Biological Control of Pests & Plant Diseases
Volom: 8 No: 1 spring and summer 2019
(1-15)
DOI: 10.22059/jbioc.2019.267141.243

Bio-fertilizer incidences on cucumber disease and defense reactions in response to *Phytophthora melonis*

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(Received: February 14, 2018- Accepted: May 15, 2018)

ABSTRACT

In this work, the effects of two commercial bio-fungicides - Biosubtyl (*Bacillus subtilis)* and Biophosphorus *(Pseudomonas* sp.) - on disease incidence and plant cell defense mechanisms were investigated. Changes in disease severity, plant defense enzyme activity, phenolic compounds content and representative defense-related genes expression were measured in cucumber plantlets infected with *Phytophthora melonis* at 24, 48, 72 and 96 hours post inoculation (hpi). The incidence rate of disease decreased in treated plants with both bio-fertilizers. The highest reduced rate of disease (60%) was noted for Biosubtyl application compared to control treatment. Biochemical analyses showed that both bio-fertilizers are able to increase total protein and phenolic compounds in infected cucumber plants. Highest activities of Peroxidase (PO) and β-1,3-glucanase enzymes were measured for bio-phosphorus application at 72 hpi while maximum expression of Polyphenol oxidase (PPO) was observed at 96 hpi for this application. Transcriptional activities of ,Lipoxygenase (Lox) showed an optimum at 72hpi (p-value < 0.01, 9.5 fold), while genes coding for cucumber pathogen-induced 4 (*Cupi4*), Phenylalanine ammonia lyase (PAL) and Galactinol synthase (Gal) presented high expression levels at 48 hpi in infected cucumber plants treated with Biophosporus. These results suggest that both bio-fertilizers could improve enhanced disease resistance in infected cucumber by inducing basal resistance mechanisms and can be used as a natural *alternative* to conventional *fungicides* for *sustainable* disease control.

Keywords: *Cucumis sativus*, Biological control, Plant immunity.

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is a major vegetable crop cultivated in greenhouse condition. Due to ambient air temperature and high humidity in greenhouse, crop production must be protected plants against different diseases caused by soil born pathogens (Hausbeck and Lamour 2004; Ho 1986). In Iran, *Phytophthora* species causing damping-off disease are one of the major limiting factor in vegetable production for approximately 75% of greenhouses (Esmaeili Shirazifard and Banihashemi 2009). Among Phytophthora species, *Phytophthora melonis* is the dominant species causing pre- and post-emergence damping off diseases (Khosrowfar and Banihashemi 2004). Actually, numerous fungicide classes are used to prevent such parasitic development. All fungicides have some major drawbacks including: application difficulties, non-target effects, phytotoxicity and side-effects, control cost, residue accumulation, and environmental health hazards (Dordas 2009). The use of biological agents to control soil-borne plant pathogens offers a non-polluting complement and could be applied as a natural alternative to current fungicides. Plant growth-promoting rhizobacterias (PGPR) could competitively colonize plant soil and roots and stimulate plant growth and/or decrease plant disease incidences (Ramjegathesh et al. 2013). The ability of bacterial strains to induce resistance in stressed plants by biotic stress is directly related to the impact of plant genetics basis and could lead to systemic acquired resistance (Van Loon et al. 1998). The detrimental effects from one or more phytopathogenic agent and abiotic stressors could be prevented by PGPR (Kloepper and

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Schroth 1978). Control of *Phytophthora* root and crown rot of cucumber has been demonstrated using some *Pseudomonads fluorescens* isolates (Shirzad et al. 2012). Biological control of soybean *phytophthora* root rot (Lifshitz et al. 1987), *Rhizoctonia solani* damping off of bean (Ahmadzadeh and Tehrani 2009), and *Sclerotinia* wilt of sunflower (Expert and Digat 1995) has been shown by application of selected isolates of PGPRs bacteria. The enzymes *β*-1,3-glucanase (β-1,3-glucanase, EC 3.2.1.6) peroxidase (PO, EC 1.11.1.7) and polyphenol oxidase (PPO, EC1.10.3.1) were reported as the main enzymatic systems for plant protection against pathogenic agents (Arfaoui et al. 2007; Paul and Sarma 2005). The capacity of different bio-fertilizers to boost defense responses against the attacks of pathogen and pests, and environmental stresses have been studied in some plant varieties (Conrath et al. 2002). In Iran, there is very little information on the physiological and molecular mechanisms underlying these phenomenons. The aim of this study was to investigate the effect of two bio-fertilizers on systemic acquired resistance in infected cucumber plants by *Phythophtora melonis* thought molecular and biochemical analysis.

MATERIALS AND METHODS

Growth condition and bio-fertilizer treatments

Plant materials

Seeds of cucumber (*C. sativus,* cv. Yalda F1) were surface sterilized in 1% chloramines-T (Sigma) for 3 min and washed several times by sterile distilled water. Surface sterilized seeds were planted into 15 cm diameter plastic pots containing autoclaved loamy sand soil (54% sand, 21% silt, 25% clay, pH 0.7).

Bio-fertilizers

Tow commercial Phosphate-Solubilizing bacteria (PSB) and Bio-subtyl inoculates were tested on infected cucumber plants with *Phythophtora melonis.* Phosphate-Solubilizing bacteria (PSB) containing *Pseudomonas* and *Bacillus* genera and Bio-subtyl containing *Bacillus subtilis* (10^8 CFU mL^{-1}) were purchased from Mehr Asia Biotechnology Company, Iran. Root of three weeks-old cucumbers plants were immerged into the cell suspension of each two fertilizers for 5 min and then were precisely transferred into pots and were maintained in greenhouse (12 h of light per day , 25 to 27 C,). Metalaxyl G 5% was used as fungicide control. Seeds planted in pots containing culture mix without bio-fertilizers were used as non-amended control.

Fungal material

Phytophthora melonis isolate was purchased from Iranian research institute of plant protection. The oomycete was cultivated on PDA medium and an active mycelial plug was transferred on V-8 juice agar medium and was maintained in the dark at 25°C for 2 week. Zoospores production was carried out by adding a plug of oomycete colony to 15mL of a soil suspension obtained from 10g soil. Final volume of suspension was adjusted to 1 liter with distilled water (Li et al. 2009).

Concentration of zoospore was calculated using a glass hemocytometer. Inoculation was done 1 week after bio-fertilizer application via adding 1 ml of a suspension (2×10^5 zoospores/ml) at the vicinity of root of cucumber plants (Abkhoo and Sabbagh 2015). The experiments were performed based on a completely

randomized design with three replicates.

Disease severity

Severity of root rot was measured by a scale in which 1: symptomless; 2= mild root rot (less than one- third of root rotted), 3= moderate root rot (one to two- thirds of root rotted), 4= sever root rot (more than two- thirds of root rotted or plant dead), The index of disease percentage was calculated as sum of disease ratings of individual roots / total number of roots ×100 (Zhang et al. 1996).

Enzyme extraction

Root samples were collected from infected and control plants (non-inoculated with bio-fertilizers) at 24, 48, 72 and 96 hours post inoculation (hpi) and were grounded and homogenized in a chilled pestle and mortar in 1 ml of 0.5 M sodium phosphate, at 0 °C (pH 7.0, 1% PVPP, 0.5 mM EDTA). A volume of two milliliters of homogenate was transferred into micro tube and then was centrifuged at 1400 rpm for 15 min at 4 °C (Gupta et al. 2013). The upper liquid phase was maintained at –20 °C for further enzyme assay.

Total phenol content and protein assays

Bradford protein assay was done for measuring the total quantity of protein in plant tissues (Bradford 1976). Bovine serum albumin (BSA) fraction was used as a standard. The solution absorbance was measured at 590 wavelength nm using a UV-Vis spectrophotometer (Unico, USA). Each protein sample was measured three times and the standard curve was made for each sample. To estimate total phenolic content, 1 gram of root sample was homogenized in 10 mL of 70% ethanol and agitated continually for 5 min in bain-marie kettle at 60 °C for about 20 minutes. Two milliliters of the ethanol extract was diluted in 10 mL of sterile distilled water and then, mixed thoroughly with 500 µL of Folin–Ciocalteu reagent for 5 min. Finally 2 mL of 25% Na_2Co_3 (w/v) sodium carbonate was added (Karthikeyan et al. 2013). The mixed solution was continuously maintained in darkness for 30 min. The absorbance was measured at 725 nm using an UV-Vis spectrophotometer (Unico, USA). The total phenolic compounds was estimated as Catechol equivalent and was expressed as 1 µg Catechol equivalents per mg fresh weight (Rahman and Punja 2005).

Enzyme activity

Peroxidase enzyme measurement was performed in the reaction mixture containing 1mL plant extract, 1 ml of 25 mM citrate–phosphate buffer (pH 5.4) and 50 µl of 200 mM guaiacol. The reaction was initiated by adding 500 µL of 500 mM H_2O_2 (Reuveni et al. 1991). Absorbance increase was measured at 475 nm four time intervals during 180 seconds using an UV-Vis spectrophotometer (Unico, USA). Potassium cyanide was used as an inhibitor for peroxidase activity.

Catechol as a substrate was used for polyphenol oxidase (PPO) activity. Plant extract containing 200 µg of protein was added to 500 ml of 0.5% catechol solution at the room temperature. Absorbance was measured at 400 nm wavelength using an UV-Vis spectrophotometer (Unico, USA).

β-1, 3-Glucanase activity was measured using Laminarin as substrate. A total volume of 500 µL reaction mixture containing 400 µL plant extract, 50 mM Na acetate buffer (pH 7.0) and 3 mg of Laminarin reagent was gathered. The amount of enzyme that

produced 1µm L^{-1} glucose equivalents under experimental condition (pH 7.0, 25 °C) was defined as one unit (U) of enzyme activity.

RNA extraction and qReal-time RT-PCR

For total RNA extraction, sampling was done at 24, 48, 72, and 96 hpi. The quality of extracted RNAs was checked by 1.5 % agarose gel electrophoresis and quantified using a spectrophotometer Scandrop (AnalyticaJena, Germany). One microgram of total RNA was used to first-strand cDNA synthesis using random hexamers, following the manufacturer's instructions (Sinaclone Co, Iran). Transcription level of some defense genes including *cucumber* pathogen-induced 4 (Cupi4), Lipoxygenase (LOX), phenylalanine ammonia lyase (PAL), and Galactinol synthase (GolS) was calculated in treated cucumber with two bio-fertilizers at different time interval after inoculation using qRT-PCR method. The Real time reaction mixture was contained 100 ng of cDNA template, 5 µl of SYBR Green PCR MasterMix,and 1 µl of 10 µM of each forward and reverse primer. The program of amplifications cycles as an initial denaturation at 95 °C for 15 min, following 39 cycles consisting 94 °C for 30 s, 61 °C for 30 s and 72 °C for 50 s, and a final extension step at 72 °C for 15 min was used. The qRT-PCRs were performed in duplicate using a Corbett Rotor-Gene 3000 lightcycler (Corbett Life Science, Australia). Melting curve analysis and electrophoresis pattern was used to determine the specificity of the PCR products. Table 1 show the characterization of primers used in this study. The Actin gene was used as the house-keeping gene (Wan *et al*., 2010).

Table 1: Primers used for PCR amplifications of defense and reference genes in cucumber .

TABLE 1 - Primers used in the quantitative real-time RT-PCR studies

Name	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon length (bp)	PCR efficiency (%)	Tm (ºC)	Accession number
LOX	ACTCTTTGAGCATATGGTTGGC	CCAAGAGTAGCTAAGGCTCCA	112	100	61	U36339
Cupi4	TCACTGTGGTGTGTGCTCTC	ACTCAAGCCATTGCCTTCCA	180	100	61	DQ482461
PAL	TCCACTCAACTGGGGTTTGG	TCTTCCACCATCCGCTTGAC	75	100	61	JN675927
GolS	CTTTGTTTGCTGAGCAGGACT	CAATGTTCTCGGGATGACGC	114	100	61	AY237112
Actin	GAAGGAATAACCACGCTCAG	ACACAGTTCCCATCTACGAG	117	100	61	

Statistical analysis

Analysis of variance (ANOVA) and the least significant difference (LSD) test, using SPSS software v.20; was used to analysis of obtained biochemical data. Relative expression software tool (REST[a] software) was used to analysis of qRT-PCR data.

RESULTS

Impact of bio-fertilizers on the incidence of disease

Cucumber plants treated with both bio-fertilizers and fungicide were monitored for disease phenotyping 3 weeks after inoculation. Disease severity was considerably reduced in treated plants with both bio-fertilizers, although Biophosphorus was more efficient than biosubtyl (Fig.1).

Although less active than methalaxyl,we suggest that the application of these bio-fertilizers as a natural alternative for fungicides could improve plant diseases controlling and induce systemic resistance especially in greenhouse conditions

Total protein and total phenolic compounds

As described in Fig. 2, the total phenolic contents in infected cucumber plants were significantly increased after application of both fertilizers, from the first time after infection until 72 hpi. The highest increase of total phenolic contents was observed at 72 hpi by application of biophosphorous (Fig. 2).

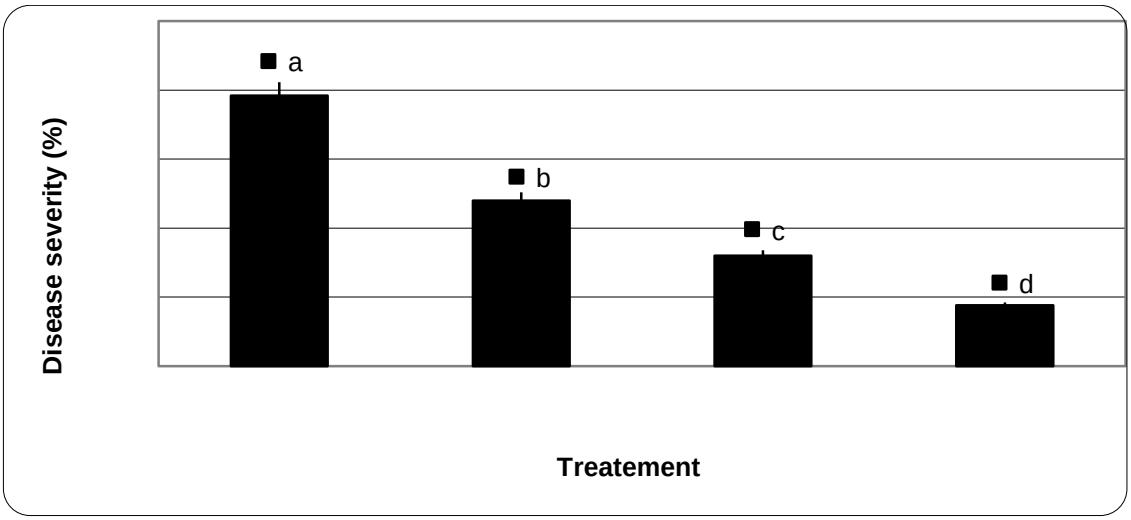

Figure 1**:** Effect of, different treatments on the incidence of damping-off idisease of cucumber caused by *Phytophthora melonis.* See methods for dose and treatment condition. Values followed by the same letter do not differ significantly ($P \leq 0.05$) according to the least significant difference test. Bars indicate the standard deviations (±SD). Data are means of three replicates.

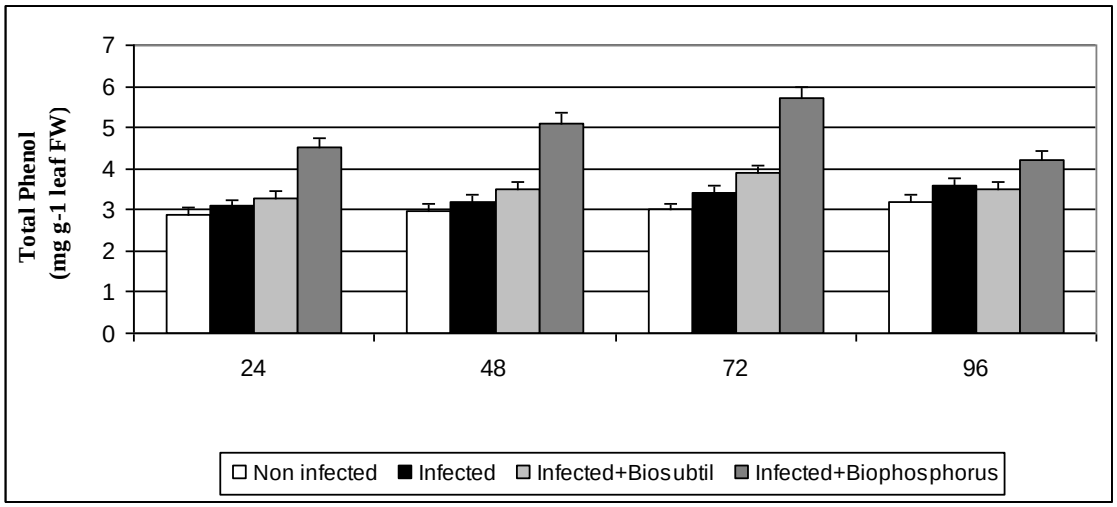

Figure 2: effect of Biosubtyl and biophosphorus bio-fertilizers on accumulation of phenolic compounds in cucumber plants treated or not with *Phytophthora melonis*

At 96 hpi, there was a decrease in phenolic containts compared to the other time points. Total phenol compounds were not significantly increased compared to the control plants by biosubtyl treatment. Accumulation of high levels of Phenolic compounds could participate to antioxidative defense system in the stressed plants at early times for plant systemic acquired resistance against pathogen. As showed in figure 3, a high quantity of protein was detected in plant extract at 72 hpi by biophosphorous

treatment. This amount was reduced at 96 hpi compared to 72 hpi. At 96hpi, the total protein level was higher than at 24 and 48 hpi for both treatments. Based on these results we suggest the potential role of plant proteins and total phenolic contents as a part of the systemic acquired resistance.

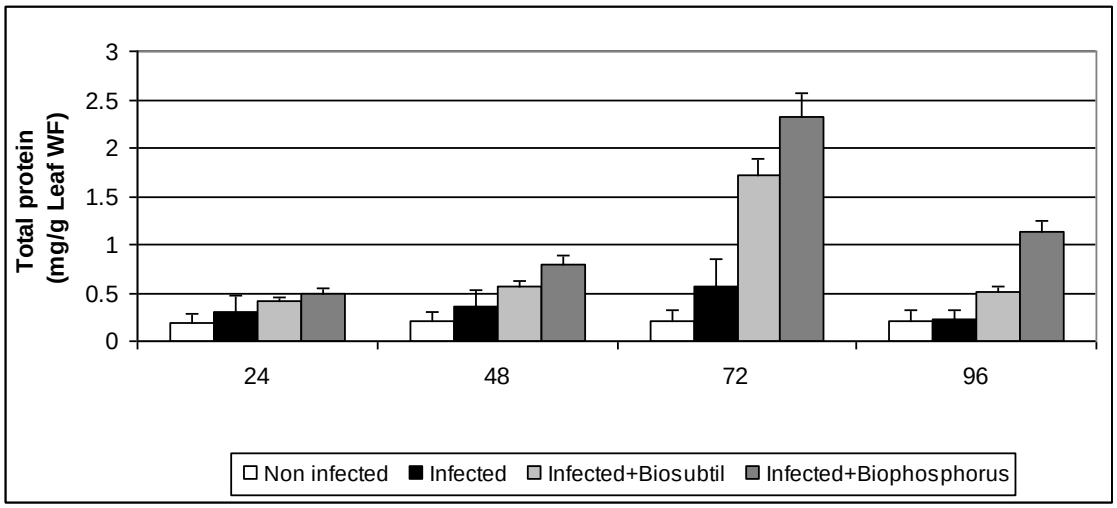

Figure 3: effect of Biosubtyl and biophosphorus bio-fertilizers on total protein in cucumber plants treated or not with *Phytophthora melonis.*

Bio-fertilizers and antioxidant enzyme activity

The activities of peroxidase, polyphenoloxydase and β-1,3-glucanase in the treated plants with bio-fertilizers was increased compared to non-treated plants(Fig. 4). The maximum peroxidase activity was measured at 72 hpi before decreasing at 96 hpi .

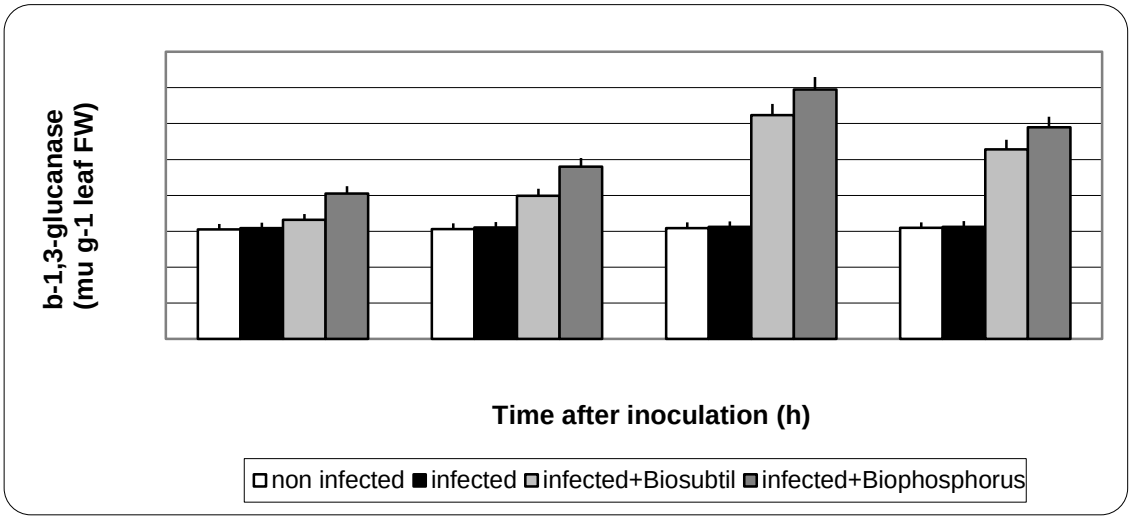

Figure 4: The effect of Biosubtyl and biophosphorus bio-fertilizers on peroxidase activity in cucumber plants challenged with or without pathogen *Phytophthora melonis*

Biophosphorous effect on the proxidase activity was higher than biosubtyl. Polyphenoloxydase activity was increased 3- and 2.2-times in the infected cucumber plants induced with biophosphorous at 72 and 96 hpi,

respectively when compared to the control plants. The highest Polyphenoloxydase activity in the treated cucumber plants with biosubtyl and biophosphorus was observed for the 3th day after application (Fig. 5).

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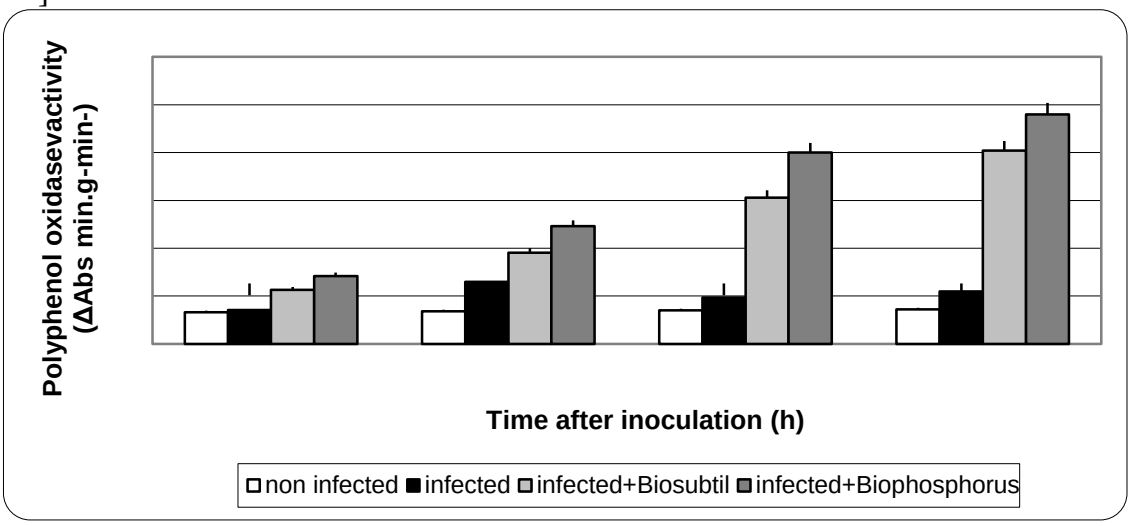

Figure 5: The effect of Biosubtyl and biophosphorus bio-fertilizers on polyphenol oxidase activity in cucumber plants treated or not with *Phytophthora melonis*

The -1,3-glucanase enzyme activity was not significantly change in non-treated infected plants compared to control for any time after inoculation (Fig. 6). But in infected plant treated with both bio-fertilizers this enzyme was significantly increased until 72hpi and then, was gradually decreased at 72 and 96 hpi (Fig.6). However, the quantity of enzyme was reduced at 96 hpi compared to 72 hpi but the enzyme activity was higher than the first time interval after inoculation (24 and 48hpi). These results indicate that this enzyme can continuously increase in infected plants treated with bio-fertilizer and could lead to plant protection.

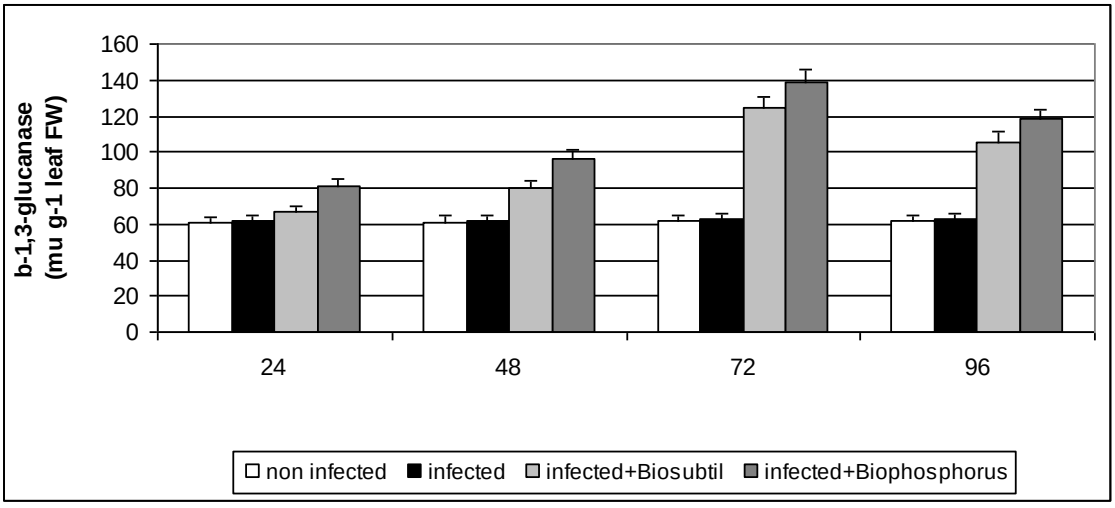

Figure 6: effect of Biosubtyl and biophosphorus bio-fertilizers on β-1,3-glucanase activity in cucumber plants challenged with or without pathogen *Phytophthora melonis*.

Peroxidase enzyme assay showed a constant amount in control plants throughout four days after plant inoculation without using bio-fertilizer.

Expression of defense-related genes in infected cucmber

The expression levels of *Cupi4* gene were elevated in treated cucumbers compared to water control plants at 24 to 48 hpi (Table 2). The highest expression level of *Cupi4* gene was found at 48 hpi (8.4 fold COMPARE TO WHAT? UNTREATED ? RATIO COMPARED TO REFERENCE GENE ?). This increase was followed by a reduced transcription level in the treated plants at 72 to 96 hpi. For bio-phosphorus treatment a decreased of *Cupi4* gene expression was quickly occurred at 72hpi (5.2 fold) and 96hpi (3 fold) هدin infected plants while this reduction was quickly occurred in bio-subtyl fertilizer application at 72hpi (3.7 fold) and 96hpi (2 fold).

Table 2 Mean comparison of *Cupi4* gene expression level (UNIT?) in infected cucumber plants with *Phytophthora melonis* in the present of two biosubtyl and biophosphorus bio-fertilizers

	Time after inoculation (h)			
Treatment	24	48	72	96
Non infected	0.89^{j}	0.90^{j}	0.90^{j}	$.90^{j}$
Infected	1.03^{g}	1.04^{g}	1.02^{g}	1.06^{g}
Infected+Biosubtyl	1.8^{f}	6.93^{b}	3.7^{d}	2^{f}
Infected+Biophosphorus	2.8^{e}	8.4^{a}	5.2^{b}	$3d^{f}$

Expression level of *Lox* gene was affected by both in bio-fertilizers treated cucumber plants. As shown in table 3, unlike Cupi4 gene the expression levels of Lox gene was significantly increased at 72hpi for bio-phosphorus (9.5 fold) and bio-subtyl (7.7 fold). The expression level of Lox gene was approximately diminished at 96hpiin compared tor 72hpi (Table 3).

Table 3: Mean comparison of *Lox* gene expression level in infected cucumber plants with *Phytophthora melonis* in the present of two biosubtyl and biophosphorus bio-fertilizers.

	Time after inoculation (h)			
Treatment	24	48	72	96
Non infected	1^{j}	$1/01^{j}$	$1/02^{j}$	1^{j}
Infected	1.04^{i}	1.05^{i}	1.06^{i}	1.03^{i}
Infected+Biosubtyl	1.8^{h}	3.4^{e}	7.7^{b}	3^{f}
Infected+Biophosphorus	2.7^{g}	6.6^{c}	9.5^{a}	4^{f}

The expression a level of PAL and Gal genes was also influenced in both bio-fertilizers treated cucumbers. A 7.5 and 6.5 fold change of *PAL* and *Gal* genes, respectively, was recorded at 72 hpi in the treated cucumber plants. The expression of both genes was approximately diminished for fourth day after inoculation in the treated cucumber plants with bio-fertilizers. The maximum value in the *Pal* (7.5 fold).and *Gal* (6.56 fold) genes expression was observed in biophosphorus application (Table 4-5). The low value of three last genes (*Lox, Pal and Gal*) expression level in compared to *Cupi4* gene. indicate the dilatory role of these gene to induce systemic resistance in infected plant.

Table 4: Mean comparison of *Pal* gene expression level in infected cucumber plants with *Phytophthora melonis* in the present of two biosubtyl and biophosphorus bio-fertilizers.

	Time after inoculation (h)			
Treatment	24	48	72	96
Non infected	1^{j}	$1/01^{j}$	$1/02^{j}$	1^{j}
Infected	1.03^{g}	1.06^{g}	1.01^{g}	1.02^{g}
Infected+Biosubtyl	1.4^{f}	2.4^{d}	5.7^{b}	2^{e}
Infected+Biophosphorus	1.93^{e}	3.4^{c}	7.5^{a}	3.5^{c}

Table 5: Mean comparison of *Gal* gene expression level in infected cucumber plants with *Phytophthora melonis* in the present of two biosubtyl and biophosphorus bio-fertilizers.

	Time after inoculation (h)			
Treatment	24	48	72	96
Non infected	1^{j}	$1/01^{j}$	$1/02^{j}$	1j
Infected	1.05^{f}	1.07^{f}	1.02^{f}	1.01^{f}
Infected+Biosubtyl	1.1^{f}	4.3^{c}	3.5^{d}	2.4^{e}
Infected+Biophosphorus	2.8^{e}	8.3^{a}	6.56^{b}	3.26^{d}

DISCUSSION

The use of bio-fertilizers, increasing access of nutrients to plant and also presenting anatagonist activities to other soil microorganisms, could elicit systemic acquired resistance to overcoming pathogens attacks. Actually in Iran, biosubtyl and bio-phosphorus are commercially used in open fields and greenhouse farming systems. However, to our knowledge, no report has been published about the role and effects of these bio-fertilizers on molecular and physiological changes in infected cucumber with phytophthora species. To monitor disease development disease severity was measured in treated cucumbers with two bio-fertilizers in comparison to control plants. Our results indicated that both bio-fertilizers are active against *Phytopthora melonis* during damping off disease of cucumber.

Use of different bacterial species including: *B. amyloliquefaciens, B. subilis, B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus* with antagonistic activity has been led to reduce a significant reduction in the incidence of disease or diseases severity on a variety of plants host (Kloepper et al. 2004). The ability of the two bio-fertilizers to reduce disease was compared to metalaxyl as a fungicide. Fungicide application could inhibit disease development temporarily just after relevance of disease symptoms but its affect will not persist during plant growth while bio-fertilizer application could protect plant against pathogens by inducing resistance mechanisms . Different phenolic compounds have been reported for their protective effect in a wide range of different types of plant (Cowan 1999; Balasundram et al. 2006; Harborne 1984). An increase in total phenolic content in plants infected with a variety of pathogenic agents has been demonstrated (de Ascensao and Dubery 2003; Matern et al. 1995; Nicholson and Hammerschmidt 1992). Based on our data, the increase in the total protein and phenolic contents in response to bio-fertilizer application could be involved in the systemic resistance observed in plants infected. It has been assumed that hypersensitive reaction in TMV-infected tomato leaf which leads to leaf necrosis is related to activation of antioxidant enzymes at the infection site (Simons and Ross 1970). Oxidative stress has been frequently indicated by modulation and concentration of antioxidant enzyme activity (Mittler 2002).

Application of *P. fluorescens* as plant growth-promoting in tomato plant has

been led to increase in expression rate of phenylalanine ammonia-lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO) as defense-related proteins against tomato wilt disease caused by *Fusarium oxysporum* f. sp. *lycopersici* (Ramamoorthy et al. 2002). A crucial role of peroxidase and polyphenol oxidase in the biological control and plant resistance to pathogen invasion has been demonstrated (Chérif et al. 1994; Šukalović et al. 2010). A positive correlation has been shown between peroxidase and resistance progress in inoculated broad bean (*Vicia faba* L) leaves with *Botrytis fabae* (Nawar and Kuti 2003). Systemic acquired resistance was increased in infected cucumber with foliar pathogen *Botrytis cinerea* treated with *Trichoderma sp.* as a bio-fertilizer (Elad 2000). They also concluded that in groundnut plants treated with *T. harzianum* after inoculation with *Macrophomina phaseolina,* the expression rate of defense related enzymes such as peroxidase and polyphenol oxidase were increased which led to profitable control of groundnut root rot disease. In this work, resistance was associated with early accumulation of *β*-1,3-glucans. These results are in agreement with other works which showed high activity of *β*-1,3-glucanase and subsequent decrease of disease incidence in infected wheat with *septoria tritici* (Collinge and Lyngs Jrgensen 2009). In rice plant, spray of leafs with *P. fluorescens* bacteria has been resulted in increased phenylalanine ammonia-lyase, phenolic contents, chitinase and ß-1,3-glucanase activity 1,-4, 3 and 7 days after treatment (Meena et al. 2000). The present results show that both bio-fertilizers cause the accumulation of β-1,3-glucanase in infected cucumber plants with lower disease index. These results indicate that increase of β-1,3-glucanase might play an important role in weakening and decomposing of *P. melonis* fungal cell walls and has consequently inhibited disease development in infected plants. Here, we analyzed the expression levels of some defense-related genes in treated cucumber with two fertilizers to elucidate the role of these bio-fertilizers in induced systemic resistance and to confirm the results of biochemical analysis. Lipoxygenase enzymes play an important role in defense reactions by pathogens growth inhibiting through phytoalexin production (Choi et al. 1994; Nemati and Navabpour 2012). Peroxidase, lipoxygenase, and phenylalanine amonialyase are linked to the ISR pathways regulated by jasmonates and ethylene and can be activated by saprophytic micro-organisms including rhizobacteria (Van Loon et al. 1998). Association of Lox gene activity with plant resistance induced by PGPR has been also reported in some plant-pathogen interactions (Blée 2002; Ongena and Jacques 2008; Silva et al. 2008). Lipoxygenases are nonheme iron-containing dioxygenases which are widely distributed in plants and animals (Porta and Rocha-Sosa 2002). They are related to substrates for other enzymes which lead to jasmonic acids production involved in signaling events during plant defense responses (Feussner and Wasternack 2002; Shah 2003). High level expression of *Lox-2* gene, involved in linolenic acid metabolism bas been reported in olive plant treated with PGPR bio-fertilizer (Siedow 1991). This fact further supports the possible role of *B. subtilis* in triggering cucumber defense response. In cucumber plant, production of phenolics and phytoalexins

compounds is closely related to PAL enzyme (Daayf et al. 1997) and could be induced by different type of inducers as wounding, low temperature, pathogen attack, and other pathogenic stress (Collinge and Slusarenko 1987; Wu et al. 2002). In this study, expression analysis of *Pal* gene showed a high level gene expression at 2-3 days after inoculation. These results indicate the role of *Pal* gene after the early stage of infection. Cucumber roots inoculated with *Pythium aphanidermatum* showed a high levels of induced PAL while roots treated with *Pseudomonas corrugata* had initially higher levels of *Pal* and this induction levels was slowly reduced after challenging the plant with pathogen (Chen et al. 2000). Application of *P. fluorescens* isolate Pf1-on tomato roots infected with the *Fusarium oxysporum* f. sp. *lycopersici*, leads to increase activity of *Pal* gene at the 4th day after challenge inoculation and this amount was reduced at with increasing **time** *after inoculation* (Ramamoorthy et al. 2002). In this work, Pal activity was increase in treated cucumber plants with both bio-fertilizers- with high activityat the 2th and 3th day after inoculation. These observations suggest the important role of Pal in resistance during early time after infection.

Application of various inducers such as salicylic acid, 2,6-dichloroisonicotinic acid and benzothiadiazole on infected cucumber plants with several fungi and bacterial agents was resulted to increase in mRNA transcripts accumulation of *Cupi4* gene, locally and systemically. These results demonstrated the association of systemic acquired resistance with *Cupi4* gene in cucumber (Phuntumart et al. 2006). We have previously investigated the role of different plant defense inducers on systemic acquired resistance following *Cupi4* gene expression analysis. In all our experiments a high expression levels of *Cupi4* gene was significantly observed when compared to control plants (Abkhoo and Sabbagh 2015; Sabbagh et al. 2017; Sabbagh and Valizadeh 2016). Based on these results, we suggest to use *Cupi4* gene as a molecular marker to investigate the effect of different bio-fertilizers on induce resistance in infected plants. The influence of galactinol (galactosyl-myo-inositol) as a soluble sugars on plant immune system and resistance pathway has been recognized in attacked plants by pathogens and insects (Moghaddam and Van den Ende 2012). Increased accumulation of galactinol has been found in syncytia cells during nematode infection (Hofmann et al. 2010). In infected cucumber with *Corynespora cassiicola,* root priming by *Pseudomonas chlororaphis* O6 elicited induced systemic resistance (ISR) and caused increased accumulation of Gal gene in treated during the early stage of inoculation compared with non treated plants (Kim et al. 2008).

Conclusion 2nd conclusion?

In conclusion, the present results indicate that both bio-fertilizers could be used as biological control agents for the control of damping off disease caused by *Ph. melonis* in cucumber. The reduction of disease incidence by bio-fertilizers application is probably due to a combined mode of action and can be connected to induce systemic resistance. Based on our finding, we suggest use of antioxidant enzymes and defense related genes as primary markers to screening different varieties of cucumber in the defense responses induced by bio-fertilizers application to damping-off disease caused by different soil born fungal pathogens. .Array analysis to investigate the role of all

pathogenesis related gene in data base is recommended.

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