

## Evaluation of the Accumulation of Pathogenesis Related (PR) Proteins and Phenolic Compounds in Response to Biotic and Abiotic Elicitors as Mechanism for Immune Response to Fusarium Wilt Disease in Faba Bean

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### Abstract

Induction of plant defense against pathogen attack is regulated by a complex network of different signals. In the present study interaction between bio-agents (*Bacillus megaterium*, *Pseudomonas fluorescens*) and abiotic inducers (Bion and Chitosan) was used as new strategy to enhance Faba bean defense responses against wilt disease caused by *Fusarium oxysporum*. Thus, changes in various physiological defenses including pathogenesis related (PR) proteins, phenolic compounds, flavonoids compounds and lignin contents were investigated in Faba bean plants. Results appeared that all treatments significantly reduced wilt incidence under greenhouse and field conditions and increased fresh and dry weights of survival plants compared with untreated seeds. The combination between biotic and abiotic inducers more effective than used individually of them. The treatment *B. megaterium*+Bion recorded the highest reduction of wilt incidence and fresh, dry weights of survival plants. On the other hand, all treatments significantly increased growth and yield parameters compared with control during both growing seasons (2013-2014 and 2014-2015). Combined *P. fluorescens*+Bion and *P. fluorescens*+Chitosan recorded the highest all growth and yield parameters in both growing seasons. Under *in vitro* conditions, all tested biotic and abiotic inducers individually or in combination reduced leaner growth of the pathogen *F. oxysporum*., *P. fluorescens*+Bion followed by *P. fluorescens*+Chitosan recorded the highest inhibition percentage in this respect.

On the other hand, all treatments increased activity of Chitinase and  $\beta$ -1,3-glucanase, phenolic, flavonoids compounds and lignin contents in Faba bean plants inoculated with *F. oxysporum* compared with untreated inoculated plant (control) during the experimental period. The combination between biotic and abiotic gave highly activities of chitinase,  $\beta$ -1, 3-glucanase enzymes and contents of phenols, flavonoids compounds and lignin than applied individually of them. Higher increase of PR proteins, phenolic and flavonoids compounds and lignin contests was obtained in plants treated with combined *B. megaterium*+Bion.

On the light of the present study, it could be suggested that the use of Bion and Chitosan as natural safe materials alone or in combination with bio agents (*P. fluorescens* and *B. megaterium*) is considered one of low cost and effective applicable methods for controlling *F. oxysporum* causing wilt disease in Faba bean.

**Keywords:** Faba bean; Fusarium wilt; Pathogenesis related (PR) protein; Phenolic compounds; Flavonoids; Lignin

### Introduction

Faba bean (*Vicia faba* L.) is one of the earliest domesticated food legumes and is now cultivated on large areas in many countries. Faba bean is used as an important human food in developing countries and as an animal feed, mainly for pigs, horses, poultry, and pigeons in industrialized countries. Feeding value of Faba bean is high and this legume has been considered as a meat extender or substitute due to its high protein content (20% to 41%) [1]. In addition, its beneficial effects in improving soil fertility and thus sustainability and profitability of production systems. Production of the crop is, however, constrained by several disease infections including fungal diseases. Wilt disease caused by *F. oxysporum* f. sp. *fabae* became serious in recent years and it is considered one of the main constraints for Faba bean production in Egypt, especially in the new reclaimed soil.

Traditional methods used to control the disease including seed treatment with fungicides. The hazardous effect of fungicides or their degradation products on the environment and human health strongly necessitates the search for new, harmless means of disease control. Induction of resistance by application of biotic and abiotic compounds as elicitors is one of the alternatives, either alone or as a part of an integrated control strategy. Biotic and abiotic stimulate the natural defense mechanisms in plants. Commonly tested chemical elicitors are salicylic acid, methyl salicylate, benzothiadiazole (Bion), benzoic acid, Chitosan, and so forth which affect production of phenolic compounds

and activation of various defense-related enzymes in plants [2]. In plants, a complex array of defense response is induced after detection of microorganism via recognition of elicitor molecules released during plant-pathogen interaction. Following elicitor perception, the activation of signal transduction pathways generally lead to the production of active oxygen species (AOS), reinforcement of plant cell wall associated with phenylpropanoid compounds, deposition of callose, synthesis of defence enzymes [2], phytoalexin biosynthesis [3] and the accumulation of pathogenesis-related (PR) proteins, some of which possess antimicrobial properties [4].

On the other hand, plant growth-promoting rhizobacteria (PGPR), viz. *P. fluorescens* and *B. megaterium* are among the various groups of plant-associated microorganisms that can reduce the activity of pathogenic microorganisms by microbial antagonism through

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competition for nutrients, production of antibiotics and secretion of lytic enzymes [5-7]. Furthermore, PGPR can reduce the severity of disease or pathogen multiplication by indirectly eliciting the plant defense system [8], which has evolved from a long-term interaction with microorganisms [9].

The objective of this investigation was to study the use of single and mixed combinations of abiotic and biotic inducers to control Faba bean wilt disease under greenhouse and field conditions and to determine resistance-related compounds and enzymes associated with plant resistance.

## Materials and Methods

### Source of fungal pathogen

*F. oxysporum* was isolated from wilted Faba bean plants grown in New Valley Governorate. It was microscopically identified on the basis of cultural and micropic characteristics to the species level according to the descriptions of Nelson et al. [10] and was confirmed in Assiut Univ., Mycol. Centre (AUMC). Pathogenicity of the isolate toward Faba bean plant was estimated Abdel-Monaim [6]. The highly pathogenic isolate was maintained on PDA medium at 4°C. Artificial soil infestation by *F. oxysporum* by using homogenized culture technique according to Muthomi et al. [11].

### Source of Faba bean seeds and growth of plants

Faba bean (*Vicia fabae* L.) cultivar Misr 1 used in this study was obtained from Legume Crop Res. Dep., Field Crop Res. Inst., Agric. Res. Center, Ministry of Agric., Giza, Egypt. Seeds were planted in plastic pots 30 cm in diameter (2.4 kg soil), filled with a pasteurized mixture of soil and sand (4:1 w/w). Five seeds were sown in each pot and these pots were irrigated every three days.

### Source of bio-agents

Bio control agent *P. fluorescens* and *B. megaterium* was isolated previously from rhizosphere soil in New Valley Governorate and antagonistic ability against most soil borne pathogenic fungi was recorded in previous studies [6].

### In vitro studies

The used highly activity antagonistic organisms *P. fluorescens* and *B. megaterium* in this study. While, chemical inducers used were Bion (Benzothiadiazole or BTH (benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester) and Chitosan (Sigma Chemical, St. Louis, MO, USA) at concentration 0.5 gm and 1 gm/L, respectively. The effect of bio-control agents and chemical inducers individually and/or in combination on *F. oxysporum* was studied as follows:

**Antagonistic bio-control agents:** *P. fluorescens* and *B. megaterium* isolates were streaked at opposite ends of PDA plates near edge and incubated at 25°C ± 1°C for 24 h. Then a mycelial disc (0.7 cm) of the pathogenic fungus *F. oxysporum* was placed in the center of each plate.

**Chemical inducers:** The effect of chemical inducers on the growth of pathogenic fungi was evaluated in PDA medium. 20 ml of PDA medium containing 0.5 gm/L Bion, 1.0 gm/L Chitosan was poured in the plates and inoculated with the pathogenic fungus *F. oxysporum*.

**Combination between bio-control agents and chemical inducers:** Flasks (250 ml) containing 200 ml PDA medium were amended with 0.5 gm/L Bion and 1 gm/L Chitosan individually, then each flask was poured in 10 plates. These plates were inoculated with antagonistic isolates and pathogenic fungus *F. oxysporum* as before.

For control treatment, the agar plug of only pathogen isolates was placed on PDA plates. The inoculated plates incubated at 25°C ± 1°C until colony of control grew to full plate. At this point, colony diameter was measured using ruler. Percentage of growth inhibition of pathogen was calculated using the formula below:

$$\% \text{ Inhibition} = (A - B) / A \times 100$$

Where:

A = Colony diameter of pathogen in control

B = Colony diameter in treated plates

### In vivo studies

**Greenhouse experiments:** The effects of bio-control agents (*P. fluorescens* and *B. megaterium*) and chemical inducers (Bion and Chitosan) individually or in combination against Faba bean wilt disease incited by *F. oxysporum* were evaluated under greenhouse conditions. Cell suspension of *P. fluorescens* and *B. megaterium* grown on nutrient broth medium for 3 days at 25°C ± 1°C were adjusted to 2.5 × 10<sup>8</sup> cfu/ml. Bion and Chitosan were prepared as solutions at 0.5 and 1 gm/L, respectively. The combination between bio-control agents and chemical inducers prepared with dissolving chemical inducers in suspension of bio-control agents.

Faba bean seeds (cv. Misr 1) soaked for 6 h in the following treatments: 1-*P. fluorescens* (Ps); 2-*B. megaterium* (Bm); 3-Bion 4-Chitosan; 5-Ps+Bion; 6-Ps+Chitosan; 7Bm+Bion; 8-Bm+Chitosan and 9-control. Plastic pots were filled with sterilized soil and mixed with *F. oxysporum* inoculum at rate 100 ml homogenized culture per pot, seven days before planting and then were sown by 5 seeds of each treatment. Four replicates were used for each treatment. In control treatment, Faba bean seeds soaked in water for 6 h. and sown at same rate. Pots were irrigated as needed.

Disease severity for *F. oxysporum* isolate was estimated after 90 days as a wilting percentage, on the basis of root discoloration or leaf yellowing. Plants with typical Fusarium wilt symptoms were assessed according to the type of symptoms that was observed using a numerical grades ranging from 0 to 5 as follows: (0)=No visible symptoms; (1)=1% to <20% of plant leaves are yellow and of the vascular systems are light brown (discoloration); (2)=20-<40% of plant leaves are yellow and of the vascular systems are brown (discoloration); (3)=40% to <60% of plant leaves are yellow and of the vascular systems are dark brown (discoloration); (4)=60% to <80% of plant leaves are yellow and of the vascular systems are dark brown (discoloration); (5)=80% ≤ 100% of plant leaves are yellow and of the vascular systems are dark brown (discoloration) or completely dead plants.

Disease severity index (DSI) described by Liu et al. [12] was adopted and calculated as follows:

$$DSI = \sum d / (d_{\max} \times n) \times 100$$

Where: (d) is the disease rating of each plant, (d) max the maximum disease rating and (n) the total number of plants examined in each replicate.

Also, at the end of experiment, the survived plants were weighted to record fresh weight per plant then dried at 80°C for 24 h to record the dry weight per plant.

**Field experiments:** Field experiment was carried out at New Valley Res. Station Farm during 2013-2014 and 2014-2015 growing seasons, to evaluate the efficiency of the tested bio-control agents (*P. fluorescens* and *B. megaterium*) and chemical inducers (Bion and

Chitosan) individually or in combination for controlling wilt disease and its effect on growth and yield parameters under field conditions. The experimental design was a complete randomized block with three replicates. The experimental unit area was 10.5 m<sup>2</sup> (3.5 × 3 m). Each unit included 5 rows; each row was 3.5 m in length and 60 cm width. Faba bean seeds (cv. Misr 1) were soaked in treatments described above for 6 h. The seeds treated were sown in hills 25 cm apart on both sides of 6 cm ridges in both seasons, 2 seed per hill. In control treatment, Faba bean seeds were soaked in water for 6 h and were sown in the same rate. The normal cultural practices of growing Faba bean were followed. Wilt incidence were recorded as above. At harvest, plant height (cm), number of branches, pods, and seeds/plant, 100-seed weight and total yield (kg/ feddan) were measured.

**Chemical analysis:** To observe the accumulation of pathogenesis related protein (chitinase and  $\beta$ -1,3-glucanase) as well as content each of phenolic compounds (free, conjugated and total phenols), total flavonoids and lignin, 15 days old seedlings were injected with *P. fluorescens* ( $2.5 \times 10^8$  cfu/mL), *B. megaterium* ( $2.5 \times 10^8$  cfu/mL), Bion (1 gm/L), Chitosan (1 gm/L), Ps+Bion, Ps+Chitosan, Bm+Bion, Bm+Chitosan and sterilized distilled water (SDW), 50  $\mu$ l /plant. Two days after treatment, pot soils infested were inoculated with 100 ml of *F. oxysporum* homogenate suspension per pot. The chitinase and  $\beta$ -1,3-glucanase activities as well as content of phenolic compounds (free, conjugated and total phenols), total flavonoids and lignin were estimated 0, 2, 4, 6, 8, 10 and 12 days after inoculation.

One gram of plant tissue was homogenized in 10 ml of ice-cold 50 mM potassium phosphate buffer (pH 6.8) containing 1 M NaCl, 1% polyvinyl pyrrolidone, (PVP), 1 mM EDTA and 10 mM  $\beta$ -mercaptoethanol [13]. Sample was filtrated through cheesecloth; the homogenates were centrifuged at 8000 rpm at 4°C for 25 min. The supernatants (crude enzyme extract) were stored at -20°C or immediately used for determination chitinase and  $\beta$ -1,3-glucanase enzymes activities and total protein. In the case of every enzyme under investigation, each treatment consisted of four replicates (3 plants/replicate) and two spectrophotometric readings using Milton Roy Spectrophotometer (Milton Roy spectronic1201) were recorded for each per replicate. The experiment for bioassays was repeated twice in time.

### Chitinase activity

The chitinase activity was determined using the method described by Wirth and Wolf [14]. High polymeric carbomethyl-substituted chitin labeled covalently Remazol Brilliant Violet 5R (CM-Chitin\*-RBV. Comp. Loewe Biochemica) was used as substrate. The reaction mixture consisted of 0.50 ml 0.01 M Na-Acetate buffer pH 5.2 with 5% (v/v) glycerin, 0.25 ml plant extract and 0.25 ml dye labeled substrate CM-\*RBV solution (2 mg/ml). Tested samples were incubated in a water bath at 37°C for 120 min. The enzyme reaction was terminated by adding 0.25 ml 2 N HCl. After centrifugation (8000 rpm; 25 min), supernatants containing soluble, dye labeled degradation products were transferred to cuvet. Absorbency was measured spectrophotometrically at 550 nm; sodium acetate buffer was added to blanks instead of plant extract. Enzyme activity was expressed as enzyme unit/mg protein.

### $\beta$ -1,3-Glucanase activity

$\beta$ -1,3-glucanase activity was assayed by the laminarin dinitrosalicylic acid method [15]. Plant samples (1 g) were homogenized with 2 ml of 0.05 M sodium acetate buffer (pH 5.0) and centrifuged at 16000 g for 15 min at 4°C. The supernatant was used in the enzyme assay. The reaction mixture consisted of 62.5  $\mu$ l of 4% laminarin and 62.5  $\mu$ l of enzyme

extract. The reaction was carried out at 40°C for 10 min. The reaction was then stopped by adding 375  $\mu$ l of dinitrosalicylic acid and heating for 5 min on boiling water, vortexed and its absorbance was measured at 500 nm. The enzyme activity was expressed as  $\mu$ g glucose released/min/mg protein.

### Protein concentration

Total protein content of the samples was quantified according to the method described by Bradford [16].

### Phenolic contents

To assess phenolic content, 1 g fresh plant sample was homogenized in 10 ml 80% methanol and agitated for 15 min. at 70°C. The total phenolic contents was determined by mixed 0.5 ml of the sample extract with 0.25 ml HCl and boiled in water bath for 10 min then left to cool. One ml of the folin ciocateu's reagent and 6 ml of Na<sub>2</sub>CO<sub>3</sub> 20% were added. The mixture was diluted to 10 ml with warm distilled water (30°C to 35°C). After 30 min standing in dark, the optical density of the developed blue colour was measured at 520 nm using a spectrophotometer against a reagent blank.

Free phenols content was determined by adding 1 ml of the reagent and 3 ml of 20% Na<sub>2</sub>CO<sub>3</sub> solution to 0.5 ml of the sample diluted to 10 ml with warm distilled water, let to stand for 30 min in dark and the optical density of the developed blue colour was measured at 520 nm using a spectrophotometer against a reagent blank.

Conjugated phenols content was determined by subtracting the amount of free phenols content from that of total phenols. The total phenolic contents and free phenols content were calculated on the basis of the calibration curve of catechol and expressed as catechol equivalents in milligrams per gram fresh weight.

### Estimation total flavonoids

Half (0.5 g) gram of fresh plant tissue was ground using a pestle and mortar with 10 ml of 80% ethanol and centrifuged at 10,000 rpm for 20 min. The supernatant was evaporated to dryness and preserved. The residue was dissolved in 5 ml of distilled water and used as the extract. To 2 ml of the extract, 0.3 ml of 5% sodium nitrate was added to the tubes. After 5 min, 0.3 ml of aluminum chloride (AlCl<sub>3</sub>) (10%) was added to all the tubes. At the 6 min, 2 ml of sodium hydroxide (1 M) was added to the mixture. Immediately, the contents of the reaction mixture were diluted with 2.4 ml of distilled water and mixed thoroughly. Absorbance of the mixture was determined at 510 nm versus a prepared blank immediately. Gallic acid was used as the standard compound for quantification of total flavonoids [17].

### Determination of lignin

One gram plant tissue from each treatment was mixed with 10 g of trichloroacetic acid (TCA) and incubated at 90°C. Delignification was stopped by cooling the reaction mixture after 240 min of reaction time. The reaction vessel was immersed in cold water and 5 ml of cold acetone were added. Suspension was filtered and liquor was evaporated until dark, high consistency liquid without smell of acetone was obtained. Lignin was precipitated by pouring the liquid in 200 ml cold water. Lignin was filtered and washed with warm water several times. After that, lignin was air-dried overnight at 4°C then weight [18].

### Statistical analysis

Analyses of variance were carried out using MSTATC, 1991 program (Ver. 2.10). Least significant difference was employed to test for significant difference between treatments at  $p \leq 0.05$  [19].

Treatments	% Wilt severity	% Reduction	Fresh weight (gm/plant)	Dry weight (gm/plant)
<i>Pseudomonas fluorescens</i>	23.51 c	67.50	13.25 cde	2.75d e
<i>Bacillus megaterium</i>	17.25 de	76.15	16.53 ab	3.35 abc
Bion	36.16 b	50.01	a10.41 e	2.10 f
Chitosan	32.38 b	55.23	12.42 de	2.42 ef
<i>P. fluorescens</i> + Bion	16.96 de	76.55	16.10 abc	3.15b cd
<i>P. fluorescens</i> + Chitosan	19.78 cd	72.65	14.25b cd	2.77 cde
<i>B. megaterium</i> + Bion	10.42 f	85.59	18.69 a	3.82 a
<i>B. megaterium</i> + Chitosan	13.42 ef	81.45	17.85 a	3.63 ab
Control	72.33 a	0.00	6.36 f	1.49 g

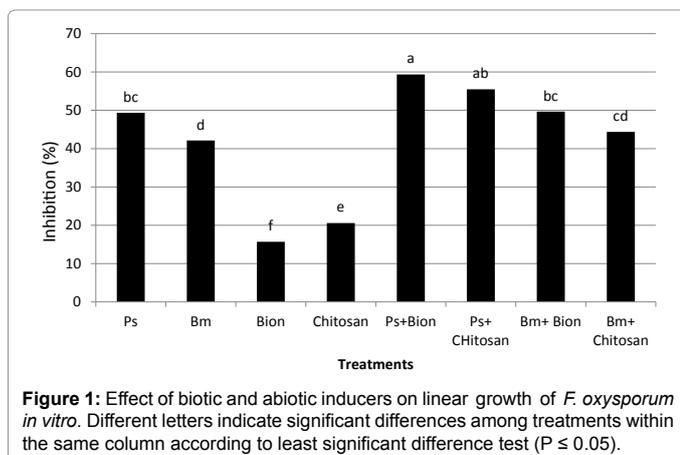
Different letters indicate significant differences among treatments within the same column according to least significant difference test ( $P \leq 0.05$ ).

**Table 1:** Effect of biotic and abiotic inducers individually and/or in combination on wilt disease caused by *Fusarium oxysporum*, fresh and dry weights of faba bean under greenhouse conditions.

Treatments	Wilt severity %			
	Season 2013-2014	% Reduction	Season 2014-2015	% Reduction
<i>Pseudomonas fluorescens</i>	14.15 bc	57.75	15.13 cd	60.11
<i>Bacillus megaterium</i>	12.37 cd	63.06	13.43 cde	64.59
Bion	17.11 b	48.91	19.24 b	49.27
Chitosan	14.76 bc	55.93	16.09 bc	57.58
<i>P. fluorescens</i> + Bion	10.15 de	69.69	11.33 ef	70.13
<i>P. fluorescens</i> + Chitosan	11.31 cde	66.23	12.24d ef	67.73
<i>B. megaterium</i> + Bion	5.89 f	82.41	6.37 g	83.21
<i>B. megaterium</i> + Chitosan	8.03 ef	76.02	9.49fg	74.98
Control	33.49 a	-	37.93 a	-

Different letters indicate significant differences among treatments within the same column according to least significant difference test ( $P \leq 0.05$ ).

**Table 2:** Effect of biotic and abiotic inducers on wilt severity under field conditions during growing seasons 2013-2014 and 2014-2015.



## Results

### Effect of biotic and abiotic inducers on wilt incidence under greenhouse and field conditions

Data present in Tables 1 and 2 shows that all treatments significantly reduce wilt severity under artificial infection with *F. oxysporum* in pots and/or natural infection in field during growing seasons 2013-2014 and 2014-2015. The combination between biotic and abiotic inducers more effective for controlling wilt disease than used individually of them either in pots or in field. The combination between *B. megaterium* and Bion recorded the highest reduction of wilt severity followed by *B. megaterium*+Chitosan under greenhouse and field conditions. However, gave 85.89% and 81.45% reduction under artificial infection with *F. oxysporum* in pots, respectively. While these treatments recorded 82.41%, 83.21% protection in case of *B. megaterium*+Bion and 76.02%, 74.98% protection in case of *B. megaterium*+Chitosan under field conditions in both growing seasons (2013-2014 and 2014-2015),

respectively. Faba bean seeds treated with Bion alone recorded the lowest protection against wilt disease in pots and field.

On the other hand, all treatments increased fresh and dry weights of survival plants compared with control under artificial infection with *F. oxysporum* in pots. The combination between biotic and abiotic inducers gave highly fresh and dry weights of survival plants more than usage individually of them. *B. megaterium*+Bion and *B. megaterium*+Chitosan recorded the highest fresh (18.69 and 17.85 gm/plant) and dry weights (3.82 and 3.63 gm/plant), respectively compared with 6.36 gm/plant fresh weight and 1.49 gm/plant dry weight in control.

### Effect of biotic and abiotic inducers on linear growth of *F. oxysporum*

Data in Figure 1 reveal that all treatments have significantly reduced linear growth of pathogenic fungus *F. oxysporum* either individually or in combination. In general, biotic inducers more effective for suppressing linear growth of pathogenic fungus than abiotic inducers and *P. fluorescens* was able to inhibit growth of *F. oxysporum* more than *B. megaterium*. The combination between of biotic and abiotic inducers was more effective than any of them individually tested. On the other hand, the combination *P. fluorescens* and Bion recorded the highest inhibition of *F. oxysporum* (59.36%) followed by *P. fluorescens*+Chitosan (55.47%). While Bion gave the lowest inhibition percentage for pathogenic fungus (15.96%) followed by Chitosan (20.59%).

### Effect of biotic and abiotic inducers on growth and yield parameters under field conditions

Data present in Tables 3 and 4 indicate that all treatments significantly increase plant growth (plant height and number of branches) and yield parameters (number of pods, seeds /plant, weight of 100 seeds and total yield/feddan) in both growing seasons (2013-2014 and 2014-2015). The combination between biotic and abiotic

Treatments	Plant height (cm)	No. of branches/plant	No. pods/plant	No. of Seeds/plant	Weight of 100 seeds	Total Yield Kg/feddan
<i>Pseudomonas fluorescens</i>	105.2 ab	3.1 ab	22.9 c	75.4 d	77.2 abc	1825.3 c
<i>Bacillus megaterium</i>	95.6 cd	2.8 bc	20.1 cd	62.4 e	74.6 bc	1699.6 de
Bion	90.2d	2.5 cd	18.6 d	60.4 e	73.2 c	1585.3 e
Chitosan	93.2 cd	2.7 bc	19.3 d	61.9 e	76.5abc	1702.3 d
<i>P. fluorescens</i> + Bion	112.3 a	3.6 a	31.5 a	102.3 a	79.6 ab	2109.4 a
<i>P. fluorescens</i> + Chitosan	108.9 a	3.2 ab	29.6 ab	92.5 b	81.2 a	2002.0 ab
<i>B. megaterium</i> + Bion	100.2 bc	3.0 bc	26.8 b	84.7c	77.1 abc	1902.3 bc
<i>B. megaterium</i> + Chitosan	98.7 bc	3.0 bc	27.3 b	85.3 c	77.9 abc	1925.9 bc
Control	74.69 e	2.1 d	12.4 e	30.5 f	71.9 c	1054.6 f

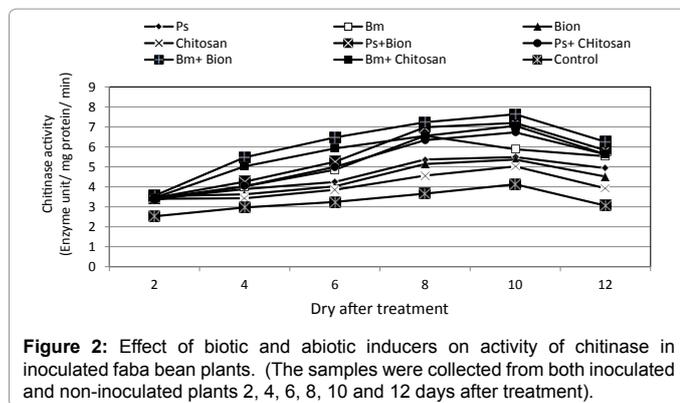
Different letters indicate significant differences among treatments within the same column according to least significant difference test ( $P \leq 0.05$ )

**Table 3:** Effect of biotic and abiotic inducers on plant growth and yield parameters under field conditions during growing season 2013-2014.

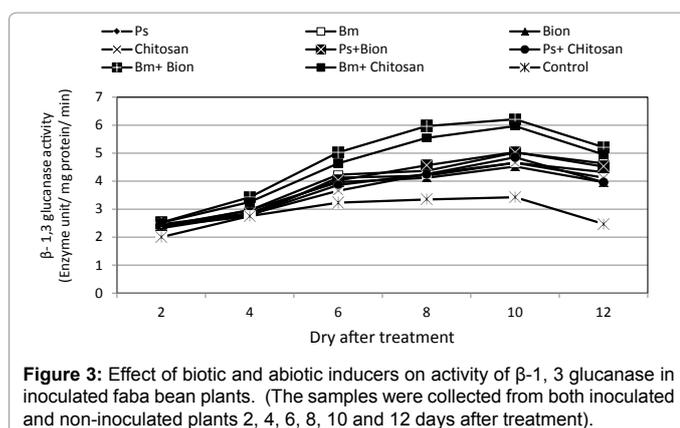
Treatments	Plant height (cm)	No. of branches/plant	No. pods/plant	No. of Seeds/plant	Weight of 100 seeds	Total Yield Kg/feddan
<i>Pseudomonas fluorescens</i>	101.7 abc	2.9 abc	21.2 d	72.3 d	76.2 ab	1798.3 d
<i>Bacillus megaterium</i>	91.4 de	2.6 cd	18.7 de	60.1 e	75.6 ab	1675.2 e
Bion	86.3 e	2.3 de	16.8 e	56.9 e	74.1 bc	1541.3 f
Chitosan	94.2 cde	2.7 bcd	18.7 de	60.1 e	75.8 ab	1655.4 e
<i>P. fluorescens</i> + Bion	110.5 a	3.3 a	29.8 a	99.6 a	78.6 ab	2029.6 a
<i>P. fluorescens</i> + Chitosan	104.5 ab	3.1 ab	28.4 ab	90.1 b	80.6 a	1989.3 ab
<i>B. megaterium</i> + Bion	95.6 bcde	2.8 bc	24.6 c	81.4 c	75.0 abc	1875.4 cd
<i>B. megaterium</i> + Chitosan	98.7 bcd	3.0 abc	25.6 bc	83.2 c	75.6 ab	1901.5 bc
Control	70.1 f	1.9 e	10.9 f	27.5 f	69.3 c	1020.6 g

Different letters indicate significant differences among treatments within the same column according to least significant difference test ( $P \leq 0.05$ )

**Table 4:** Effect of biotic and abiotic inducers on plant growth and yield parameters under field conditions during growing season 2014-2015.



**Figure 2:** Effect of biotic and abiotic inducers on activity of chitinase in inoculated faba bean plants. (The samples were collected from both inoculated and non-inoculated plants 2, 4, 6, 8, 10 and 12 days after treatment).



**Figure 3:** Effect of biotic and abiotic inducers on activity of beta-1, 3 glucanase in inoculated faba bean plants. (The samples were collected from both inoculated and non-inoculated plants 2, 4, 6, 8, 10 and 12 days after treatment).

inducers increased all growth and yield parameters more than usage of biotic or abiotic individually in both growing seasons. Faba bean seeds treated with Ps+Bion recorded the highest all growth and yield parameters except weight of 100 seeds in both growing seasons. While, Bion treatment recorded the lowest increase of all growth and yield parameters in both seasons.

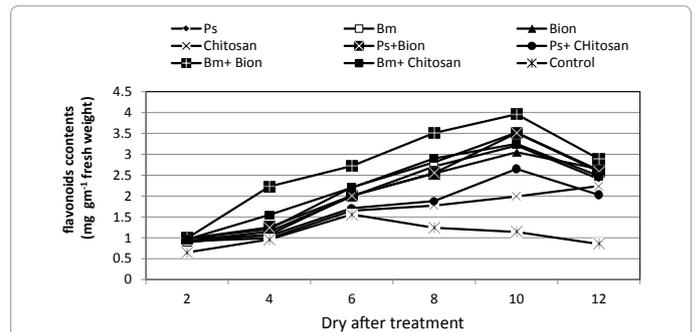
### Accumulation of pathogenesis related (PR) protein, phenolic, flavonoids compounds and lignin contents in Faba bean plants associated with biotic and abiotic inducer treatments

Accumulation of pathogenesis related (PR) protein (chitinase and beta-1,3-glucanase), phenolic compounds (total, free and conjugated phenols content), total flavonoids and lignin contents in Faba bean plants inoculated with *F. oxysporum* treated and untreated with biotic (*B. megaterium* and *P. fluorescens*) and abiotic (Bion and Chitosan) inducers were studied.

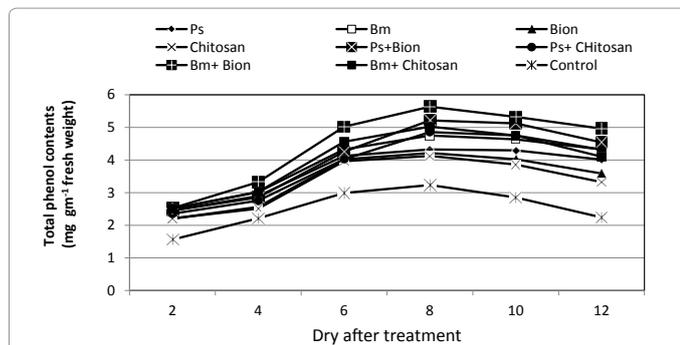
**Chitinase activity:** Data in Figure 2 show that Chitinase activity of inoculated Faba bean plants treated with biotic and abiotic inducers was higher than that of inoculated plants (infection control plants) after all time from application. Maximum increase was recorded at 10 days after treated with biotic and abiotic either individually or in combinations except in case of *B. megaterium* while the maximum activity recorded after 8 days from treatment then all cases the activity of Chitinase enzyme was decreased. Combined application of biotic and abiotic inducers increased the activity of Chitinase more than the applied of them individually. The combination between *B. megaterium* and Bion recorded the highest increase of Chitinase activity after all time from application.

**$\beta$ -1,3-Glucanase:** Data present in Figure 3 show that all treatments increased activity of  $\beta$ -1,3 glucanase in Faba bean plants inoculated with *F. oxysporum* after all time of application compared with untreated inoculated plants (control). The increased of  $\beta$ -1,3-glucanase activity was reached maximum levels at 10 days after treatments then decreased progressively. Combination between *B. megaterium* and Bion recorded the highest level of  $\beta$ -1,3-glucanase activity followed by *B. megaterium*+Chitosan at after all time of application.

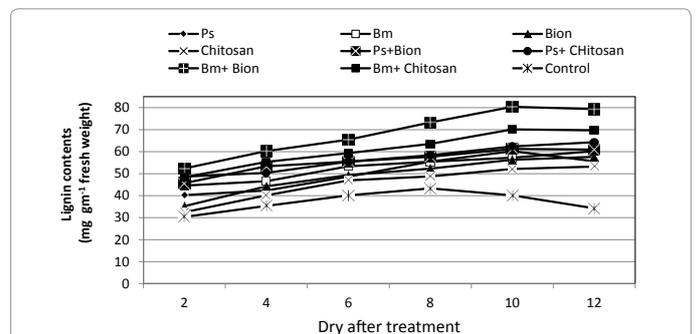
**Phenolic compounds:** The content of phenols (free, conjugated and total phenols content) was greatly increased in Faba bean plants treated with biotic and/or abiotic inducers either individually or in combined compared with untreated plants (Figures 4-6). The increase of phenolic contents (total and free phenols) was increased until 8 days after of



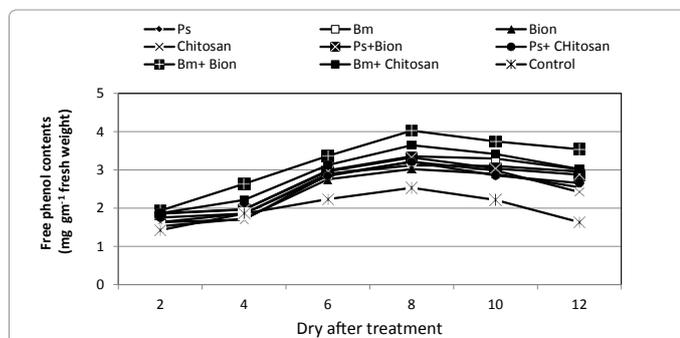
**Figure 7:** Effect of biotic and abiotic inducers on flavonoids contents in inoculated faba bean plants. (The samples were collected from both inoculated and non-inoculated plants 2, 4, 6, 8, 10 and 12 days after treatment).



**Figure 4:** Effect of biotic and abiotic on total phenols in inoculated faba bean plants. (The samples were collected from both inoculated and non-inoculated plants 2, 4, 6, 8, 10 and 12 days after treatment).



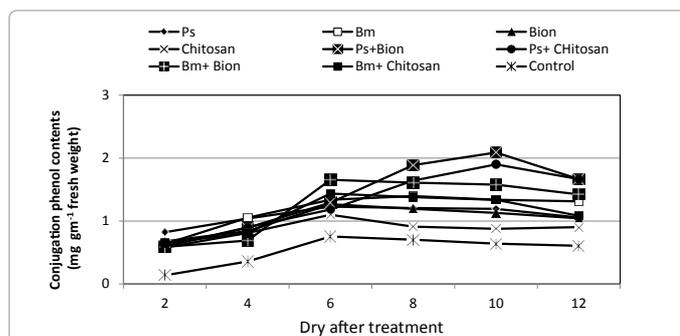
**Figure 8:** Effect of biotic and abiotic inducers on lignin contents in inoculated faba bean plants. (The samples were collected from both inoculated and non-inoculated plants 2, 4, 6, 8, 10 and 12 days after treatment).



**Figure 5:** Effect of biotic and abiotic inducers on free phenols in inoculated faba bean plants. (The samples were collected from both inoculated and non-inoculated plants 2, 4, 6, 8, 10 and 12 days after treatment).

application treatments then decreased progressively. While the highest level of conjugated phenol contents was differed according to type of treatment (6-10 days from application of treatments) then decreased progressively. The Combination between *B. megaterium* and Bion recorded the highest levels of phenolic compound contents either total or free phenols) after 8 days from application, while the highest levels of conjugated phenols were recorded after 10 days from application of *P. fluorescens*+Bion.

**Total flavonoids:** The accumulation of total flavonoids in infected plants was increased after treated with biotic and/or abiotic inducers either applied individually or in combination under infection with *F. oxysporum* as compared with infection plant alone (Figure 7). The highest level of flavonoid contents was recorded after 10 days from treatments application except Chitosan treatment whereas the highest contents recorded after 12 days from Chitosan application. The combination treatment *B. megaterium* and Bion recorded the highest levels from flavonoid contents after all time of application.



**Figure 6:** Effect of biotic and abiotic inducers on conjugation phenols in inoculated faba bean plants. (The samples were collected from both inoculated and non-inoculated plants 2, 4, 6, 8, 10 and 12 days after treatment).

**Lignin contents:** During examination period, a remarkable increase in lignin content of Faba bean plants treated with biotic and/or abiotic either individually or in combination under infected with *F. oxysporum* was recorded as compared with untreated infected plants (Figure 8). The increase was much pronounced at 10-12 after application of treatments. *B. megaterium*+Bion treatment recorded the highest increase in lignin content in Faba bean plants infected with *F. oxysporum*, followed by combined application with *B. megaterium* and Chitosan. In addition, Chitosan alone was the least effective ones during after all period of application.

## Discussion

Faba bean (*Vicia fabae* L.) is one of the most important food crops

in Egypt and many other parts of the world. Faba bean production has been reduced greatly due to the attack by wilt disease caused by *F. oxysporum*. The control of this disease is very difficult. Application of fungicides and fumigants have provided a good control of soil pathogens and a broad spectrum of activity and have been popular with growers all over the world although they are cheap, but hazardous, and sometimes difficult to apply [20]. Nowadays, the application of biological control using antagonistic microorganisms and chemical inducers has proved to be successful for controlling various plant diseases. However, it is still easy and not costly in application. It can serve as the best control measure under greenhouse and field conditions. In this study, I am focused on the use of two biotic inducers viz. *B. megaterium* and *P. fluorescens* and two chemical inducers (Bion and Chitosan) either applied as individually and/or in combinations under artificial infection with *F. oxysporum* in greenhouse and natural infection in field. Added to interpretation of the obtained results by estimating the accumulation of pathogenesis related protein (Chitinase and  $\beta$ -1,3-glucanase) as well as content each of phenolic compounds (free, conjugated and total phenols), total flavonoids and lignin. Results of this investigation showed that, application of biotic inducer and abiotic inducer alone or in combination effectively reduced wilt severity as compared with untreated infected control under greenhouse and field conditions. However, combined application was superior; it recorded maximum reduction wilt severity. Also, all biotic and abiotic inducers reduced mycelial growth *in vitro* either used individually or in combination. The biotic inducers reduced growth of *F. oxysporum* more than abiotic inducers. The reduction in disease severity as a result of application of biotic (*B. megaterium* and *P. fluorescens*) and abiotic (Bion and Chitosan) may be due to their well-known property of inducing resistance and inhibiting the mycelia development by triggering the various defense related pathways [21,22]. In addition, the inhibition of mycelial growth in a dual culture assay is due to the production of either siderophore, HCN [23] or hydrolytic enzymes, i.e. Chitinase and  $\beta$ -1,3-glucanases [24] as mechanisms for biological control. On the other hand, all treatments improved Faba bean growth and yield components under field conditions. The combination of between biotic and abiotic inducers increased growth and yield parameters more than when applied individually. The plant growth promoting effects of biotic and abiotic inducers observed in the present study are consistent with the result of Abdel-Monaim [6] found that the combination of bio-control agents and chemical inducers were more effective than using them individually and SA+*T. viride* was the best treatment in this respect. The underlying mechanisms for this plant growth promoting action may be attributed to effects on plant physiological processes such as nutrient uptake, cell elongation, cell division, enzymatic activation, and protein synthesis [25].

The exogenous application of biotic and abiotic inducers trigger the SAR signal transduction pathway in several plant species by accumulation of pathogenesis-related (PR) proteins, phenolic and flavonoid compounds and increased of lignin, some of which possess antimicrobial properties [2] when applied as seed soaking. In this study Faba bean treated with biotic and abiotic inducers either individually or in combination due to increase of accumulation pathogenesis-related (PR) proteins (Chitinase and  $\beta$ -1,3-glucanases), phenolic compounds (total, free, and conjugated phenols content), flavonoid compounds and lignin contents compared untreated seeds during all time after application. Increased accumulation of pathogenesis-related proteins such as  $\beta$ -1,3-glucanases and Chitinase that may contribute to the resistance *via* hydrolysis of the pathogen cell wall; an oxidative burst, which triggers the elicitation of phytoalexins with antifungal properties; and the phenylpropanoid pathway by activation of PAL, which leads

to the formation of phytoalexins, lignins, and SA. Lignification of the plant cell walls is a mechanism to resist pathogen invasion [26].

In conclusion, induction of resistance by some biotic (*B. megaterium* and *P. fluorescens*) and/or abiotic (Bion and Chitosan) inducers, might provide a practical supplement to environment-friendly disease management of wilt disease in Faba bean when they are combined with appropriate integrated agronomic practices. Depending on the results of the current study, it is recommended to use combination between biotic and abiotic inducers especially combined *B. megaterium* and Bion to control wilt disease in Faba bean plants as seeded soaking for 6 h.

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