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# INDUCTION OF DEFENSE RESPONSES IN FABA BEAN PLANTS AGAINST CHOCOLATE SPOT DISEASE CAUSED BY *BOTRYTIS FABAE* USING CHITOSAN

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## ABSTRACT

**Chocolate spot disease caused by *Botrytis fabae* is a major problem that limits faba bean production in Egypt. The *in vitro* antifungal activity of chitosan and its ability to elicit defense responses in two faba bean cultivars Giza 416 (resistant) and Giza 40 (susceptible) against this fungus were evaluated. Seven isolates of *B. fabae* were cleaned from infected leaves. All isolates were pathogenic to Giza 40 cultivar, with isolate 3 (Bf-3) was the most virulent. Chitosan inhibits the mycelial growth of *B. fabae* (Bf-3) in concentration-dependent manner. At 0.75 % and 1 %, chitosan completely inhibits the growth of *B. fabae*. Under greenhouse conditions, two fab bean cultivars, Giza 416 (resistant) and Giza 40 (susceptible) were sprayed with 0.5% chitosan. Treatment with chitosan 0.5% reduced the chocolate spot disease severity for both cultivars compared with untreated infected control. Plants of both cultivars treated with chitosan showed a lower rate of lipid peroxidation and higher levels of chlorophyll and total phenols compared with untreated infected control. Chitosan stimulated the activity of defense enzymes such as peroxidase (POD), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL). Furthermore, chitosan treatment increased the expression levels of three pathogenesis**

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**related (PR) genes including PR1, PR2 (β-1,3-glucanase), and chitinase. The resistant cultivar (Giza 461), whether treated or untreated with chitosan displayed weaker symptoms, and enhanced values for all physiological and molecular responses than the susceptible cultivar (Giza 40). The findings show that chitosan has a high potential for protecting faba bean plants from *B. fabae* by regulating biochemical, physiological and molecular responses in faba bean plants under infection.**

**Keywords:** Chocolate spot, *Botrytis fabae,* Chitosan, Resistance, Defense response

## INTRODUCTION

Faba bean (*Vicia faba* L.), also known as fava bean, horse bean, and broad bean, is one of the world's oldest legumes crops, and is mainly grown in cool and moist areas, which are suitable for the development of several foliar diseases that cause severe crop losses **(Omar, 2021)**. In many countries, faba bean seeds is mainly used as human food whether fresh, dried or canned, while, in several other countries, it mostly used as livestock feed **(Dhull *et al*., 2021)**. Faba bean is one of the most economic legume crops in Egypt, as a cheap protein-rich staple food that might be used in place of animal protein in human diets **(El-Hendawy *et al*. 2010; Dhull *et al*., 2021)**.

Faba bean productivity is affected by a variety of yield-limiting factors including fungal diseases **(Torres *et al*., 2006; Omar, 2021)**. Chocolate spot disease, caused by *Botrytis fabae* Sardina and to some extent *B. cinerea*, is one of the most devastating fungal diseases that cause a significant loss in the yield in Egypt and worldwide, especially when environmental conditions are favorable **(Abd El-Karem *et al*., 2013; El-Kholy, 2014; Beyene *et al*., 2018)**. Chocolate spots disease causes yield loss of 25-85% in the Nile Delta region of Egypt **(Mohamed, 1996)**. *B. fabae* could infect leaves, stems, flowers, and pods. Symptoms begins as reddish-brown spots on leaves and progresses to chocolate-colored lesions, which may develop into large necrotic zones. If the disease appears during

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flowering period, it might abort flowering before developing pods resulting in crop failure **(Hanounik and Hawatin, 1982; El-Metwally *et al*., 2010)**.

Fungicides are mainly used to control chocolate spots disease **(Sahile *et al*., 2008; Sahar *et al*., 2011)**. However, alternative methods are required due to public health concerns, protection of the environment, and the emergence of resistant isolates **(El-Hendawy *et al*., 2010; Mahmoud *et al*., 2011)**. Chitosan is a natural, non-toxic, biodegradable polymer derived from deacetylation of chitin **(Jitareerat *et al*., 2007)**. Chitosan protects plants against fungal diseases in three ways: (1) as an antifungal compound that inhibits mycelial growth and sporulation **(Rinaudo, 2006, Basit *et al*., 2020; Kumaraswamy *et al*., 2018; Abou-zeid *et al*., 2020)**, (2) by inducing a wide range defense responses in plants **(Xing *et al*., 2015; Carmona *et al*., 2021)**, and (3) formation of film on the treated surfaces **(Romanazzi *et al*., 2018)**. Chitosan has been used to manage a wide range of pre- and post-harvest diseases in various crops **(Ge *et al*., 2010; Meng *et al*., 2010; Li *et al*., 2016; Hassan and Chang 2017; Xoca-Orozco *et al*., 2018)**.

Chitosan is an efficient elicitor and induced systemic acquired resistance (SAR) in plants exposed to pathogens **(Xing *et al*., 2015; Orzali *et al*., 2017)**. Chitosan stimulates host production of defense-related proteins and enzymes **(Lin *et al*., 2005; Hadwige, 2013; Xing *et al*., 2015; Orzali *et al*., 2017)**. Chitosan and its derivatives have also been shown to raise the amount and activity of enzymes and genes expression of peroxidase (POD), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), catalase (CAT) and superoxide dismutase (SOD) in a variety of crops **(Xing *et al*., 2015; Li *et al*., 2016)**, which is correlated with induced resistance in these crops. Pathogenesis related (PR) proteins were also enhanced as a result of chitosan treatments. *PR1*, *PR2* (β-1,3-glucanase), *PR5* (thaumatin-like proteins), and *PR8* (chitinase) genes expression were induced in the chitosan treated plants under phytopathogenic fungi infection **(Sathiyabama *et al*., 2014; Beatrice *et al*., 2017; Carmona *et al*., 2021)**. Therefore, current study aimed to investigate the effect of chitosan on the growth of *B. fabae in vitro* and to control chocolate spot disease in the greenhouse. Additionally, its effect on

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some physicochemical parameters, defense related enzymes (POD, PPO, and PAL) as well as expression of *PR1*, *PR2*, and *CHT* (chitinase) genes.

## MATERIAL AND METHODS

### Materials

Two Faba bean (*Vicia faba* L.) cultivars with different levels of resistance to *Botrytis fabae*, Giza 461 (Resistance) and Giza 40 (susceptible) were provided by Field Crops Research Institute, Agriculture Research Center, Giza, Egypt. Chitosan (Fluka) was obtained from Sigma-Aldrich, Switzerland. Naturally infected faba bean leaves with typical symptoms of the chocolate spot were collected from different location at El-Beheira Governorate, Egypt for isolation of *B. fabae*.

### *Botrytis fabae* Isolation

*B. fabae* pathogen was isolated from collected naturally infected faba bean leaves. Infected leaflets were cut into small pieces, surface sterilized with 1%sodium hypochlorite (NaOCl) for 2 min, rinsed twice with sterilized distilled water, dried between two layers of sterilized filter papers, and placed on potato dextrose agar (PDA) media in Petri dishes and incubated for 7 days at 20±1°C. Pure isolates were obtained using single spore or hyphal tip techniques and identified based on the microscopical and the morphological characteristics according to **Morgan (1971)** and **Jarvis (1977)**.

### Inoculum Preparation

Cultures were grown on faba bean leaf agar media (FBLA) medium (Leach and Moore, 1966) and incubated at 20±1°C for 12 days. Inoculum suspension was made by adding sterile distilled water and conidia were collected by scraping the surface of the medium with a needle. Spore suspensions were counted using the haemacytometer slide and adjusted to $2.5\times10^5$ spores/ ml with sterile distilled water **(Abd- Rabh *et al*., 2013)**.

### Pathogenicity test

The Pathogenicity of the *B. fabae* isolates were investigated on the susceptible cultivar, Giza 40 in the greenhouse. Seeds were sterilized with

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2% sodium hypochlorite for two minutes, rinsed with sterile water and planted in 20cm diameter plastic pots filled with autoclaved sandy clay soil (1:2, v/v). Five seeds were sown per pot and thinned after germination to three plants per pot. Plants were fertilized with N: P: K (20:20:20) fertilizer every 15 days and irrigated when needed. Forty-five days after sowing, plants were sprayed with the conidial suspension of the tested isolates and covered with polyethylene pages for 24 hrs to maintain a high relative humidity and kept in the greenhouse under natural winter conditions. Control plants were sprayed with distilled water. Three replicates were used for each isolate with three pots per replicate. The Severity of chocolate spot disease were assessed 7 days post inoculation (dpi) using the disease scale (class rate 1-9) of **Bernier *et al*. (1993)**. The disease severity (%) was calculated using **Hanounik (1986)** formula:

Disease severity (%) = $\{(\Sigma n \times V) / (9N)\} \times 100$

where, n represent the number of plants in each class rate, V represent the class rate, N represent the number of infected plants and 9 is the maximum severity class rate.

### *In vitro* assay

### Chitosan preparation

Chitosan solutions were prepared by dissolving the chitosan powder in 0.5% (v/v) aqueous acetic acid to make solutions with concentrations of 0.03, 0.06, 0.125, 0.25, 0.50, 0.75 and 1.0%. The chitosan solutions were stirred for 3 hrs at 25°C before the pH was adjusted to 5.5 with NaOH (1N) **(Meng *et al*., 2010)**.

### Effect of chitosan on the linear growth of *B. fabae*

The Effect of chitosan at 0.03 to 1% on the linear growth of *B. fabae* was carried out on PDA plates using poison food technique **(Dhingra and Sinclair, 1985)**. Chitosan was mixed with autoclaved PDA media just before solidification to achieve the desired concentrations and poured in sterilized 9cm Petri dishes. Plates were inoculated with 5mm discs from the edge of 7-day-old culture of *B. fabae* aggressive isolate (Bf-3) and incubated at 20±1˚C. Inoculated PDA media plates without chitosan were used as control. Five Petri dishes were used for each concentration. The

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fungal growth diameters (cm) were measured when the control plates covered with the mycelia. The percentage of mycelia growth inhibition was estimated using **Pandey *et al*. (1982)** formula:
Mycelia growth inhibition (%) = {(dc-dt)/dc} ×100
where: dc is average diameter of fungal growth in the control, and dt is average diameter of fungal growth in the in treatments.

**Effect of chitosan on infection with *B. fabae* under greenhouse conditions**

Plants of Giza 461 (Resistance) and Giza 40 (susceptible) cultivars were grown on plastic pots in the greenhouse as mentioned in the pathogenicity test. Chitosan 0.5% was sprayed twice to run off onto the faba bean leaves of the two cultivars at 35 and 42 days after sowing. Two days after the last chitosan treatment, plants were sprayed with *B. fabae* (isolate Fb-3) conidial suspension ($2.5 \times 10^5$ spores/ ml) until leaves draining and covered with polyethylene pages for 24 hr to maintain a high relative humidity and kept in a greenhouse under natural winter conditions. Plants sprayed with sterile water used as non-infected control, while chitosan untreated plants inoculated with *B. fabae* was used as infected control. Five replicates for each treatment with three pots per replicate were used. Some leaves were collected at 72 hrs post inoculation (hpi) to determine various biochemical and gene expression changes associated with the infection process and chitosan treatment. The Severity of chocolate spot disease were assessed 7 dpi as previously mentioned in the pathogenicity test. Efficacy percentage (E%) of chitosan in reducing chocolate spot severity percentage was assessed according to **Rewal and Jhooty (1985)** as follow:
E% = (C - T/ C) x 100
Where: C = Disease severity % in control; T = Disease severity % in the treatment

**Determination of leaf chlorophyll concentration**

Total Chlorophyll contents were determined 7 dpi using portable chlorophyll meter (SPAD-502, Minolta, Japan).

**Biochemical assays**
**Determination of lipid peroxidation**

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Malondialdehyde (MDA) content was evaluated using the thiobarbituric acid (TBA) method according to **Heath and Packer (1968)**. Leaf samples (1.0g) were homogenized in 5ml of 0.1% 3-chloro acetic acid (TCA) and centrifuged at 12.000*g* for 30 min at 4$^{o}$C, then 1.0ml of the supernatant was mixed with 3ml of 0.5% TBA on 20% TCA and incubated at 95$^{o}$C for 25 min. Reaction stopped by ice cooling, and the absorbance was measured at 532 and 600nm (Jenway, Model 6305, Bibby Scientific Limited, UK). MDA content is given as mmole MDA $g^{-1}$ FW using an extinction coefficient of 155m$M^{-1}cm^{-1}$ (El-Komy, 2014).

**Determination of Total phenolic content**

Leaf samples (1.0g) was homogenized in 10ml of 80 % methanol, extracted at 70 $^{o}$C for 15 min and centrifuged at 12000*g* for 5 min. The methanolic extracts were used to determine the total phenolic content using Folin-Ciocalteu colorimetric method **(Zieslin and Ben Zaken, 1993)**. The total soluble phenol content expressed as microgram gallic acid (GA) $g^{-1}$ FW.

**Determination of defense enzymes**

Peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) activities were measured in faba bean leaves after 72 hpi with *B. fabae* isolate Bf-3. Healthy and inoculated leaves from the middle nodes of five plants (replicates) per treatment were collected for enzyme analysis.

**Enzymes extraction**

For PPO and POX extraction, one gram of faba bean leaves sample was homogenized in 5ml of a pre-cooled (4°C) 50mM potassium phosphate buffer (pH 7.0) containing 2% polyvinyl pyrrolidone (PVP), and 1mM EDTA. The homogenate was centrifuged at 12,000*g* for 20 min at 4°C (Universal 32R, Hettich Zentrifugen, Germany). The supernatant was kept at -20°C and used for PPO and POX enzyme activity assays. For PAL extraction, leaf sample (1g) was homogenized in 3 ml of 100mM ice-cold borate buffer (pH 7.0) containing 1.4 mM of 2-mercaptoethanol and 0.1g of PVP. The supernatant was kept at -20°C and used for PAL enzyme activity assay.

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**Assay of POD, PPO and PAL enzymes**

POD activity was assayed spectrophotometrically using **Hammerschmidt and Kuc (1982)** method. One hundred µl of enzyme extract were mixed with 1.5 ml of pyrogallol (0.05M), then incubated at 28±2°C for 5 min before starting the reaction with 0.5ml of 1% $H_2O_2$. The increase in the absorbance was measured at 20 seconds intervals for 3 min at 420nm (Jenway, Model 6305, Bibby Scientific Limited, UK). POD activity was presented as changes in the absorbance ($\Delta OD_{420}$) $min^{-1}$ $g^{-1}$ of fresh weight (FW).

PPO activity was measured according to **Mayer *et al*. (1965)**. The PPO reaction mixture consisted of 200µl of enzyme extract, 1.5ml of 100 mM phosphate buffer (pH 6.5), then mixture was incubated at 30°C for 5 min before starting the reaction with 200µl of catechol (10mM). The increase in the absorbance was measured at 30 seconds intervals for 3 min at 495nm (Jenway, Model 6305, Bibby Scientific Limited, UK). The increase in absorbance were recorded at 495nm. Enzyme activity was expressed as $\Delta OD_{495}$ $min^{-1}$ $g^{-1}$ FW.

PAL activity was determined using the method described by **Dickerson and colleagues (1984)**. Enzyme extract (0.4ml) was incubated with 0.5ml of 100mM borate buffer (pH 8.8) and 0.5ml of 12mM L-phenylalanine at 30°C for 30 min. The rate of conversion of L-phenylalanine to trans-cinnamic acid at 290nm (Jenway, Model 6305, Bibby Scientific Limited, UK) was used to determine the PAL activity The amount of trans-cinnamic acid produced was calculated using its extinction coefficient of 9630 $M^{-1}cm^{-1}$. The enzyme activity was calculated in nmol trans-cinnamic acid $min^{-1}g^{-1}$ FW.

**Expression analysis of *PR1*, β-1,3-glucanases (*PR2*), and Chitinase genes using Real Time-qPCR**

Samples of faba bean leaves (from the middle nodes) of healthy, untreated, and treated infected plants of both cultivars were collected after 72 hpi for gene expression analysis. Total RNA was extracted using total RNA purification kit (Jena bioscience, Germany) following the manufacturer's protocol and stored at -80◦C until needed. First strand

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cDNA was created from isolated RNA using Oligo (dT) primer and MLV reverse transcriptase enzyme (Fermentas, USA) according to manufacturer's instructions. Comparative qRT-PCR analysis was used to quantified the expression of *POX*, *PPO*, Chitinase, *PR1*, and *PR2* genes. The primers sequence of the selected genes are shown in Table 1. The faba bean elongation factor 1-alpha (*ELF1A*) gene was used as internal housekeeping (reference) gene **(Cheng *et al*., 2012; El-Komy, 2014)**.

The PCR Reaction (25µl) consists of 1µl cDNA (100 ng), 12.5µl of 2× Quantitech SYBR® Green Mix (Fermentaz, USA), 1µl of 25pM/µl of forward and reverse primers, and 9.5µl of RNase free water. The Reactions were conducted in Rotor-Gene 6000 system (Qiagen, ABI System, USA), with one cycle at 95°C for 10 min followed by 40 cycles (95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec). Comparative CT method ($2^{-\Delta\Delta Ct}$ method (**Schmittgen and Livak, 2008**) was used to determine the relative expression level. The target genes expression levels were normalized relative to *ELF1A* gene and the relative expression of non-infected control plants of each cultivar at each time point was set as 1.

Table1. Primers used in the molecular studies

| Primers | | Primer sequence 5´ →3´ | Annealing (°C) | Ref |
|---|---|---|---|---|
| Chitinase | F | 5-GAGTGGTGTGGATGCTGTTG-3 | 60 | Gorji et al., 2014 |
| | R | GCCATAACCGACTCCAAGCA-3 | | |
| PR1 | F | CAGTGGTGACATAACAGGAGCAG | 60 | Cheng et al., 2012 |
| | R | CATCCAACCCGAACCGAAT | | |
| PR2 | F | CCAATGGGTACAAAGAAACG | 60 | Chang et al., 2012 |
| | R | AAACCAAGTAACCAATGAAAGG | | |
| ELF1A | F | GTGAAGCCCGGTATGCTTGT | 60 | Cheng et al., 2012 |
| | R | CTTGAGATCCTTGACTGCAACATT | | |

**Statistical analysis**

Collected data were analyzed by one-way analysis of variance using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Significant

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differences between means were determined using Tukey's HSD test at a probability level ≤ 0.05.

## RESULTS AND DISCUSSIONS

### Isolation, identification and Pathogenicity test of *B. fabae*

Seven isolates of *B. fabae* were obtained and identified from the naturally infected faba bean leaves collected from four locations at El-Beheira Governorate, Egypt. Pathogenicity tests were performed on these isolates to determine their virulence.

The seven obtained isolates of *B. fabae* were pathogenic and able to infect faba bean Giza 40 cultivar, and caused different degrees of disease severity, with significant differences between the seven isolates (Table 2). *B. fabae* isolate 3 (Bf-3) isolated came from Kafr El-Dawar locality was the most virulent with 61.20% chocolate spot severity. While, isolate 2 (Bf-2) from Abou El-Matamer locality gave the lowest disease severity (34.30%). The most virulent isolate (Bf-3) was chosen for further studies. These findings are in harmony with those obtained by several reports **(Mahmoud *et al*., 2012a; Abd- Rabh *et al.*, 2013; El-Kholy, 2014)** which found that *B. fabae* was the most frequently recovered fungus from chocolate spot affected samples, with high variability in virulence across isolates.

Table 2: Pathogenicity test of the isolated *Botrytis fabae* showing the disease severity

| Isolate | Location | Disease severity % |
|---|---|---|
| Bf-1 | Hosh Issa | $37.60^{d}$ |
| Bf-2 | Abou El-Matamer | $34.30^{d}$ |
| Bf-3 | Kafr Edawar | $61.20^{a}$ |
| Bf-4 | Abou Hommos | $45.60^{c}$ |
| Bf-5 | Hosh Isaa | $55.00^{ab}$ |
| Bf-6 | Hosh Issa | $41.80^{cd}$ |
| Bf-7 | Kafr Eldawar | $47.60^{bc}$ |

Different letters indicate significant differences between treatments according to the Tuckey HSD test (P < 0.05). Values are the mean of three replicates.

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**Effect of chitosan on the linear growth of *B. fabae in vitro***

Different concentrations of chitosan (0.03- 1.0%) were tested for their inhibitory effect on linear growth of *B. fabae in vitro*. The results in Table (3) indicated that all tested chitosan concentration had significant inhibitor effect on tested FB-3 isolate of *B. fabae*. Chitosan inhibits the mycelial growth in concentration-dependent manner, whereas the lowest growth inhibition (16.33%) was observed at 0.03% chitosan and the highest (92.55%) was recorded at 0.50% chitosan. Meanwhile, at 0.75 and 1 %, chitosan completely suppresses the fungal growth. These results support previous reports on the antifungal effect of chitosan on mycelial growth of several plant pathogenic fungi, including *Botrytis cinerea*, *Rhizoctonia solani* and *Fusarium solani*, *F. oxysporum*, *Macrophomina phaseolina*, *Alternaria solani*, *Sclerotium rolfsi*i, and *Phytophthora infestance* **(Rabea and Steurbaut, 2010; Reglinski *et al*., 2010; Park and Chang, 2012; Rabea and Badawy, 2012; Ahmed, 2017; El-Mohamedya *et al*., 2019; Mohammed *et al*., 2019; Thabet, 2019; Carmona *et al*., 2021)**.

Table 3: Effect of Chitosan on the Mycelial linear growth (cm), Mycelial growth inhibition (%) of *B. fabae in vitro*.

| Treatment | | Mycelial linear growth (cm) | Mycelial growth inhibition (%) |
|---|---|---|---|
| Control | | 9.00[a] | 0.00 |
| Chitosan (%) | 0.03 | 7.53[b] | 16.33 |
| | 0.06 | 5.70[c] | 36.66 |
| | 0.125 | 3.67[d] | 59.22 |
| | 0.25 | 2.23[e] | 75.22 |
| | 0.50 | 0.67[f] | 92.55 |
| | 0.75 | 0.00[g] | 100 |
| | 1.00 | 0.00[g] | 100 |

Different letters indicate significant differences between treatments according to the Tuckey HSD test ($P < 0.05$). Values are the mean of five replicates.

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Chitosan inhibits fungal development by interacting with the negative charge of cell membrane components on fungal surfaces, resulting in formation of polyelectrolytic complexes, altered membrane permeability, and causing leakage of intracellular electrolytes and proteinaceous components **(Bautista-Baños *et al*., 2006; Coqueiro and Piero, 2011)**. Furthermore, chitosan can inhibit Pathogen DNA transcription to RNA **(Li *et al*., 2008)**. Because of its non-toxic and biodegradable characteristics, chitosan and chitosan derivates have become well-known biological control agents **(El-Mohamedya, 2019)**.

**Effect of chitosan on infection with *B. fabae* under greenhouse conditions**

Effect of Chitosan at 0.5% on the severity of chocolate spot disease in two faba bean cultivars, Giza 461 (R) and Giza 40 (S), was investigated under the greenhouse conditions (Fig. 1). The results indicated that there were significant differences in the susceptibility of the two tested faba bean cultivars to *B. fabae* at 7 dpi. Giza 40 was the most sensitive to chocolate spot disease with a disease severity of 62.6%, while, Giza 461 was resistant to the disease with a severity of 9.12% (Fig. 1). Foliar spray applications of the chitosan at 0.5% significantly reduced the severity of the disease to 3.62 in Giza 461 and 21.32% in Giza 40 compared with the untreated infected control. The efficacy (E%) of chitosan in reducing chocolate spot severity percentage were 60.3 and 65.9% in Giza 461 and Giza 40 cultivars, respectively (Fig. 1).

These findings agreed with **Faoro *et al.* (2008)** who found that spraying chitosan reduced infection with powdery mildew infection in barley. Application of chitosan control potato late blight **(Chang and Kim, 2012)**. Chitosan (5 mg/ml) reduced early blight disease severity by nearly 75% **(Sathiyabama *et al*., 2014)**. Spraying of chitosan extract decreased the incidences of early blight and bacterial spot blight in tomato grown in greenhouse and field **(Ramkissoon *et al*., 2016)**. Cucumber seedlings from chitosan-treated seeds showed enhanced resistance to damping-off disease caused by *P. capsici* **(Zohara *et al*., 2019)**. The reduction in disease

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incidence might be attributed to the fact that chitosan treatment induces SAR responses and regulates several defense genes, including the activation of defense-related enzymes, phytoalexins and PR proteins which results in induced resistance **(Xing *et al.*, 2015; Sharif *et al.*, 2018; Carmona *et al.*, 2021)**. Also, Chitosan has direct effect as antifungal compound by inhibiting mycelial growth, sporulation **(Kumaraswamy *et al.*, 2018)**

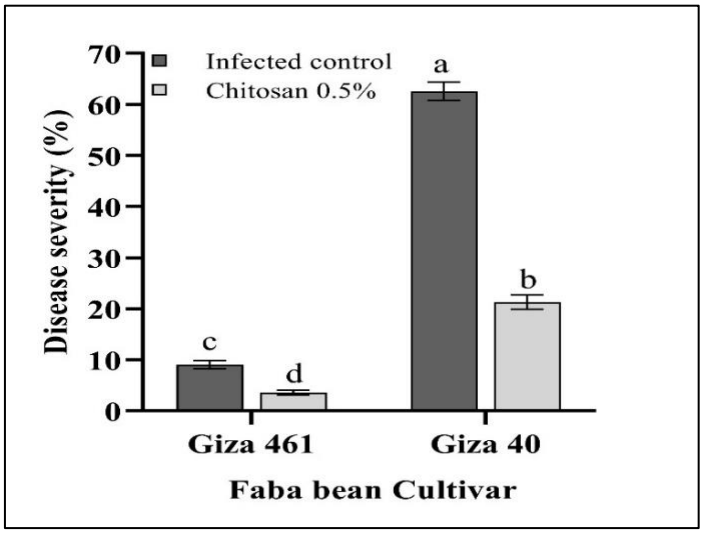


Fig. 1. Effect of the chitosan (0.5%) in chocolate spot disease severity of two faba bean cultivars infected with *B. fabae* under the greenhouse condition. Different letters indicate significant differences between treatments according to the Tuckey HSD test ($P < 0.05$). Values are the mean of five replicates ± SE.

**Leaf chlorophyll content (SPAD)**

The data in Table (5) demonstrated that there was no significant difference in chlorophyll concentration between the non-infected control of both cultivars. Chitosan untreated plants infected with *B. fabae* of the two faba bean cultivars showed a significant decrease in chlorophyll contents.

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The susceptible cv. Giza 40 exhibited the highest decrease in chlorophyll contents at 7 dpi with 21.9% reduction compared to the non-infected control, while the resistant cv. Giza 461 had 11.1% reduction. Chitosan treatment at 0.5% enhanced chlorophyll content in infected plants of both cultivars as compared to untreated infected plants. Chitosan treatment increased the chlorophyll content of the infected Giza 461 and Giza 40 plants by 12.3 and 15.8%, respectively, as compared to untreated infected plants. Giza 461 infected plants treated with chitosan exhibited the same level of chlorophyll content as the healthy non-infected plants.

Table 5: Effect of chitosan (0.5%) on chlorophyll content, total phenol and Malondialdehyde (MDA) contents in the leaves of two faba bean cultivars infected with *B. fabae*

| Treatment | Chlorophyll content (SPAD) | Malondialdehyde content (mmol MDA $g^{-1}$ FW) | Total phenols content (µGA $g^{-1}$ FW) |
|---|---|---|---|
| **Giza 461** | | | |
| Non-infected control | 45.78$^{a}$ | 3.02$^{d}$ | 35.27$^{e}$ |
| Infected control | 40.76$^{b}$ | 4.12$^{b}$ | 53.91$^{c}$ |
| Chitosan 0.5% | 45.71$^{a}$ | 3.45$^{c}$ | 69.20$^{a}$ |
| **Giza 40** | | | |
| Non-infected control | 45.91$^{a}$ | 2.88$^{d}$ | 32.80$^{e}$ |
| Infected control | 35.86$^{c}$ | 6.30$^{a}$ | 38.20$^{d}$ |
| Chitosan 0.5% | 41.53$^{b}$ | 4.34$^{b}$ | 59.13$^{b}$ |

Different letters indicate significant differences between treatments according to the Tuckey HSD test ($P < 0.05$). Values are the mean of five replicates ± SE.

Chlorophyll is a good indicator of the health condition of the plant, and several reports demonstrated that chitosan application increased total chlorophyll and photosynthesis which promotes and enhances plant growth under biotic and abiotic stresses **(Farouk *et al.*, 2011; Zong *et al.*, 2017; Carmona *et al.*, 2021)**. **Limpanavech *et al.* (2008)** demonstrated that

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chitosan treatment induced chloroplast gene expression and chloroplasts expansion, which may have contributed to its efficiency in avoiding reduction in chlorophyll synthesis and photosynthesis suppression associated with *B. fabae* infection.

**Effect of chitosan on biochemical changes of faba bean plants**

**Lipid peroxidation**

The lipid peroxidation levels in faba bean leaf tissues were measured as the malondialdehyde (MDA) concentration. MDA content is regarded one of the most critical markers of plasma membranes integrity in plant cells. MDA is considered as cytotoxic and has a deleterious impact on the structure and function of the cell membrane (Shi et al., 2013).

The results in Table (4) indicated that no significant in the MDA levels were detected in non-infected leaves of the two tested cultivars. Inoculation with *B. fabae* significantly increased the MDA levels in both cultivars compared with non-infected controls. The MDA levels were significantly higher in susceptible cv. Giza 40 than in resistant cv. Giza 416. The MDA contents in the tissues of the susceptible cultivar was 6.3 mmol/g FW, which was 1.53-fold higher than that of the resistant cultivar (Table 4). Such findings consistent with those obtained by **El-Komy (2014)** who found a higher level of MDA in susceptible cultivar (Giza 40) than in resistant one (Nubaria), indicating that oxidative damage helps *B. fabae* to overcome the plant defense system and facilities pathogenesis process **(Govrin and Levine, 2000)**.

Treatments with 0.5% chitosan reduced MDA contents in both cultivars compared to the untreated control. The MDA content of infected Giza 40 and Giza 461 plants treated with chitosan decreased by 31 and 16%, respectively, compared to untreated infected plants. This finding is supported by results of **Youwei and Yinzhe (2013)**, **Zong *et al*. (2017)**, and **Farouk *et al*. (2011)** which found a reduction of MDA contents in chitosan treated plants under stressed conditions compared with untreated ones. Chitosan application significantly reduced lipid peroxidation by

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triggering antioxidant enzymes resulting in decreased membrane permeability **(Guan *et al*. 2009; Farouk *et al*., 2011)**.

**Total phenols content**

The total phenol content was significantly increase in leaves of faba bean plants of the two tested cultivars as a result of infection by *B. fabae* (Table 4). The untreated infected plants of resistant cv. Giza 461 had total phenol content higher than the susceptible cv. Giza 40 by 1.41-fold. Chitosan treatments at 0.5% significantly increased total phenols content in the plant leaves of both cultivars. Infected Giza 461 plants treated with chitosan had the highest level of total phenols content (69.20 µGA $g^{-1}$ FW), while the untreated non-infected plants of cv. Giza 40 had the lowest (32.80 µGA $g^{-1}$ FW).

These findings are in harmony with those of **El-Shafey *et al*. (2020)** who found that a resistant cultivar (Giza 716) had a higher level of total phenol than a susceptible one (Giza 40) after *B. fabae* infection. Several studies have indicated that a higher phenolic content was positively related to plant resistance to various fungal diseases **(Abo-Elyousr *et al*., 2009; Mahmoud *et al*., 2012b; El-Shafey *et al*., 2020)**. Plants produce phenols as an early response to pathogen infection. Because phenols are toxic to pathogens, rapid accumulation of phenols at the infection site inhibits the pathogen's development rate. Phenols play an important role as signal molecules, cell wall strengthening factor, and antimicrobial compound **(Kruger *et al*., 2002; Usha and Jyothsna, 2010)**. Chitosan treatments boosted the level of phenols in plants **(Kahromi and Khara, 2020; Attia *et al*., 2021)**. Chitosan increased phenols level by activating relevant enzymes involved in the phenol syntheses pathway, such as PAL enzyme **(Romanazzi *et al*., 2017)**.

**Defense enzymes activities**

Data in Table 5 showed that POD, PPO and PAL enzyme activities of the non-infected control plants were not significantly differed between the resistant cv. Giza 461 and the susceptible cv. Giza 40. However, after inoculation with *B. fabae* (Bf.3 isolate) the enzyme activity of POD, PPO

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and PAL in leaves of the two cultivars increased compared with non-infected control and it was clear that the resistant cv. Giza 461 showed a higher activity than the susceptible cv. Giza 40 at 72 hpi. Similar results were obtained by **Mahmoud *et al*. (2012b)** who found that POD and PPO enzyme activities were elevated in faba bean leaves after inoculation with *B. fabae*, with the resistant cv. Giza 461 showed a higher activity than the susceptible cv. Giza 429. POD activity in *B. fabae* infected leaves of faba bean resistant cultivars was ten times greater than in susceptible cultivars **(Nawar and Kuti, 2003)**. According to **Aldesuquy *et al*. (2015)**, *B. fabae* infection increased the activity of POD, PPO and PAL in infected faba bean plants.

Treatments with chitosan at 0.5% led to a significant further increase in POD, PPO and PAL enzyme activities in both cultivars compared to the untreated infected control (Table 5). Meanwhile, the rate of increase was higher in cv. Giza 461 compared to cv. Giza 40. Infected plants of the resistant cultivar treated with chitosan had the highest activity level for the three assayed enzymes with approximately 1.43-, 1.51- and 1.67-fold higher than that in untreated infected plants, and 2.86-, 2.21- and 2.71-fold that in non-infected control plants for POD, PPO, and PAL activities respectively (Table 5). Induction of defense related enzymes is one of the main induced plant defense responses that prevent or reduced pathogen invasion **(Walters *et al*., 2005; Ebrahim, 2012; Nisha *et al*., 2012)**. Many investigators demonstrated that treatment with chitosan enhanced the activity of defense enzymes in infected plants. POD and PPO activities were increased in chitosan treated in okra plants inoculated with *Erysiphe cichoracearum* **(Soliman *et al*., 2017)**, strawberry fruits inoculated with *B. cinerea* and *R. stolonifera* **(Thabet, 2019)**, and tomato inoculated with *A. solani* **(Adss *et al*., 2021)**. POD, PPO, and PAL were elevated in chitosan (0.5%) treated potato tubers infected by *R. solani* **(Mohammed *et al*., 2019)**.

POD and PPO enzymes catalyze the oxidation of phenolic substances to quinones which are toxic to the invading pathogens. POD and PPO involved in plant cell lignification, cell wall strengthening, induced hypersensitive reaction, triggering SAR response, and synthesis of

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phenolics and phytoalexins **(Campos *et al*., 2004; Gozzo, 2004; Constabel and Barbehenn, 2008),** which enhance plant resistance against pathogens. PAL is a critical enzyme in the phenylpropanoid pathway and it thought to be the primary enzyme in the biosynthesis of phenolic compounds **(Ali *et al*., 2007)**. PAL was induced as a result of pathogen attack and treatment with elicitors **(Mandal and Mitra, 2007; Aldesuquy *et al.*, 2015)**. The current results revealed that *B. fabae* infection and chitosan treatment enhanced PAL activity (Table 5) resulted in formation of phenolic compounds which provide appropriate substrate for POD and PPO catalysis and production of fungal toxic quinones.

Table 6: Effect of chitosan (0.5%) on peroxidase (POD), polyphenol (PPO) and phenylalanine ammonia lyase (PAL) enzymes activities in the leaves of two faba bean cultivars infected with *B. fabae*.

| Treatment | POD activity $\Delta OD_{420}$ $min^{-1}$ $g^{-1}$ FW | PPO activity $\Delta OD_{495}$ $min^{-1}$ $g^{-1}$ FW | PAL activity nmol trans-cinnamic acid $min^{-1}g^{-1}$ FW |
|---|---|---|---|
| **Giza 461** | | | |
| Non-infected control | $0.327^{d}$ | $0.563^{d}$ | $2.15^{e}$ |
| Infected control | $0.653^{b}$ | $0.823^{b}$ | $3.48^{b}$ |
| Chitosan 0.5% | $0.937^{a}$ | $1.243^{a}$ | $5.84^{a}$ |
| **Giza 40** | | | |
| Non-infected control | $0.313^{d}$ | $0.550^{d}$ | $2.09^{e}$ |
| Infected control | $0.437^{c}$ | $0.680^{c}$ | $2.66^{d}$ |
| Chitosan 0.5% | $0.666^{b}$ | $0.819^{b}$ | $3.06^{c}$ |

Different letters indicate significant differences between treatments according to the Tuckey HSD test ($P < 0.05$). Values are the mean of five replicates ± SE.

**Expression analysis of PR genes**

To investigate the molecular role of chitosan at 0.5% on induction of defense responses, the relative expression level of *PR1* and *PR2*, and *CHT* defense genes was quantified in leaves of the faba bean resistant cv.

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Giza 461 and susceptible cv. Giza 40 plants inoculated with *B. fabae* using RT-qPCR. Expression profile of these genes was analyzed 72 hpi (Fig. 2).

Results show that expression *PR1*, *PR2* and *CHT* genes were induced by the infection with *B. fabae*. The untreated infected plants of both cultivars showed a significantly higher expression level compared to non-infected plants. In general, the expression levels were higher in infected resistant cultivar than the susceptible one for all analyzed genes. Compared with non-infected control, the highest defense gene expression was achieved in *B. fabae*-infected leaves of the resistant cultivar for *PR1* (4.1-fold) and *PR2* (3.16-fold), and *CHT* (5.06-fold), indicating that these genes were induced in response to infection and their induction was implicated in the resistance to *B. fabae*. These results agreed with that obtained by **Cheng *et al*. (2012)** and **Elkomy (2014)** who found that the expression levels of the *PR1* and *PR2* genes were higher in *B. fabae* infected leaves and higher in the faba bean resistant cultivar compared to the susceptible one. *PR1* gene expression was also elevated in host-pathogen interaction of faba bean -*Puccinia striiformis* f. sp. *tritici* **(Cheng *et al*., 2012)**, faba bean -*Uromyces fabae* **(Rauscher *et al*., 1999)**, and tomato -*F. oxysporum* f. sp. *lycopersici* **(Aimé *et al*., 2008)**. *PR2* gene (β-1,3-glucanase) was deferentially expressed in potato *A. solai* interaction **(El-Argawy *et al*., 2017)**. *CHT* (chitinase) gene expressed at early stages in faba bean -*B. fabae* interaction **(Attia *et al*., 2006)**, and in *Brassica juncea* -*A. brassicae* interaction **(Rawata *et al*., 2017).**

However, treatments with chitosan at 0.5% led to a significant further increase in *PR1*, *PR2* and *CHT* genes expression levels in both cultivars compared to the untreated infected control. Meanwhile, the rate of increase was higher in resistance cv. Giza 461 compared to susceptible cv. Giza 40 for the three genes. Infected plants of the resistant cultivar treated with chitosan had the highest activity level for the three genes with approximately 2.06- 2.22-, and 1.47-fold higher than that in untreated infected plants, 7.8-, 8.8-, and 7.46-fold that in non-infected control plants for *PR1*, *PR2* and *CHT* genes, respectively. Several Reports documented the positive effect of chitosan in inducing the PR genes in several crops. Chitosan increased *CHT* and *PR2* expression in wheat **(Díaz-Martínez *et***

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***al.*, 2018)**. *PR1* gene expression was upregulated when chitosan or oligochitosan were used to induced resistance against *P. syringae* pv. *actinidiae* in kiwifruit plants **(Beatrice *et al.*, 2017)**, *tobacco mosaic virus* (TMV) in *Arabidopsis* **(Jia *et al.*, 2016)**, and *F. oxysporum f. sp. Lycopersici* in tomato **(Carmona *et al.*, 2021)**.

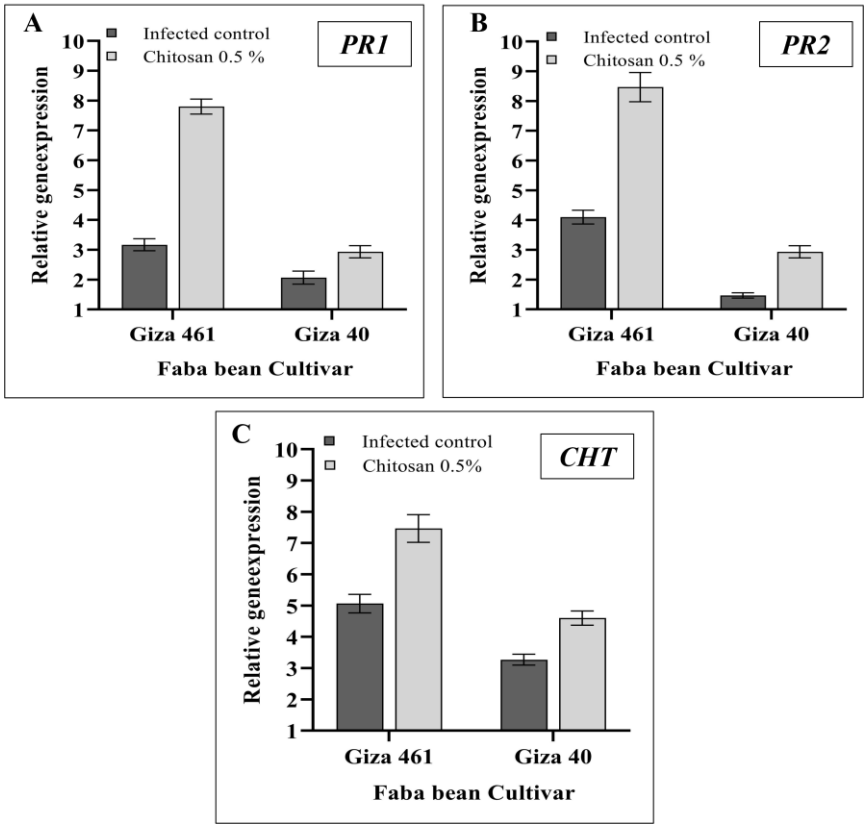


Fig. 2. Effect of chitosan (0.5%) on relative gene expression of *PR1* (A), *PR2* (B), and *CHT* (C) genes in leaves of two faba bean cultivars at 72 hpi with *B. fabae.* The expression levels of the target genes were normalized relative to *ELF1A* gene and relative expression of untreated control plants at each time were set as 1. Each value represents mean ± SE (n = 3).

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PR1 proteins are salicylic acid signaling pathway markers that are produced in plants as part of the SAR response, which is responsible for the salicylic acid accumulation **(Jia *et al*., 2016; Beatrice *et al*., 2017)**. PR1 proteins have antimicrobial properties against various plant pathogens and involved in plant cell-wall thickening **(Linthorst *et al*., 1989; Kattupalli *et al*., 2021).** Chitinases and β-1,3-glucanases are important plant enzymes that degrading the fungal cell-wall, suppressing mycelial growth and sporulation, and releasing oligosaccharide elicitors (β-1,3-glucans) that activate plant defense-related genes **(Ebel and Cosio, 1994; York *et al*., 2004; Tobias *et al*., 2017; Kumaraswamy *et al*., 2018)**. Transgenic plants containing β-1, 3 glucanases and/or chitinases were found to be more tolerant to infection than the wild type **(Zhu *et al*., 1994; Cletus *et al*., 2013; Durechova *et al*., 2019)**. The antifungal activity of these PR proteins makes them promising targets in plant breeding programs.

## CONCLUSIONS

The results of this study indicated that the application of chitosan protected faba bean plants against chocolate spot disease, mostly by inducing systemic resistance. Chitosan could be used as a natural and environmentally safe alternative to a synthetic fungicides and growth promoters in management of chocolate spot disease and sustainable production of faba bean.

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## إستحثاث الإستجابات الدفاعية في نباتات الفول البلدي ضد مرض التبقع الشيكولاتي المتسبب عن فطر *Botrytis fabae* بإستحدام الشيتوزان

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### الملخص العربي

يعتبر مرض التبقع الشيكولاتي (البني) في الفول الناتج عن الإصابة بفطر *Botrytis fabae* أحد أهم المشكلات التي تحد من إنتاجية الفول في مصر والعالم. تم تقييم قدرة الشيتوزان كمضاد للفطريات معمليا وكذلك قدرته علي إستحثاث الإستجابات الدفاعية في صنفين من الفول، جيزة 461 (مقاوم) و جيزة 40 (حساس)، ضد هذا الفطر. تم عزل سبعة عزلات من العينات المصابة وتم تعريفها علي أنها فطر *Botrytis fabae*. جميع العزلات كانت قادرة علي إحداث المرض علي صنف جيزة 40 وكانت العزلة رقم 3 (Bf-3) هي الأكثر قدرة مرضية علي إحداث المرض. المعاملة بالشيتوزان ثبطت النمو المسيليومي في فطر *B. fabae* بمعدل يرتبط بالتركيز وعند تركيزات 0.75 و 1 % من الشيتوزان تم تثبط نمو الفطر تماما. تحت ظروف الصوبة، تم رش نباتات الفول لكلا الصنفين بالشيتوزان 0.5 % ثم عمل عدوي بالعزلة Bf-3 لفطر *B. fabae*. الرش بالشيتوزان خفض شدة مرض التبقع البني في كلا الصنفين بالمقارنة بالكنترول الغير معامل والمعدي بالفطر. نباتات كلا الصنفين المعاملة بالشيتوزان أظهرت معدلات أقل لأكسدة الدهون الخلوية Lipid peroxidation ومستويات أعلي من الكلوروفيل والفينولات الكلية مقارنة بالكنترول الغير

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معامل والمعدي بالفطر. وكذلك فإن الشيتوزان قام بتحفيز نشاط الإنزيمات الدفاعية مثل البيروكسيديز والبولي فينول أوكسيديز و الفينيل ألانين أمونيا لاييز. بالإضافة لذلك فإن المعاملة بالشيتوزان أدت لزيادة في مستوي التعبير الجيني في نباتات الفول البلدي لكلا الصنفين لثلاثة من الجينات المتعلقة بالمقاومة وهي *PR1* و *PR2* (الجلوكانيز) و *CHT* (الشيتينيز). الصنف المقاوم (جيزة 461) سواء كان معامل أو غير معامل بالشيتوزان أظهر أعراض مرضية أضعف، ومستويات محسنة في جميع الاستجابات الفسيولوجية و الجزيئية بالمقارنة بالصنف الحساس (جيزة 40). هذه النتائج تظهر أن الشيتوزان يمتلك قدرة عالية علي حماية نباتات الفول من الإصابة بفطر *B. fabae* عن طريق التحكم في الإستجابات البيوكيميائية والفسيولوجية والجزيئية في نباتات الفول تحت ظروف الاصابة.

**الكلمات الدالة:** التبقع البني، *Botrytis fabae*، الشيتوزان، المقاومة، الاتجابة الدفاعية