Systemic Acquired Resistance Induced by Some Biotic Agents against Downy Mildew of Cucumber Disease

Mostafa A. Amer, Sawsan M. El-Abd, Sahar F. Deraz, and Nehal A. Zaid

***Abstract*—** Systemic acquired resistance (SAR) is known to play an important role in plant disease and pest resistance. In this study the interactions between cucumber downy mildew disease caused by *Pseudoperonospora cubensis* and some biological control agents (*Trichoderma harazianum*; *Pseudomonas fluorescen*; *Ampelomyces quisqualis*) and its consequences changes on pathogenesis related (PR) proteins activity such as: Peroxidase (PO), β-1,3-glucanase (GLU) and Chitinase (CHI) were investigated. As a result of plant infection with *P. cubensi*, defensive responses are mediated by hypersensitivity reaction (HR), SA signal pathway and PR-proteins.

The control of downy mildew on cucumber by the fungus *T. harzianum* under greenhouse conditions led to increase of both peroxidase and β-1, 3-glucanase activities. Such activities were used as a marker for resistance to *P. cubensis*. While biological treatment with *P. fluorescens* did not induce peroxidase activity but it was a good inducer to β-1,3-glucanase activity. On the other hand, treatment with *A. quisqualis* did induce neither peroxidase nor β-1,3-glucanase (Although it gave a good result in SA signal pathway induction). All bio-agents showed no or less activation for chitinase. After (HR) response plant protection extended to 7 days. These results prove that all biotic inducers have the ability to induce (SAR) against cucumber downy mildew and give higher protection.

Keywords*—Pseudoperonospora cubensi*, Systemic acquired resistance, biological control, *Trichoderma harazianum*, Downy milde, *Pseudomonas fluorescen*, *Ampelomyces quisqualis*.

I. Introduction

In Egypt, cucumber (*Cucumis sativus L.*) is one of the most important vegetable crops grown under protected cultivations in greenhouse. Downy mildew of cucumber, caused by *Pseudoperonospora cubensis* (Berk and Curtis), is one of the most prevalent and distributed foliar diseases of protected cultivation, that reduce the production considerable from early spring until autumn seasons [1], [2] greatly affects both yield and quality.

Induced resistance can be split broadly into systemic acquired resistance (SAR) and induced systemic resistance (ISR). ISR is phenotypically similar to pathogen- SAR in that it confers an enhanced defensive capacity against diseases caused by fungi, bacteria, viruses, and nematodes [3]. Both localized acquired and systemic acquired resistance (ISR, SAR) were extensively studied by Ross [4], [5], who was the first-to introduce definitions of these phenomena. Now it is well documented that treatments of plants with various agents (e.g., virulent or a virulent pathogens, non pathogens, cell wall fragments, plant extracts, and synthetic chemicals) can lead to the induction of resistance to subsequent pathogen attack, both locally and systemically [6].

The accumulation of PR proteins upon infection with microbial pathogens is well documented in plants [7]. Pathogenesis related (PR) proteins are plant proteins that are induced in pathological situations [8]. Usually they are produced via the salicylic-dependent pathway and are considered a part of the multiple defense systems of plants [9]. For example, chitinase and β-1,3-glucanase have the ability of degrading fungal and bacterial cell walls.Peroxidases are key enzymes in lignification and hypersensitive responses in plants, which limit disease spread [8].

β-1,3-glucanases which are capable of hydrolyzing the β-1,3-glucans found in the cell walls of several genera of fungi [10]. Induction of β-glucans has been demonstrated in many plant-fungal pathogen interactions and they are thought to play several roles in plant defense. Firstly, they can degrade the cell wall of pathogen or disrupt its deposition, contributing to pathogen death [11]. Secondary, they can release cell wall fragments that act as elicitors of active host defense response [12].

There is a link between Pathogenesis-Related proteins (PRs) and acquired resistance in virus-infected tobacco [13], [14], [15]. Fraser [16] was pointed out that PRs became apparent in non-inoculated leaves distinctly later than acquired resistance appeared manifest. However, in tissues already primed to express PRs, challenge inoculation might lead to their earlier and faster accumulation. Van loon and Bakker, [17] reported that non- pathogenic, root colonizing bacteria, notably of the genus pseudomonas, are able to suppress various diseases by competition, antibiosis, or producing enzymes direct against soil- borne pathogens, or by induction of systemic resistance in the plant, extending protection to foliar pathogens. *Trichoderma* spp. have been reported induce systemic and localized resistance to a variety of plant pathogens. A subsequent challenge of *Trichoderma* pre- inoculated plants with the leaf pathogen *Pseudomonas syringae* pv. *lachrymans* resulted in higher systemic expression of the pathogenesis related genes encoding for enzymes relative to non- inoculated ,challenged plants. This indicates that *Trichoderma* induce a potentiated state in the plant enabling it to be more resistant to subsequent pathogen infection, this fact recorded by [18]. Angeli, *et al*. [19] presented a new mycoparasite, as he reported that a limited

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amount of natural parasitism of *Erysiphe necator* by *Ampelomyces* spp. (0.17% to 3.51%) was observed in all of the years of the survey. Some of the isolated ampelomyces strains have conidia that are shaped differently than those of the commercial *A. quisqualis* strain (AQ10) and are phylogenetically different from AQ10.

Abdulgader [20] found that activity of β-1,3-glucanase chitinases in tomato plants, inoculated with *A. solani*, pre-treated with *P. fluorescens* and *T. harzianum* inducers, showed gradual increase and attained maximum values 48 hours after inoculation in both resistant and susceptible cvs. whereas increase in enzyme activities, induced by *P. fluorescens* treatments were 1.00, 1.14, and 1.07-fold, respectively, compared with enzyme activities in susceptible Castle Rock cv.

The aim of the present work is to study the role of different biological control agents (instead of fungicides and other chemicals) as safety alternatives against cucurbit downy mildew in relation to their ability to induce systemic resistance mechanism in cucumber plants.

II. Material And Methods

A. Cucumber plant

Seeds of El-Safa 62 cucumber cultivar used in these experiments were obtained from Vegetable Crop Research Institute El- Dokki, Egypt.

B. Source of Pathogen and planting materials

During summer season 2011 infected leaves of cucumber with typical angular lesion of downy mildew were collected from El-Bosely location in El-Behera and used directly for artificial infection. Biocontrol agent-treated plants were challenged with pathogen spores suspension (4600 Sporangia/ml).

III. Preparation Of Bio-Inducers

A. Trichoderma harazianum

T. harazianum isolate was brought from (Bio-Fertilization Institute, Ain-Shams University, Egypt). Mass culturing of the biocontrol agents was done on potato dextrose agar (PDA) medium in Petri plates. Fifty ml of potato dextrose broth was taken in 250 Erlenmeyer flask and sterilized. A 4 mm diameter disk of 10 days old culture of each biocontrol agent was aseptically transferred to cooled broth and incubated at 28 ± 2 ºC for 10 days. On the 10^{th} day, culture filtrate of these biocontrol agents were harvested by filtering through Whatman filter paper No. 42 and repeatedly centrifuged (9000 rpm) to obtain a cell free culture filtrate [21]. Spores containing suspension with 10^6 CFU/ ml were used for plant treatment.

B. Bio-bacteriocide (Bio- cure- B)

Bio-bacteriocide was brought from (T. STANES & Company Limited-India). The bioagent was *Pseudomonas fluorescens* bacterium contain 10^9 CFU/ml, the dosage.

C. Bio-fungicide (Stanes bio-Dewcon)

Bio-fungicide was brought from (T. STANES & Company Limited-India). The bioagent was *Ampelomyces quisqualis* AQ10 fungus contain 10^9 CFU/ml, the dosage/ Ha was 1liter/ 500 liter of water as a foliar spray.

The effect of treatment was evaluated by treating plants once per day for one, two or three days before pathogen inoculation. A compressed air hand sprayer was used for all of the leaf applications and each treatment was applied at a rate of 20-40 ml for each plant, depending on the number of leaves. Plant leaves were sprayed on both upper and lower leaf surfaces with biotic inducers before inoculation, in order to evaluate the effect of application time prior to inoculation.

IV. Spectrophotmetric Assay

A. Enzyme extraction from plant tissues

Inoculated leaves were harvested at 3, 6, 9, 24, 48, 72 hrs post-inoculation and stored at -80°c for subsequent analysis. Samples (1gm fresh weight) were grounded to a fine powder in liquid nitrogen and used for extraction of individual enzymes by homonization in sodium acetate buffer 50mM pH5.2 (3ML/GM) AT 4°C. The homogenate were centrifuged at 10,000 g for 15 min at 4°c and the supernatent were used for enzyme assay [22].

B. Peroxidase activity assay:

Peroxidase activity was assayed spectrophotmetrically according to the method described by Hammarschmidt *et al*. (1982). The reaction mixture for the peroxidase assay included 25 µl of enzyme solution, 1ml of potassium phosphate buffer (10 mM pH 6.9), 1ml of 25% guaiacol and 1ml of 100 mM H_2O_2. One unit of the peroxidase activity has been defined as the change of 1.0 absorbance unit at 470 nm per minute per gram fresh weight of leaf tissues**.**

C. Chitinase activity assay:

Chitinase activity was measured by reduction of 3,5-dinitrosalicylic acid, in presence of the amino- sugar N-acetyl-D-glucosamine (NAG) released the by enzymatic hydrolysis of chitin [23]. One ml of 10% wet colloidal chitin, suspended in 0.2 M phosphate buffer pH 6.5, and 1 ml of the enzyme extract, were mixed and incubated for 30 min at 50 °C. Enzymatic action was stopped by adding 1 ml 1% NaOH and the mix was then centrifuged (7000 rpm/min for … min). One ml supernatant and 1 ml of 1% 3,5- dinitrosalicylic acid (dissolved in 30% sodium potassium tartrate in 2 M NaOH) were mixed, to measure soluble NAG produced by chitinases present in culture supernatants. Tubes were incubated for 5 min in boiling water, and their absorbance (535 nm) was recorded. Readings were interpolated in a standard curve prepared with a series of dilutions of NAG (0 to 10 µM/ml) and 3,5-dinitrosalicylic acid. A chitinase activity unit (CU) was defined as the amount of enzyme required to produce 1 µmol of NAG in 1 h.

D. β-1,3-glucanase activity assay

β-1,3-Glucanase activity was assayed using the method of Abeles and Forrence [24]. In this colorimetric assay laminarin was utilized as a substrate and the dinitrosalicyclic reagent (prepared by adding 300 ml of 4.5 % NaOH to 880 ml of a solution containing 8.8 g of dinitrosalicylic acid and 255 g of potassium sodium tartrate $6H_20$) was used to measure the reducing sugars produced. Experiments revealed that optimum conditions for assay were pH 5 and 50 ºC, 0.5 ml of enzyme in 0.05 M, pH 5, potassium acetate buffer was routinely added to 0.5 ml of 2% (w/v) laminarin in water and incubated at 50 ºC for 60min. The laminarin was dissolved by heating the 2%,, solution briefly in a boiling water bath before use. The reaction was stopped by adding 3 ml of the dinitrosalicylic reagent and heating the tubes for 5 min at 100 ºC. The tubes were then cooled to 25 ºC, the contents were diluted 1:10 with water, and the optical density was read at 500 nm. .

V. Total protein content assay

Total protein content was determined according to (Bradford [25] using bovine serum albumin as a standard.

VI. Statistical analysis

Two- ways analysis of variance (ANOVA) was used to compare differences treatments and taking sample times, means were separated using least significant difference (L.S.D) test [26].

VII. results and discussion

1. Hyper Sensitivity Reaction (HR) Response In Biotic-Induced Plants

As presented in Table (1) and Figure (1), biotic-induced plants showed obviously necrosis symptoms that express hypersensitivity reaction. The symptoms appeared for days after pathogen challenge in all induced plants and also in untreated control (infected control). Symptoms in biotic-induced plants appeared more rapidly than challenged control. Additionally, number of necrotic lesions was less in the biotic-induced plants than in challenged control. Furthermore, plants protection extended to 7 days after necrosis appearance. This results coincide with high enzymatic activation as shown later. The response of plants to infection pathogens are complex and involve the elevated expression and activity of a whole range of defense responses. The response include structural defense which includes like the pathogen recognition leading to development of a rapid, localized cell death, termed the hypersensitive response (HR), at the site of attempted infection in the plant, thus including production of anti-microbial compounds [27]. In our study, our results showed that (HR) response induced in all plants even non-treated ones (challenged control). Several studies [28], [29], [30], [31], [32] reported that tissues of plants attacked by necrotizing pathogens including *Rhizoctonia* and *Colletotrichum* species have been shown to accumulate substantial amounts of these enzymes not only at the sites of infection but in distant tissues. In another study by Funnell, *et al*. [33] reported that systemic acquired resistance (SAR) is induced following inoculation of *Peronospora tabacina* (the causal organism of downy mildew of *Nicotiana tabacina*) into the stems of *Nicotiana tabacina* plants highly susceptible to the pathogen. The presence of these enzymes in plants infected by *Rhizoctonia* and *Colletotrichum* species has been associated with lesion development and limiting the spread of disease. The data recorded in this study suggested that biotic-treated plants produced necrotic lesions before challenged control and these results may be due to the ability of bio-agent to enhance plant priming which defined by Conrath, *et al*. [34] as the capacity of a plant to express a faster and stronger basal defense response upon pathogen infection. We can also consider that 7 days protection with bioagent- treated plants is a good results and it is in agreement with Harman *et al*. [35] that induced resistance last between 4-14 days.

Table I
Percentage of resistance and susceptibility to downy mildew according to hypersensitivity reaction:

Treatments	DMDP** (%)	DMDS* (%)
T. harazianum	76.90	18.5
P. fluorescense	37.69	50.0
A. quisqualis	6.50	75.0
Control	0	80.25

*DMDP= downy mildew disease resistance, **DMDS= downy mildew disease severity. L.S.D .05 %= 7.135.

Fig. 1 Effects of different bio-inducers in Downy mildew disease in cucumber, (A) challenged control, (B) *A. quisqualis*, (C) *P. fluorescens* and (D) *T. harazianum* treated plants.

2. Peroxidase activity

A. Effect of T. harazianum as a biotic inducer on peroxidase activity

Results indicated that, in all the treatments, there was no significant difference in the activity of peroxidase between three times treated sample (T.H3) and control. They increased peroxidase activity by 104% as compared with control. However two time treated sample (T.H2) and one time treated sample (T.H1) were significantly increased peroxidase (PO) activity compared with control as it reached 132-134%, respectively (Table 2). Peroxidase activity reached its

maximum activity after 72 hrs plants develop a complex variety of events that involve synthesis and accumulation of new proteins that can have direct or indirect action during pathogenesis. Similar trend was obtained by Abdulgader [20] in *F. solani* experiment, where peroxidases activities were 18.93 to 39.67% more than that of untreated tomato plants. The coordinate induction of several PR- proteins that may act synergistically is part of defense strategy that plants activate against the invading pathogen and may limit the colonization of the plant by inhibiting pathogen growth [22]. The oxidative enzymes play an important role in induced resistance by the oxidation of phenols to oxidized toxic products (quinone) which limit fungal activity. Also, peroxidase catalyze the final polymerization step of lignin synthesis, which increase the ability of tissue to lignify which may restrict the fungal penetration [36], [37], [38], [39]. Marra, *et al*. [40] observed that, when both *Trichoderma* and the pathogen were interacting with the plant, several PR-proteins were up-regulated less than by the pathogen alone. Our results suggested that significant increase in peroxidase activity in T.H1 and T.H2 coupled to the increase of SA levels in the same treated plant (data not shown). This is compatible with the observations of others [41], [42] that the infection with *P. cubensis* caused an increase in activity of oxidative enzymes such as peroxidase and polyphenoloxidase as compared with healthy cucumber leaves. Additionally, Alkahtani *et al*. [43] reported that treating cucumber plants with different tested abiotic inducer showed significant reduction in powdery mildew disease severity as well as there was increasing in enzymatic activities such as peroxidase, polyphenoloxidase, chitinase and β-1,3-glucanase. Another study by Hamiduzzaman *et al*. [44] indicated that some biochemical changes at the cellular level upon infection with *P. viticola* (the causal organism of downy mildew in grapevine) were observed. Callose deposition and lignification at the cellular level could contribute to prevent the infection of *P. viticaola* in β-aminobutyric acid (BABA) - treated plants. In the current study the highest peroxidase activity detected at 72hrs, whereas Nandeeshkumar *et al*. [22] showed that peroxidase in chitosan- treated plants significantly increased after 12 hrs with *Plasmopara halstedii* (the causal organism of downy mildew in sunflower). In pearl millet, peroxidase activity has already been described to be associated with reduction in the rate of pathogen multiplication and spread [45].

TABLE II
EFFECT OF T.HARAZIANUM AS A BIOTIC INDUCER ON PEROXIDASE ACTIVITY

Peroxidase activity 470 nm/mg protein							
T. harazianum	**3h**	**6h**	**9h**	**24h**	**48h**	**72h**	**Mean**
T.H3	2.04	1.64	1.73	2.13	1.95	3.01	2.08
T.H2	2.48	2.28	2.76	3.01	2.58	2.96	2.68
T.H1	2.58	2.81	1.93	2.48	3.01	2.99	2.63
Control	2.32	2.13	2.08	2.66	2.60	1.14	1.99
Mean	2.40	2.22	2.13	2.57	2.50	2.53	
L.S.D 0.05%	Treatments (A)=0.453 Time intervals (B)=0.351 A*B=1.11						

*(T.H3) three times, (T.H2) two times, and (T.H1) one time induced plants with *T. harazianum*.

B. Effect of P. fluorescence as a biotic inducer in peroxidase activity

Results in Table (3) indicated that no significant differences in detected peroxidise activity between treatments and control. It was inferred that application of *P. fluorescence* led to an increase in peroxidise activity up to the 24 to 48 hrs after challenge inoculation with *P. fluorescence* when compared to control and declined thereafter. Our results showed that no significant increase in PO activity in P.F treated plants challenged with pathogen compared with control. Abdulgader [20] found that *P. fluorescence* gave the highest enzyme activity induction (0.580 units/mg protein) in resistant tomato Riogrand cv. The study by Ramos, *et al*. [46] suggested that two different responses to pathogen challenge are possible which are dependent upon the specific PGPR strain. One of them is very effective in decreasing disease severity which involves the Ethylene (ETH) pathway with an increase in phenylpropanoid metabolism. The second response involves SA coupled to an increase in PO, which has little effect on disease protection. The previous data assumed our results that showed a decrease on PO activity coincide with a decrease in SA levels (data not shown).

TABLE III
EFFECT OF P.FLUORESCENS AS A BIOTIC INDUCER ON PEROXIDASE ACTIVITY

Peroxidase activity (470 nm) gm/ml							
P.fluorescens*	**3h**	**6h**	**9h**	**24h**	**48h**	**72h**	**Mean**
P.F3	1.71	1.92	1.86	2.80	2.78	1.01	2.01
P.F2	2.38	1.83	2.62	2.87	3.01	1.79	2.42
P.F1	2.53	0.66	1.83	2.70	2.69	1.33	1.96
Control	2.32	2.13	2.08	2.66	2.60	1.14	1.99
Mean	2.24	1.64	2.10	2.80	2.80	1.32	
L.S.D 0.05%	Treatments (A)= 0.453 Time intervals (B)=0.351 A*B= 1.11						

*(P.F3) three times, (P.F2) two times and (P.F1) one time induced plants with *P. fluorescens*

C. Effect of A. quisqualis as a biotic inducer on peroxidase activity

Ampelomyces quisqualis (AQ10) was very effective against powdery mildew, achieving up to 98% of control [47]. However, among all the treatments, there was no significant differences in peroxidise activity between treatments and control as indicated in Table (4). Three times treated plants with *A. quisqualis* increased peroxidise activity by 109% compared with untreated control, while two times treated plants increased only by 101.5% of untreated plants. On the other hand, one time treated plants showed an obvious decrease of activity reached to 83.9% of untreated control. Peroxidase activity reached its maximum increase at 6 hrs and continued until the end at 72 hrs with three time treated plants. However, It was inferred that two and on time application of *A. quisqualis* led to an increase in peroxidise activity to its maximum after 24 and 3 hrs, respectively, when compared to control and declined thereafter. Although the unsignificant increase in PO activity in A.Q- treated plants, there was a significant increase A.Q1– treated plants challenged with the pathogen in SA level (Data not shown) that must be associated with PO activity. This result may due to an increase in PR- proteins depend on SA pathway.

TABLE IV
EFFECT OF A.QUISQUALIS AS A BIOTIC INDUCER ON PEROXIDISE ACTIVITY

A. quisqualis	β-1,3-glucanase activity mM/60min/mg protein						
	3h	6h	9h	24h	48h	72h	Mean
A.Q3	2.04	0.75	2.25	3.01	2.37	2.63	2.17
A.Q2	2.38	0.60	2.41	2.85	2.22	1.66	2.02
A.Q1	2.08	1.59	1.92	1.30	1.71	1.40	1.67
Control	2.32	2.13	2.08	2.66	2.60	1.14	1.99
Mean	2.21	1.27	2.17	2.50	2.23	1.71	
L.S.D 0.05	Treatments (A)= 0.453 Time intervals (B)=0.351 A*B= 1.11						

*(A.Q3) three times, (A.Q2) two times and (A.Q1) one time induced plants with *A. quisqualis*.

3. B-1,3-GLUCANASE ACTIVITY

A. Effect of T. harazianum as a biotic inducer on β-1, 3 glucanase activity

Results in Table (5) indicated that β-1, 3-glucanase activity increased only with two times treated plants with *T. harazianum* by more than 240% compared with untreated control, whereas no significant differences between T.H1 and T.H3 relative to control. β-1,3-glucanase activity reached its maximum activity in T.H1, T.H2 and T.H3 after 24, 3 and 6 hrs, respectively. Expression of higher levels of hydrolases such as β-1,3-glucanases and chitinases has been shown to provide enhanced resistance to fungal pathogens [48], [49]. Their induction after pathogen infection conferes protection by directly degrading fungal cell wall components and indirectly by releasing some elicitors from the decaying fungal cell wall that might stimulate other plant defense mechanisms like phytoalexin accumulation in the host plant [50]. In the current study, T.H2 plants recorded higher increase in β- 1, 3-glucanase activity which are in agreement with the results obtained by Kini *et al*. [51]. In this study, seed treatment with synthetic jasmonate analogon resulted in increased level of β- 1, 3-glucanase activity following the pattern of induction similar to that observed in resistant cultivar. Also, we observed in our study that β-1,3-glucanase activity reached its maximum activity at 3 hrs. These data are in contrast with data reported by Tian *et al*. [52], [20] in which β-1, 3-glucanase activities was significantly induced by *Cryptococcus laurentii* after 24 hrs of inoculation with pathogen. However, three times and one time treatment did not lead to significant increase in enzyme activity. These results could be explained by some previous reports which gave some reasons including: the switch off of plant resistance mechanisms and a progressive decrease of the bioagent elicitation [53].

TABLE V
EFFECT OF T.HARAZIANUM AS A BIOTIC INDUCER ON B-1,3-GLUCANASE ACTIVITY

T. harazianum *	β-1,3 glucanase activity mM/ mg protein/60min**						
	3h	6h	9h	24h	48h	72h	Mean
T.H3	3.90	6.6	6.7	6.90	6.70	2.70	5.60
T.H2	14.4	9.3	13.1	9.99	9.60	11.4	11.3
T.H1	3.90	3.9	2.6	3.20	2.97	2.32	3.20
Control	1.51	4.1	3.8	4.60	8.20	5.90	4.70
Mean	5.93	5.99	6.55	6.92	6.87	5.60	
L.S.D 0.05%	Treatments (A)=1.77 Times intervals (B)=1.37						

*(T.H3) three times, (T.H2) two times, and (T.H1) one time induced plants with *T. harazianum*. **Enzyme activity expressed as mM glucose released /mg protein/ 60 min.

B. Effect of P. fluorescens as a biotic inducer on β-1, 3-glucanase activity

The increased activity of β-1, 3-glucanase in one time and three times treated plants were significantly with increased activity by 182.9% and176.6%, respectively compared with control (Table 6). On the other hand, there were no significant differences between two times treated plants with control. In the two times treated plant, β-1, 3-glucanase activity reached its maximum at 6 hrs and declined there after. Basically, proteins extracted from bacterized pre-treated plants challenged with the pathogen showed the highest antifungal activity against these pathogens [54]. ISR by *Pseudomonas* spp. Involves increasing physical and mechanical strength of the host cell wall and causing biochemical and physiological changes leading to synthesis of PR-proteins [55], [56]. Tian *et al*. [52] reported that the induction of resistance was positively correlated with the concentration of biocontrol agents; however, we observed in our study that there was a significant increase in P.F3 and P.F1 plants. In another study by Saikia *et al*. [54] reported that *P. fluorescens* pre-treated plants challenge - inoculated with the pathogen resulted in an additional increase in PR-proteins, chitinase and β-1,3-glucanase. Furthermore, in this study there was no significant increase in two times treated plant in β-1,3-glucanase activity compared to control. Our findings in the current study are in agreement with Ji and Kuc [57] reported antifungal activity of cucumber β-1, 3-glucanase and chitinase.

TABLE VI
EFFECT OF P. FLUORESCENS AS A BIOTIC INDUCER ON B-1,3-GLUCANASE ACTIVITY

P. fluorescens	β- 1,3-glucanase activity mM/60min/mg protein						
	3h	6h	9h	24h	48h	72h	Mean
P.F3	10.6	14.4	8.1	5.7	7.1	5.5	8.6
P.F2	4.6	5.8	4.1	3.5	3.7	3.2	4.2
P.F1	7.4	11.5	5.8	9.3	8.1	7.7	8.3
Control	1.51	4.1	3.8	4.6	8.2	5.9	4.7
Mean	6.03	8.95	5.5	5.8	6.8	5.6	
L.S.D .05%	Treatments (A)=1.77 Times intervals (B)=1.37						

*(P.F3) three times, (P.F2) two times and (P.F1) one time induced plants with *P. fluorescens.*

C. Effect of A. quisqualis as a biotic inducer on β- 1, 3-glucanase activity

In all treatments, there were no significant differences in the β-1,3-glucanase activity compared with control and the maximum activity reached at 6hrs (Table 7). Although A.Q1 successes in inducing SA signal pathway, no significant increase in β-1,3-glucanase activity was observed in *A. qusqualis* treated plants similarly to peroxidase activity. These results may be due to the ability of the biocontrol agent to induce other PR- proteins that depends on SA signal pathway. In general, *A. quisqualis* did not prove any significant effects to downy mildew control although *A.quisqualis* parasitism reduces powdery mildew sporulation as well as the production of chasmothecia and may eventually kill the entire mildew colony [58] , [59] , [60], [61], [62].

TABLE VII
EFFECT OF A.QUISQUALIS AS A BIOTIC INDUCER ON B-1,3-GLUCANASE ACTIVITY

A. quisqualis *	β- 1,3-glucanase activity mM/60min/mg protein						
	3h	6h	9h	24h	48h	72h	Mean
A.Q3	3.3	5.1	3.8	3.3	3.2	2.6	3.5
A.Q2	4.8	6.5	5.8	4.4	4.1	3.5	4.9
A.Q1	5.4	6.8	6.7	4.1	4.1	3.4	5.1
Control	1.5	4.1	3.8	4.6	8.2	5.9	4.7
Mean	3.8	5.6	5.0	4.1	4.9	3.9	
L.S.D 0.05%	Treatment (A)=1.77 Times intervals (B)=1.37						

*(A.Q3) three times, (A.Q2) two times and (A.Q1) one time induced plants with *A. quisqualis*.

4. EFFECT OF T. HARAZIANUM, P. FLUORESCENS AND A. QUISQUALIS AS BIOTIC INDUCERS ON CHITINASE ACTIVITY

Results in Figure (2) indicated that all treatments with with tested bio-inducers did not stimulate chitinase activity except for the three times treated plant with *P. fluorescens* in which chitinase activity reached its maximum activity at 9 hrs, this results can be explained by that all members of class Oomycetes (including downy mildew causal pathogen) are containing little of chitin [63]. Similar results were reported in which that no or little chitinase activity was detected in plants after infection with downy mildew causal pathogen *S. graminicola*. However, Kini *et al*. [51] showed an increase in activity after challenge with *S. graminicola*. In another study by Saikia *et al*. [54] reported that chitinase exhibited more antifungal activity in comparison to β-1,3-glucanase *in vitro*. Mathivanan *et al*. [64] reported that purified chitinase of *F. chlamydosporum* inhibited the germination of uredospores of *puccinia arachidis* and lysed the walls of uredospores and germ tubes. Furthermore, Jeun *et al*. [65] proved that the hyphal growth of two anthracnose pathogens growing on the agar medium were inhibited by *Bacillus amylolquefaciens* EXTN1 indicating a direct antifungal effect of EXTN-1 to anthracnose pathogen, however, EXTN-1 did not suppress the hyphal growth of the Oomycetes fungus *Phytophthora capsici*.

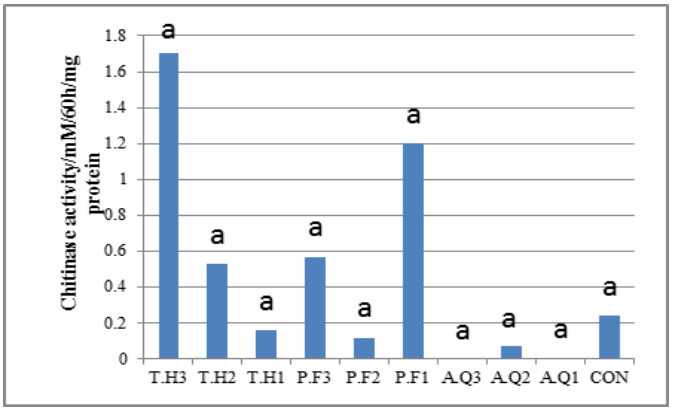

Fig. 2 Time course changes in chitinase activities in cucumber leaves after inoculation with *T. harazianum* (T.H), *P. fluorescence* (P.F) and *A. quisqualis* (A.Q).

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