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INDUCE SYSTEMIC RESISTANCE IN CUCUMBER BY SOME BIO-ELICITORS AGAINST ALTERNARIA LEAF BLIGHT DISEASE CAUSED BY *ALTERNARIA CUCUMERINA* FUNGUS

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Abstract

Three bacterial and fungal bio-elicitors agents have been evaluated *in vitro* for their antagonistic potential against *Alternaria cucumerina* anecrotrophic pathogen caused alternaria leaf blight disease on cucumber (*Cucumis sativum* L.). Two bacterial namely *Pseudomonas fluorescens* isolate PF83, *Bacillus subtilis* isolate BS87 and a fungal namely *Trichoderma viride* isolate TV10. They are cell-free (CF) strongly inhibited the growth of pathogen in dual culture tests. The isolates of bio-elicitors are completely inhibited the pathogen growth of 100%. No inhibitory effect was observed on cucumber seedlings. They were artificially inoculated with bio-elicitors. On the contrary, bio-elicitors are significantly reduced ($P \leq 0.5$). The alternaria leaf blight disease incidence in cucumber infected with pathogen and lead to increased root, shoot fresh, dry weights, height of rapeseed plants and differed significantly compared to control plants infected by the pathogen. In addition, RNAs of sentinel defense genes were monitored in *A. cucumerina* fungal infested and non-infested plants. Three plant defense-related genes were tested for gene expression, namely, *MYC-2* gene (a marker for JA signaling), *PR-3* gene (a marker for ET signaling) and *PR-2* gene (a marker for SA signaling). The qPCR results showed that the *MYC-2* and *PR-3* genes expression levels increased in infected plants by *A. cucumerina* fungal and with bio-elicitors plants. The nature of gene expression was detected in cucumber for the first time in infected plants by *A. cucumerina* or non-treated with bio-elicitors. The results indicate that the application of bio-agents to induced systemic resistance can be beneficial in protecting plants sensitive to diseases.

Key words: *A. cucumerina*, *Pseudomonas fluorescens*, *Bacillus subtilis*, *Trichoderma viride*, cucumber.

Introduction

Cucumber (*Cucumis sativum* L.) is among the most important vegetable crops produced in Iraq. Unfortunately, cucumber is infected with several pathogens and *Alternaria cucumerina* (Ell. & Ev.) pathogen is considered is one of the major pathogens infecting such plants in Iraq, which that lead to the reduction in the quantity and quality of yields (Bhargava *et al.*, 1985., Batta, 2003).

The pathogen attacks the leaves especially lower leaves causing large necrotic areas and sporulation of the pathogen as necrotic areas on the upper surface and form of brown to dark-brown growth could be observed (Bhargava *et al.*, 1985).

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Trichoderma viride, *Psedomonas fluorescens* and *Bacillus subtilis* can be used as biotic agents to plant protection from various pathogens through inducing systemic resistance (ISR), which emerged as an important mechanism by which selected Plant Growth-Promoting Rhizobacteria (PGPR)¡Fungi (PGPF) can be defined by induction of defenses in plants against many pathogens via application of plant growth-promoting microorganisms in soil, as well as direct spreading on plants (Duczek *et al*., 1999, Alkooranee *et al.*, 2015, Alkooranee *et al.*, 2017).

Several studies showed that *Psedomonas. fluorescens* has been used to induce systemic resistance in plants before infection by pathogen. Thus, it can be able to protect the plants from infection. Induce systemic resistance in *Arabidopsis thaliana* against *P. syringae*

pv. tomato, where *P. fluorescens* that produce the polyketide antibiotic 2, 4-diacetylphloroglucinol (2, 4-DAPG) are effective rhizobacteria that suppress root, crown rots, damping-off, and wilts diseases in a wide range of crops. They play a key role in the natural suppressiveness of some soils to certain soilborne pathogens (Weller *et al*., 2012).

Ability of *P. fluorescens* to induced systemic resistance in chickpea against *Fusarium oxysporum* f.sp. *Ciceri* caused Fusarium wilt disease, because of its ability to produced salicylic acid (SA) in root tissues and synthetic medium. (Saikia *et al*., 2003).

Several strains of *Bacillus* spp. can to induce systemic resistance in some of host which dependent on ethylene, jasmonic acid pathways, the regulatory gene *NPR1* and which are independent of salicylic acid These results recorded the agreement with ISR model elicited by *Pseudomonas* spp. (Kloepper *et al*., 2004).

Experiments conducted on tomato and bean plants showed that over expression of both fengycin and surfactin biosynthetic genes producer *Bacillus subtilis* strain 168 was associated with increase in the potential of the derivatives to ISR, in tomato cells, lipopeptide over producers play important role in resistant through induction of key enzymes of the lipoxygenase pathway (Ongena *et al.*, 2007). Specific strains of *Trichoderma* sp. colonize and penetrate root tissues of plant initiate a series of biochemical and morphological changes lead to defense response in the plant and to ISR (Sharma *et al.*, 2013). In previous results suggest that cell-free (CF) of *T. harzianum* TH12 plays a role in SAR in oilseed rape against *Erysiphe cruciferarum* caused powdery mildew disease and *S. sclerotiorum* caused sclerotinia stem rot disease by stimulating SA pathway (Alkooranee *et al*., 2015., Alkooranee *et al*., 2017).

Induced systemic resistance in plant involves a broad number of biochemical and physical changes such as phenolic compounds or callose deposition and lead to synthesis some defense-related enzymes or, activation some of the hormonal pathways such as Jasmonic acid (JA), Ethelyn (ET), and Salicylic acid (SA) pathways (Llorens *et al*, 2017).

The studies have been described the role of resistance genes in following a hormonal pathway in order to induced systemic resistance in plants. ISR was described as a hormonal response due to the effect of PGPR and PGPF in the soil. ISR is dependent on increased sensitivity or accumulation of jasmonic acid, (JA) and ethylene (ET) signaling pathway for resistance formation in plants (Pieterse *et al*., 2000), SAR, in contrast to ISR, did not involve the accumulation of jasmonic acid (JA) and ethylene (ET) signaling pathways but is dependent on Salicylic acid (SA), and it is induced by most pathogens that cause tissue necrosis and several chemical have been identified (Gao *et al.*, 2014). In this study, resistance genes associated with different hormonal processes were used. Through these genes and the difference between the bio-elicitors, their culture filtered and the *A. brassicae* fungus pathogen on hormonal pathways and their relationship with ISR and SAR.

The present study has three objectives:

i) To assess bio-elicitors *P. fluorescens* PF83, *B. subtilis* BS87 and *T. viride* TV10 and their culture filtered non-treated (CF) to inhibition *A. cucumerina* growth *in vitro*.

ii) To assess bio-elicitors induced protection in oilseed rape to *A.cucumerina* pathogen.

iii) To identify one or more signaling hormonal pathways involved with SAR and ISR by analyzing expression patterns of the JA-responsive *LOX-1* gene and ET-responsive *ETR-1* SA-responsive *PR-2* genes, in cucumber plants treated with bio-eliciotrs.

Materials and Methods

Plant materials, pathogen and biotic

Cucumber seeds, fungus *A. cucumerina* isolate, the biotic elicitors *P. fluorescens* PF83, *B. subtilis* BS87 and *T. viride* TV10 were obtained from the field crops department, College of Agriculture, Wasit University, Wasit, Iraq. The laboratory and greenhouse experiments were conducted in the microbiology laboratory of field crops department in the winter season of 2018.

An experiment was conducted to investigate cucumber, *A. cucumerina* and biotic elicitors materials in the greenhouse. The *T. viride* TV10 and *A. cucumerina* were maintained and cultured on autoclaved potato dextrose agar (P.D.A) medium (200 g peeled potato, 20 g dextrose, 15 g agar to 1 liter distilled water) in the dark at 20 and 25 ± 1°C. *P. fluorescens* PF83 and *B. subtilis* BS87 were grown in pour plate method on Nutrient broth medium at 28± 2°C for two days for used to inhibition of pathogen mycelia growth.

Production of spore suspensions and cell free (CF)

Five mycelial 1-cm square disks of actively growing TV10 have been inoculated separately into 100 ml of autoclaved P.D.B in 250-ml Erlenmeyer flasks and incubated with a rotary shaker (85 rpm) at 30±1°C. After 15 days, the mycelial mat was harvested and grinded to form a spore suspension. The cell concentration was then

adjusted to 1×10^7 CFU/ml (TV10). Pure cultures of bio-elicitors were maintained on respective agar slants and stored at 4°C for further use. Two days of Cell-free (CF) of *P. fluorescens* PF83 of *B. subtilis* BS87 grown on Nutrient broth medium and 20-day-old *T. viride* TV10 grown on PDB were prepared by centrifugation (12,000 × g for 15 min) followed by filter sterilisation with a 0.4-ìm filter unit; the supernatants were collected and used as enzyme solutions.

***Invitro* inhibition of mycelia length growth**

To determine the effects of bio-elicitors on mycelia length growth of the targeted pathogen *A. cucumerina* in dual-culture techniques, 1×10^7 CFU/ml of *T. viride* TV10, 1.5×10^5 CFU/ml of *P. fluorescens* PF83 and 1×10^5 CFU/ml of *B. subtilis* BS87 and their culture filtrated (CF) were added as 5 ml for each elicitor agent to 100 ml molten P.D.A media (40°C±2°C) (autoclaved at 121°C 15 psi for 30 min) separately, they were mixed properly prior to plating. The media was poured into Petri dishes at 20 ml per plate. Plates were inoculated separately with 5-mm mycelia plugs of the pathogen *A. cucumerina* placed in the centre of the plates. The inoculated plates were incubated at 24°C±1°C for 7 days. Percent mycelia growth inhibition of each pathogen was calculated using the formula:

$$\text{Percent inhibition} = C - T / C \times 100$$

Where C = control (radial growth of the pathogen) and T = treatment (radial growth of the pathogen after inhibition by the antagonist).

Preparation of pathogen inoculum

A. cucumerina was cultured on P.D.A. medium for 5 days in the dark at 24°C. Two mycelia discs (8 mm in diameter) of fungus collected from the edges of 3 day-old cultures were transferred into 100 ml of autoclaved P.D.B in 250-ml Erlenmeyer flasks and incubated for 15 days at 24°C, and shaking it every day by hand. The resulting fungal suspension was adjusted to 1×10^3 fragments mL^{-1} and used as the inoculum source.

Screening of elicitors agents on ISR-eliciting potential and disease effect

Cucumber seeds were surface disinfected using 70% ethanol for 1 min, then 1% sodium hypochlorite solution for 5 min. Sterilized seeds were planted in 15-cm-diameter pots containing 1-kg mixture of sand and peat moss at a ratio of 1:1 that had been autoclaved twice for 30 min within a 24-h interval. Five seeds of oilseed rape were sown into each pot, and grown under greenhouse conditions. Irrigation was applied by drenching twice a week.

To screen the bacterial and fungal bio-elicitors capable of eliciting ISR, two-weeks plants were treated with water (control) or inoculated with suspensions 100 ml (1.5×10^5 CFU/ml) of *P. fluorescens* PF83, (1×10^5 CFU/ml) of *B. subtilis* BS87 and (1×10^7CFU/ml) *T. viride* TV10 as well as their CF by mixed with the upper soil surface of each pot separately, 24 hours before infection cucumber seedling by spraying spores of the pathogen inoculum were prepared in 2.4. Paragraph to leaves and stems to ensure indirect contact between bio-elicitors and pathogen.Each treatment consisted divided to three replicates, three pots were left without addition elicitor agents and pathogen, and three pots were inoculated with pathogen inoculum only as a control treatment. Irrigation was applied by drenching twice a week. Three weeks after, Disease index (DI) and Disease severity (DS) were recorded on plants. The percent disease index (DI) was calculated using the following formula (Kalloo *et al.*, 1997):

$$PDI = \frac{No.of\ inf ected\ leaves}{Total\ no.of\ leaves\ exa mined} \times 100$$

Disease severity (DS) was recorded on 0 – 5 scale as follows (Singh, 1996),

Grade Leaf area affected (%)

0 = Healthy leaves

1 = 1 – 10 percent of the leaf area infected

2 = 11 – 25 percent of the leaf area infected

3 = 26 – 50 percent of the leaf area infected

4 = 51 – 75 percent of the leaf area infected

5 = more than 76 percent of the leaf area infected

The per cent disease severity (DS) was calculated as per the formula:

$$\text{per cent disease severity (DS)} = \Sigma(n\times v)/N\times S \times 100$$

Where,

Σ = Summation

n = Number of leaves in each category

v = Numerical value of each category

N = Number of leaves examined,

S = Maximum numerical value

The shoot and root lengths (cm) were measured on seedlings after three-weeks of infected with pathogen with the use of transparent meter rule. Fresh and dry (70°C for 48 h by electric oven) shoot and root plants weight (gm) were reported on a sensitive electronic weighing balance.

Gene Expression Assay

Two leaves were harvested from each plant per treatment after 24 hours of inoculated by *A. cucumerina* pathogen. It saved in liquid nitrogen to freeze. The total RNA of cucumber leaves were extracted, first-strand cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR) were carried out using the protocol described by Alkooranee *et al* (2015). Three primers (Table 1) were used for cucumber including the *LOX-1, PR-2* and *ETR-1*genes, in addition to the GAPDH gene, which uses as a housekeeping gene**.**

Table 1: qRT-PCR primers used in this study

	Gene description	Pathway	Primer sequence (5^1-3^1)
LOX-1	lipoxygenase 1	JA	CTCTTGGGTGGTGGTGTTTC TGGTGGGATTGAAGTTAGCC
PR-2	Beta 1,3 glucanase	SA	TCAATTATCAAAACTTGTTCGATGC AACCGGTCTCGGATACAACAAC
ETR-1	Histidine protein kinase	ET	GCCATTGTTGCAAAAGCAGA GCCAAAGACCACTGCCACA
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase		CGCTTCCTTCAACATCATTCCCA TCAGATTCCTCCTTGATAGCCTT

Statistical analysis

Statistical analysis was performed using GenStat software, and the means ($P \leq 0.05$) were compared between treatments using least significant difference (LSD) tests (Gomez and Gomez, 1984).

Results and Discussion

Detection ability of antagonists of bio-elicitors *invitro*

The percentage of inhibition growth of *A.* alternate when exposed directly to the double-culture technique in Petri dishes to *P. fluorescence* (PF83), *B. subtilius* (BS87) and *T. viride* TV10 treatment were reached 100% (Fig. 1). The cell free (CF) reached 100%, 93.58% and 81.14% respectively (Fig. 1). The results of bio-elicitors treatments have been significantly showed ($p = 0.05$) differences from the control treatments.

Greenhouse Experience

The results of greenhouse experiment showed that the bio-elicitors have a high ability to protect the seedlings from fungal infection by induced resistance compared with control treatments, as the results showed that the bio-elicitors reduced the percentage of the alternaria leaf blight disease degree of index (DI), disease severity (DS) and also led to a clear increase in some indicators of vegetative growth (Table 2). The results showed in Table 2 that the pathogenic fungus *A. cucumerina* is effecting significant in plants compared to non-infected plants with the fungus pathogen three-weeks old seedlings inoculated with PF83, BS87, TV10 and their cell-free (CF) resulted in a statistically significant reduction of symptoms of the disease, compared with control plants.

These results showed that the effects of the pathogenic fungus *A. cucumerina* were significant in cucumber compared to untreated plants. The symptoms of the disease. The symptoms of disease appeared as small light green flecks then turned to grayish and circular, the disease index (DI) and disease severity (DS) were 53.48 % and 36.67%, respectively, in seedling infected with pathogen, while reaching 0.00 % in seedling non-infected with pathogen (Table 2). Also, the data indicate that the pathogenic fungus causes reduced shoot height , reduced root, shoot fresh and dry weights were reached 7.46 cm, 2.93 g, 0.52 g, 0.95 and 0.052 g, respectively, while reached in without pathogen treatment 11.59 cm, 4.41 g, 0.82 g, 1.32 and 0.084 g, respectively (Table 2).

The bio-elicitors significantly reduced ($P \leq 0.5$) the *Alternaria* leaf blight disease incidence in the cucumber seedlings infected with *A. cucumerina*. The highest reduction in *Alternaria* leaf blight disease degree of index (DI) and disease severity (DS) reached 5.00 % and 2.18 %, respectively, were treated with PF83 treatment. The DI and DS of seedling treated with CF of PF83 reached 11.33% and 2.27%, respectively. On the other hand, DI and DS reached 6.67% and 4.92% of that treated with BS87, its CF reached 14.50% and 10.18%, respectively. The disease index and disease severity in cucumber seedlings treated with TV10 reached 9.33% and 6.34% respectively, and for its CF reached 10.63% and 6.67%, respectively (Table 2).

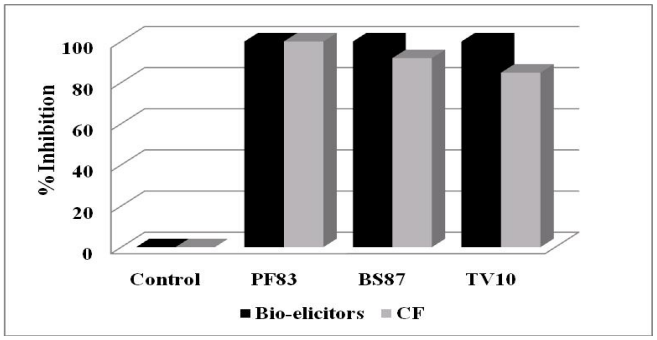

Fig. 1: Antagonistic activities of *P. fluorescence* PF83, *B. subtilius* BS87 and *T. viride* TV10 and their cell free against *A. cucumerina*

Amendment with bio-elicitors increased shoot length, root, shoot fresh and dry weights of cucumber plants differed significantly compared to control plants infected by the pathogen (Table 2). The PF83 treatment ranked as most effective on plants with 15.63 cm, 7.59 g, 0.96 g, 1.90 g and 0.176 g (high plant, root fresh weight, root dry weight, shoot fresh weight, and shoot dry weight, respectively). This was followed by BS87, which reached 14.53 cm, 7.74 g, 0.91 g, 1.70 g and 0.121 g, respectively, followed by CF of PF83, which reached 14.32 cm, 7.37 g, 0.92 g, 1.40 g and 0.147 g, respectively (Table 2).

Gene Expression

The effects of the six bio-elicitors treatments on *R-gene* expression were determined on cucumber plants in both infected or non-infected with *A. cucumerina* 24 hours post infection, by qRT-PCR we have studied the profile of three genes related to resistant defense in cucumber leaves, including *LOX-1, PR-2* and *ETR-1*. The putative marker *LOX-1* (lipoxygenase 1) gene was used as indicators for the path of the JA signal whereas the *PR-2* (Beta 1, 3 glucanase) gene was used as indicators for the path of the SA signal, while *ETR-1* (Histidine protein kinase) gene was used as indicators for the path of the ET signal.

For identification of the genes resistance response in cucumber infected or non-infected with *A. cucumerina.* The results showed that *LOX-1*gene was expressed about 6.63 fold in the leaves at 24 hours post infection with *A. cucumerina* fungal pathogen compared with non-infected plants. Meanwhile, for *PR-2* gene the expression level was regulated by 1.12 fold in infected plants from those non-infected, and the expression level of the *ETR-1*gene reached 4.83 fold at 24 dpi (Fig. 2-A).

Table 2: Effect of bio-elicitors and their CF on cucumber infected by *A. cucumerina* in green-house conditions.

Bio-elicitors and A.cucumerina		% Disease index (DI)	% Disease Severity DS	Shoot length (cm)	Shoot weight (g)		Root weight (g)	
					Fresh	Dry	Fresh	Dry
Non Pathogen		00.00	00.00	11.59	4.41	0.82	1.32	0.084
Control		53.48	36.67	7.46	2.93	0.52	0.95	0.052
T.viride	TV 10	9.33	6.34	12.73	5.39	0.46	1.39	0.090
	*CF	10.63	6.67	13.92	5.13	0.22	1.41	0.066
B.Subtilius	BS87	6.67	4.92	14.53	7.74	0.91	1.70	0.121
	CF	14.50	10.18	14.12	6.28	0.82	1.61	0.105
P. fluesorcens	PF83	5.00	2.18	15.63	7.59	0.96	1.90	0.176
	CF	11.33	2.27	14.32	7.37	0.92	1.40	0.147
L.S.D.		**11.65**	**8.92**	**3.11**	**1.62**	**0.14**	**0.22**	**0.013**

Table with the some treatments are not significantly different ($P = 0.05$).
*CF= Cell free of bio-elicitors. L.S.D = least significant difference.

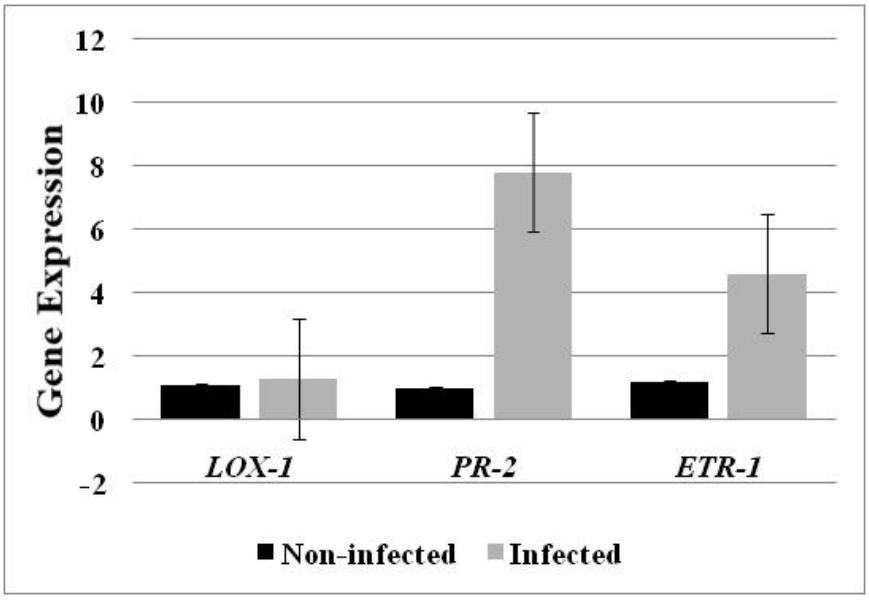

Fig. 2: qRT-PCR gene expression profile of *LOX-1, PR-2* and *ETR-1* in cucumber at 24 hour post infected and non-infected with *A. cucumerina*

Comparison of the genes resistance response in cucumber treated and non-treated with bio-elicitors

Three plant defense-related genes were tested for gene expression to identify the response of hormonal pathways to the bio-elicitors factors in addition to the effect of *A. cucumerina* fungal pathogen.

In plants treated with bio-elicitors and infected with *A. cucumerina*, the expression levels of *LOX-1* was related with JA pathway after 24 hpi which up-regulated by 28.30-fold after treated with PF83 and up-regulated by 17.46-fold and 11.69-fold after treated with BS87 and TV10, respectively at 24 hpi (Fig. 3). However, the levels expression of *PR-2* gene were down-regulated in leaves treated with bio-elicitors PF83, BS87 and TV10 reached 1.21-fold, 0.93-fold and 1.80-fold, respectively (Fig. 3).

The gene expression level of *ETR-1* gene in cucumber seedling infected with *A. cucumerina* and treated with bio-elicitors PF83, BS87 and TV10 were up regulated at 24 hpi by 15.25-fold,

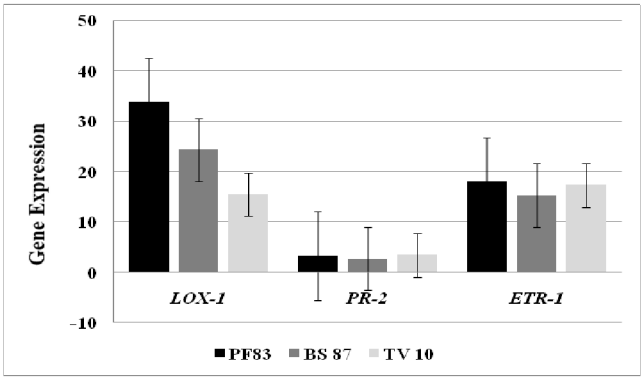

Fig. 3: qRT-PCR gene expression profile of *LOX-1*, *PR-2* and *ETR-1* in cucumber treated with bio-elicitors at 24 hour post infected and non-infected with *A. brassicae*

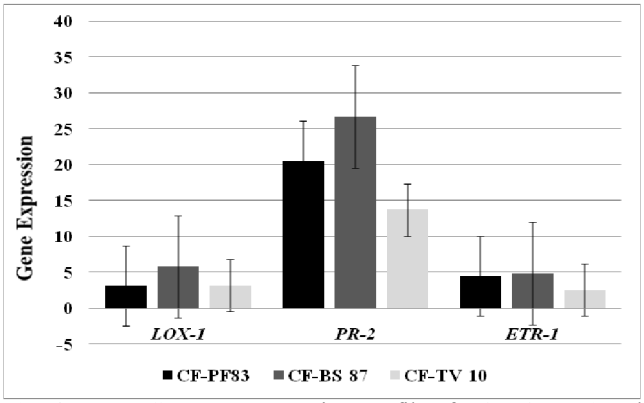

Fig. 4: qRT-PCR gene expression profile of *LOX-1*, *PR-2* and *ETR-1* in cucumber treated with cell free (CF) of bio-elicitors at 24 hour post infected and non-infected with *A. cucumerina*

10.41-fold and 11.94-fold, respectively (Fig. 3).

For Cell free of bio-elicitors treatment, 24 hours post infection by *A. cucumerina*, the expression levels *PR-2* were increased and up regulated by 17.15-fold, 23.06-fold, 11.98-fold, which treated with PF83, BS87 and TV10, respectively (Fig. 4). The levels expression of *LOX-1* and *ETR-1* genes were down-regulated in leaves treated with cell free of bio-elicitors, and expression levels of *LOX-1* gene were regulated by 2.66-fold, 4.50-fold, 2.96-fold, which treated with PF83, BS87 and TV10, respectively (Fig. 4). While the gene expression levels of *ETR-1* in plants treated with *B. subtilis* was down-regulated 3.07-fold, 3.84-fold, 1.92-fold, respectively at 24 hpi (Fig. 4).

Discussion

Three isolates of bio-elicitors agents *Pseudomonas fluorescens* isolate PF83, *Bacillus subtilis* isolate BS87 and a fungal *Trichoderma viride* isolate TV10, as well as their cell-free (CF) were used to induced systemic resistance in cucumber (*(Cucumis sativum L.*) against the *Alternaria cucumerina* caused alternaria leaf blight disease. To study the effect of bio-elicitors agents in induced resistance and their effect on the regulation of hormonal resistance pathways by selecting three resistance genes. Each gene is linked to a major hormonal pathway, through this study; we will know the relationship of hormonal pathways to systemic resistance if they are Induced Systemic Resistance (ISR) or Systemic Acquired Resistance (SAR).

As a first step, the antagonistic capability of bio-elicitors agents to *A. cucumerina* pathogen was tested in dual culture assay. The results showed that all bio-elicitors agents had an antagonistic effect on *A. cucumerina*.

The laboratory results were consistent with several studies that demonstrated the possibility of some bio-control agents in inhibiting the growth of many pathogens in dual culture. Some strains prevent the growth of fungal pathogens through the production of volatile and non-volatile antibiotics and the production of low-molecular-compounds (Saraf *et al*, 2014). *Trichoderma* isolates and other agents can produce many enzymes that inhibit the growth of fungi. They are more than 200 types of highly toxic antibiotics for any organism (Rini and Sulochana, 2007). These includ elytic enzymes proteases, β-1, 4-glucanase cellulose, chitinase, which can degradation the cell walls of pathogens (Sivasithamparam and Ghisalberti, 1998).

*Bacillus sp.*G341 have fungicidal actions and significant inhibitory activity to the mycelial growth in dual culture assay of nine fungal pathogens. The GC-MS results indicated that the G341 produced three volatile compounds: 3-hydroxy-2-butanone, 1-butanol and dimethylsulfoxide (Lim *et al*, 2017). *Bacillus* species produce several different types of antimicrobial compounds, such as antibiotics, cell wall degrading enzymes and siderophores (Grata and Nabrdalik, 2013). The ability of culture filtrated to inhibit the growth of fungi in dual culture maybe it can production some volatile compounds and release of biomolecules from extracellular enzymes, such as pectinolytic, proteolytic and amylolytic enzymes (Michalikova and Michrina, 1997).

In order to determine the effect of bio-eliciotrs and their CF on seedlings development, height, root, shoot fresh and dry weight were evaluated, and they showed significant differences in improved plant growth compared with untreated control seedlings, suggesting that bio-elicitors as a plant growth regulators.

Suppression of *Alternaria* leaf blight disease on plant leaves by cell suspensions was observed when bio-elicitor cells were applied to plant root before plants were

inoculated with *A. cucumerina*. In previous studies, it has been suggested that bio-elicitors can be used to reduce the percentage of plant infection to various diseases, De Vleesschauwer *et al* (2008) pointed out that the ability of *P. fluorescens* WCS374r to trigger ISR in rice against the leaf blast pathogen *Magnaporthe oryzae*.

Treatment with the CF of bio-elicitors significantly ISR in plants compared with control plants or infected plant by *A. cucumerina*. There were no statistically significant differences between bio-elicitors and their cell-free (CF) treatments, this indicates antifungal substances produced by bio-elicitor cells can penetrate well inside plant tissues to exhibit therapeutic effects and exert preventive effects against *Alternaria* leaf blight disease. Therefore, once *A. cucumerina* infects plant tissue, it may be not affected at all or only slightly by antifungal substances produced *in vitro* by bio-elicitors (Yang *et al*., 2007., Ismail *et al*., 2011).

Ongena *et al* (2005) found that root treatment of bean with *Pseudomonas* led to induced systemic resistance to *Botrytis cinerea* caused grey mold disease on leaves, where this bio-elicitors can producing some compounds isolated from cell-free culture, such as N-alkylated benzylamine derivative (NABD) have a role in ISR.

Gupta *et al.* (2000) have proved that *B. subtilis* strain FZB-G and thier CF can be produce probiotic substances associated with plant defence through defence gene activation to induction of systemic resistance and can to stimulation of growth of tomato seedlings against *Fusarium oxysporum* f. sp. *radicis-lycopersici* fungal pathogen.

To study the role of plant growth-promoting fungi (PGPF) to induced systemic resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (Pst), five volatile organic compounds were extracted from three fingi of PGPF which led to the significantly reduced disease severity in *A. thaliana* (Naznin *et al.*, 2014). The roots inoculated with strain of *Trichoderma* exhibited higher activities of peroxidase, β-1, 3-glucanase, cellulase and chitinase when compared to an untreated plants 72 hours post inoculation. *T. harzianum* is the most effective as bio-elicitor agent against a wide range of plant pathogens (De Meyer *et al.*, 1998., Martínez-Medina *et al.*, 2013).

After confirming the ability of these bio-elicitors to induced systemic resistance, we have examined the genetic expression of three of the most important resistance genes, which are directly related with important hormonal pathways in plants, expression levels of resistance genes were monitored by RT-PCR for three marker genes: *LOX-1* (lipoxygenase 1) gene was used as indicators for the path of the JA signal whereas the *PR-2* (Beta 1,3 glucanase) gene was used as indicators for the path of the SA signal, while *ETR-1* (Histidine protein kinase) gene was used as indicators for the path of the ET signal.

*LOX-1*and*ETR-1*expression levels accumulated in infected leaves of cucumber after 24 hpi. Contrastingly, *PR-2* expression levels were down-regulated. This result indicates that the JA and ET single pathways were stimulated in the infected leaves and that the infection of necrotrophic pathogen fungi led to minor resistance of the type ISR in sensitive plants. The JA signalling has recently been reported as a switch hormonal regulator of defense responses in Arabidopsis and sesame plants to necrotrophus pathogens (Birkenbihl *et al*., 2012., Chowdhury *et al.*, 2017).

Necrotrophy is a life-style for some fungi pathogen can act as a switch to induce resistance through stimulation JA and ET pathways in host (Chowdhury *et al.*, 2017). The low level of gene expression of *PR-2* may be due to being linked to SA pathway, which is motivated by biotrophs and in turn stimulates the so-called systemic acquired resistance (SAR) in the host.

To reduce the infection process, pathogen's lifestyle can play an important role in stimulating the defense strategy and the type of hormonal resistance pathways in the host plant where the plants rely on the activation of phytohormone pathways in a timely manner, depending on the pathogen's lifestyle, (Ding *et al*., 2011).

Increased expression of *LOX-1* and *ETR-1* genes associated with JA and ET hormonal pathways, respectively, indicating that they recorded systemic resistance of ISR type, while the *PR-2* gene did not increase significantly after treatment with bio-elicitors. Alizadeh *et al*., (2013) indicate that ISR in *Arabidopsis* and cucumber plants elicited by *T. harzianum* and *Pseudomonas* sp. fungal pathogens was not dependent on SA signalling pathway, and the both bio-elicitors were activated the same pathway. thus, they are compatible in the rhizospheres and have no enhanced effect in combination. Application of Benzothiadiazole (BTH) in the root of tomato plants against *Pseudomonas. syringae* pv. tomato led to elevated activation of SA pathway responses, in contrast, the ISR-inducing by PGPR led to elevated activation of SA pathway responses at 12 hpi to 60 hpi.

Inoculation stages of cell free (CF) in oilseed rape revealed a transition from bio-elicitors-to-chemical-elicitors switch as confirmed by transcriptional

experiments. The expression levels of *PR-2* gene were increased in plant treated with cell-free (CF) of bio-elicitors indicates that systemic resistance used the SA, JA / ET pathways were not involved in systemic resistance. The genes associated were not up-regulated in plant treated with CF, it means that JA/ET does not interact with abiotic-elicitors. Several studies have indicated that the hormonal pathway SAR is usually stimulated by treating plants with abiotic stress or environmental stress (Hofmann, 2008., Yasuda *et al*., 2008., Bektas and Eulgem, 2014., Gao *et al.*, 2014). The ET pathway was involved in ISR in large quantities in *Arabidopsis* plants treated with *P. fluorescens* WCS417r against *P. syringae* pv. *tomato* DC3000 (Pieterse *et al*., 2000), but the qRT-PCR showed the ET pathway was not involved in ISR in *Arabidopsis* plants treated with two volatile organic compounds against *P. syringae* pv. *tomato* DC3000 (Naznin *et al*., 2014). Therefore, exogenous application of bio-elicitors and their CF before the onset of *Alternaria* leaf blight disease in cucumber presents a possible means for plant protection to diseases.

Conclusion

The study signifies the better performance of bio-elicitors and their culture filtered treated and non-treated with heat. As the cucumber is economic vegetable crop such screening of cucumber to bio-elicitors can be beneficial for obtaining the higher yield. We recommend that the application of useful resistance bio-elicitors as a real alternative to classic means of control plant diseases.

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