.319	-1.69	-3567.43**
Tv+Fu	0.069	16.13ns	-201.45	0.300	4.49	-3344.44**
Fu +Tv+Rh	0.110	-33.47**	-379.71	0.346	-10.27	-3877.01**
Cont. (Healthy)	0.083			0.314		
cont. (Infected)	0.023			0.009		
LSD 5%	**0.050**			**0.056**		
LSD 1%	**0.073**			**0.083**		

Rh = *R. leguominosarum*, Tv = *T. virid* , Fu = Topsin M70 Fungicide
Over CH (%) = (CH-T)/CH)×100 Over CI (%) = (CI-T)/CI)×100
Where : CH= Control healthy CI= Control infected T= Treatment
* Peroxidase activity expressed as change in absorbance at 425 nm/g fresh weight
** Poly phenol oxidase activity expressed as change in absorbance at 490 nm/g fresh weight

activity/min. Meanwhile, mixing of Topsin M70% with Rh+Tv and Rh recorded the highest increase in peroxidase activity with the averages of.0.346 and 0.319 compared with infected control 0.009 activity/min, respectively. The least activity however, was recorded for individual Rh (PPO and PO). **Saber *et al.* (2009)** found that total phenols and polyphenol oxidase activity in faba bean plants play important roles in plant protection against *Botrytis fabae* and both of them were induced greatly because of dual application of *R. leguminosarum* and Trichoderma, compared to fungicide + *R. leguminosarum*. The suggested mechanisms for the pathogen defense of polyphenol oxidase (PPO), include; (1) general toxicity of PPO-generated quinones to pathogens and plant cell, accelerating cell death (2) alkylation and reduced bioavailability of cellular proteins to the pathogen (3) cross-linking of quinones with protein or other phenolics, forming a physical barrier to pathogens in cell wall and (4) quinone redox cycling leading to H_2O_2 and other reactive oxygen species, which are known to be important factors in plant pathogen interactions and defense signaling (**Li and Steffens, 2002; Raj *et al.*, 2006**). That is why levels of PO and PPO are naturally high in resistant varieties. The high activities of peroxidase and polyphenol oxidaese in infested soil with other treatments could be considered as an antioxidant mechanism for protecting plants against the effects of pectinase on the plant cell walls.

The relationships between peroxidase or polyphenol oxidase activities and emergence damping-off of pea plants grown in greenhouse conditions

The relationships between peroxidase, polyphenol oxidase and pre post emergence and total damping off in pea cultivar Master P are shown in Table 8 and Fig. 4. Obtained results illustrate negative correlation between peroxidase activity or polyphenol oxidase and total damping-off. In addition, results in Fig. 4 (1A, 2A and C) indicated that polyphenol oxidase is negatively associated with pre and total damping-off where the correlation coefficient are -0.864 and -0.896, respectively. The strength of these relationships were shown again in the sharpness of the regression slops.

On the other side this relationship was negative but insignificant in the post emergence damping-off with correlation coefficient of -0.474. For peroxidase activity the obtained results showed that the association between damping-off (pre, post and total damping-off) with peroxidase (Fig. 4) (1B, 2B and D) were negative and significant with correlation coefficients of -0.956, -0.563 and -0.896. The strength of these relationships were shown again in the sharpness of the regression slops. **Mandal *et al.* (2009)** mentioned that PO and PPO are important in the defense mechanism against pathogens through their role in the oxidation of phenolic compounds to quinones, causing an increase in antimicrobial activity. Therefore, they may be directly involved in stopping pathogen development. In addition, protective effect of ascorbic and salicylic acids are related to reduce active oxygen species damage to essential protein and/or nucleic acid (**Noctor and Foyer, 1998; Segarra *et al.*, 2006**).

From the afore-mentioned results an opposite correlation between the PPO and/or PO enzyme activities and pre, post emergence and total damping-off which negative was noticed between the pre and post emergence total damping-off and PPO and PO enzyme activities. These results are in harmony with the results mentioned by **Pena and Ankue (1992) and Nawar and Kuti (2003)** who indicated a positive relation between resistance and peroxidase activity. Peroxidase also produces free radicals and hydrogen peroxidase which are toxic to many microorganisms. They mentioned that chemicals play roles behind this resistance induction as accumulation of phytoalexins, lignifications of phenols and activation of chitinase, polyphenol oxidase and peroxidase.

In conclusion, the present study showed that, soaking pea seeds in rhizobial cultural filtrate and suspension of *T.viride* individually proved to have the potential to control, damping – off and to enhance plant growth of pea plants. The low rate of inhibition was obtained by the use of the tested bioagents, thus, can be integrated with the use of the resistance pea cultivars, and the proper cultural and national management or in combination with drenching fungicide can successfully managed by combined application of tested bioagents with a cheap fungicide against *R. solani* and to identify associated biochemical changes in treated plants.

Table 8. Correlation coefficient between pre, post and total damping-off and each of polyphenol oxidase and peroxidase activities

Independent variable (x)	R	R^2	F. test	P. value	Regression equation
Poly phenol oxidase (X1)					
Pre (y_1)	-0.864**	0.746	20.606**	0.003	pre = 80.42-803.66*poly
Post (y_2)	-0.474	0.224	2.025	0.198	post = 3.96-36.30*poly
Total damping-off (y^3)	-0.896**	0.803	28.481**	0.001	total = 90.25-904.24*poly
Peroxidase activity (X^2)					
Pre (y_1)	-0.956**	0.914	74.70**	0.0001	pre = 72.60-228.35*pero
Post (y_2)	-0.563*	0.317	3.256	0.114	post = 3.77-11.09*pero
Total damping-off (y_3)	-0.967**	0.936	102.16**	0.0001	total = 80.16-250.67*pero

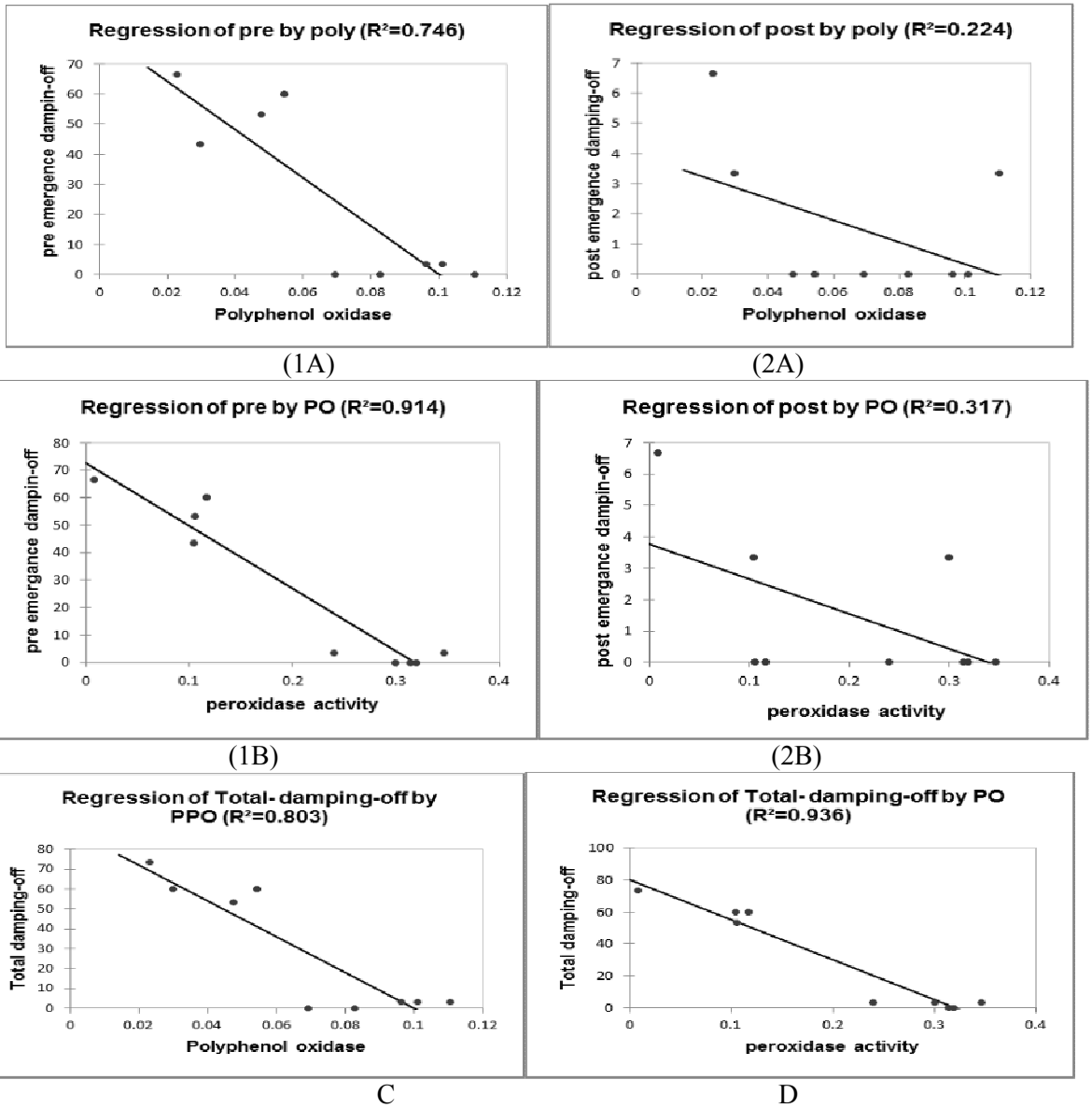

Fig. 4. Relationships between peroxidase or polyphenol oxidase activity and emergence damping off of pea plants grown in greenhouse conditions

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تأثير معاملة التريكودرما فيردى والرايزوبيم ليجيومينوزاريم مع التوبسن م٧٠ لمكافحة مرض موت بادرات نباتات البسلة المتسببة عن الفطر ريزوكتونيا سولانى

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أُختبر تأثير معاملة التريكودرما فيردى والرايزوبيم ليجيومينوزاريم مع المبيد الفطرى توبسين م٧٠ وذلك سواء كمعاملة منفردة أو مخاليط وتأثيرها لمكافحة أمراض موت البادرات وأعفان جذور نباتات البسلة وذلك تحت الظروف المعملية والصوبة، أنتج كل من الرايزوبيم ليجيومينوزاريم والتريكودرما فيردى تحت ظروف المعمل بعض المواد المضادة والمشجعة لنمو النبات وتم تقييمها ورصدها فى المعمل متضمنة الفوسفات الذائبة وإندول حمض الخليك و سيدروفورات وهيدروجين سيانيد وإنزيم الشيتينيز والبرتونيز، وجد معمليا أن استخدام التوبسين م ٧٠% بتركيزات تتراوح من ١٠ إلى ٥٠ جزء فى المليون يقلل من النمو المسليومى للفطر ريزوكتونيا سولانى وكذلك التريكودرما فيردى والرايزوبيم ليجيومينوزاريم ومخاليطهم، وجد أن أعلى نسبة تثبيط النموالفطرى ريزوكتونيا سولانى و التريكودرما فيردى كان عند تركيز ٤٠ و٥٠ جزء فى المليون مقارنة ببقية المعاملات بنسبة ٨٣,٣٠ ، ١٠٠,٠٠، ٧٦,٠٨ و١٠٠,٠٠% عل الترتيب، ووجد أن والرايزوبيم ليجيومينوزاريم يظهر ثبات وتحمل ومقاومة عند نفس التركيزات حيث كانت نسبة التثبيط ٥٢,١٩ و٥٩,٨٥% على الترتيب، فى تجربة الصوبة تم إجرائها فى محطة البحوث الزراعية بإيتاى البارود محافظة البحيرة، تم نقع بذور البسلة فى راشح التريكودرما والرايزوبيم ليجيومينوزاريم سواء كمعاملة منفردة أو مختلطة ثم معاملة المبيد بعد الزراعة مباشرة وتم تقييم تأثيرها على نسبة الاصابة بموت البادرات ونسبة النباتات المتبقية بعد الإصابة، ووجد أن معاملة البذور مع الرايزوبيم + المبيد والتريكودرما+المبيد و الرايزوبيم + التريكودرما + المبيد أظهرت أعلى نسبة فى تقليل نسبة النباتات المصابة بموت البادرات، وكانت نسبة النباتات المتبقية تصل إلى ١٠٠%، أظهرت نتائج الدراسة زيادة فى أطوال النباتات والوزن الرطب والحاف، بالإضافة إلى زيادة فى النشاط الفسيولوجى للصبغات الموجودة فى النباتات، كما أظهرت نتائج الدراسة تحفيز بعض اليات الدفاع الكيموحيوية مثل انزيمى البيروكسيديز والبولى فينيل اوكسيديز وكانت أفضل المعاملات فى هذا الصدد هى التريكودرما فيردى+ توبسين م٧٠ والتريكودرما فيردى+ الرايزوبيم ليجيومينوزاريم + توبسين م٧٠، أوضحت نتائج البحث المتحصل عليها زيادة فى النشاط الفسيولوجى المتمثل فى نشا ط إنزيمى البيروكسيديز والبولى فينيل اوكسيديز تحت ظروف العدوى الصناعية وكانت هناك علاقة عكسية مع نسبة موت البادرات الكلية والمتمثلة إحصائيا بمعامل الإرتباط بنسبة ٩٦,٧٠% للإنزيم البيروكسيديز و ٨٥,٦٠ % للبولى فينيل اوكسيديز.

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