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Research Article

Induction and expression of systemic resistance to downy mildew disease in grapevine by chitosan

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Abstract

Grapes represent a significant agricultural product globally. Despite their significance and value, grapes face numerous challenges, including disease. Grapevines are susceptible to various pathogens, with downy mildew caused by *Plasmopara viticola*, being the most damaging. In the twentieth century, contact and systemic fungicides were developed to combat plant pathogens, such as downy mildew. Recently, there has been a growing demand to minimize fungicide use and shift towards sustainability through implementing eco-friendly practices. Utilizing plant defense elicitors to stimulate disease resistance in grapevines is a crucial strategy. We conducted experiments in the field over two seasons, 2021 and 2022, at two different locations using two grape cultivars ('Flame' and 'Crimson'). Four concentrations of chitosan (1, 2, 4, and 8 mM) were applied. The results indicated that chitosan could induce systemic resistance against downy mildew caused by *P. viticola* by activating the salicylic acid (SA) pathway, increasing the plant's concentrations of SA and phenols, and augmenting the efficacy and activity of defense enzymes. The treatment also significantly enhanced the yield production of 1.3-glucanase, chitinase, peroxidase, and polyphenol oxidase. The present study assessed the efficacy of chitosan as a resistance inducer in managing grapevine downy mildew and examined its mechanism of action.

Keywords: β-1.3-glucanase; inducing resistance; peroxidase; phenols; *Plasmopara viticola;* polyphenol oxidase; salicylic

Introduction

In Egypt, grapevines are regarded as the second most important fruit crop following citrus (Ammar *et al.,* 2018). Grapevine downy mildew disease (GDM) caused by *Plasmopara viticola* (Berk. & M. A. Curt.) Berl. & De Toni is among the most devastating diseases impacting viticulture, particularly in temperate-humid

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climates. This pathogen selectively targets leaves and grapes, adversely affecting both the yield of the vines and the quality of the harvested fruits by reducing the sugar content (Ammar *et al.,* 2018; Taibi *et al.,* 2022). The pathogen of this disease is an obligate biotrophic oomycete that infects young, tender green leaf, twig, and fruit tissues, resulting in significant short-term losses. These infections can obliterate 40–90% of plants in the field under optimal humidity and temperature conditions (Toffolatti *et al.,* 2018; Koledenkova *et al.,* 2022).

The management strategy for this disease mainly depends on fungicides, which are detrimental to the environment, necessitating the search for safe alternative products to ensure more sustainable and safe agriculture (Gessler *et al.,* 2011; Koledenkova *et al.* 2022 and Taibi *et al.,* 2022). Although numerous alternative products have been investigated, utilizing inducer plant resistance remains one of the most promising methods. Resistance inducers are molecules that activate plant-specific mechanisms via a series of processes, resulting in the establishment of systemic acquired resistance (SAR) or induced systemic resistance (ISR) (Héloir *et al.,* 2019; Mahmoud *et al.,* 2021). Following the induction of resistance, the plant gains improved defensive capabilities against subsequent pathogen infections. SAR necessitates SA as a signaling molecule and is linked to the production and accumulation of pathogenesis-related (PR) proteins (Prakongkha *et al.,* 2013; Mahmoud *et al.,* 2021).

Motivated inducers may consist of both synthetic and natural compounds. Chitosan (CHT), a poly (1,4)-2-amino-2-deoxy-β-D-glucose and a deacetylation derivative of chitin, is a natural compound derived from the exoskeletons of arthropods, including crustaceans like lobsters, shrimps, crabs, mollusk radulae, cephalopod beaks, fish, and insects (Kurita, 2006). Previous research indicates that it is highly effective in promoting plant growth and enhancing disease resistance (Manjunatha *et al.,* 2009; El Hadrami *et al.,* 2010; Prakongkha *et al.,* 2013). Chitosan has recently emerged as an environmentally sustainable method for managing crop diseases (Orzali *et al.,* 2017) and has demonstrated antiviral, antibacterial, and antifungal properties (Chakraborty *et al.,* 2020; Omar *et al.,* 2021).

Numerous studies indicate that the foliar application of chitosan enhances plant growth and yield while promoting the synthesis of secondary metabolites such as polyphenolics, flavonoids, lignin, and phytoalexins in plants (Emami-Bistgani *et al.,* 2017; Xoca-Orozco *et al.,* 2017). It also influences seed plasma membrane permeability, the generation of reactive oxygen species, the expression of unique early responsive and defense-related genes, peroxidase, phenylalanine ammonia-lyase, tyrosine ammonia-lyase, chitinase, 1.3-glucanase and catalase activities (Roby *et al.,* 1987; Kuchitsu *et al.,* 1997; Kaku *et al.,* 1997; Nishizawa *et al.;* 1999 Guan *et al.,* 2009).

This research aimed to evaluate the effectiveness of chitosan as a resistance inducer in controlling GDM compared with common fungicides (Amistar) and investigate their mode of action.

Materials and Methods

Experimental condition and treatments

The field experiments were conducted in a private farm at Nobaria, Behera, Governorate (5-year-old 'Crimson') and Somesta, Beni-Suef, Governorate (3-year-old 'Flame') at 2022 and 2023 growing seasons (Figure 1). The plants were spaced by 2 m in the rows, with 3 m between the rows. Five treatments were tested, compared with untreated control; the application rates are listed in Table 1.

Somesta, Beni-Suef Nobaria, Behera, Governorate

Figure 1. Representative field sites for grapevine downy mildew control experiments in Behera and Beni-Suef

Table 1. Treatment application rates and concentrations for tested products

Trt.	Product name	Product concentrate or rate
1	Chitosan	1 mM
2	Chitosan	2 mM
3	Chitosan	4 mM
4	Chitosan	8 mM
5	Amistar SC 25% (Azoxystrobin)	50 ml /100 L
6	Control	

A complete randomized block design system with four replicates per treatment was used. Each replicate consisted of ten grapevines along a row. A drop irrigation system and the recommended fertilization were applied.

The plant was left to its natural inoculation condition. The treatments were applied two times with an interval period of 30 days, the first treatment in the second half of March when the plants started to be sensitive to GDM infection. The treatments were distributed by spraying 20 litres using a motorized backpack sprayer.

Diseases assessment

The disease severity scale used in this study was adapted based on the methodology described by Bennett and Westcott (1982), with modifications to suit the specific conditions of grapevine downy mildew evaluation, GDM infections were evaluated in all vines of each treatment on 100 leaves randomly selected per vine. During 2022 and 2023, GDM symptoms were recorded for the grapevine leaves on the first of June, July, and August. The disease severity on the leaves was assigned to 11 levels, according to the percentage of surface covered by the GDM symptoms, from 0 (uninfected leaf) to 10 (over 90% of leaf surface). The infection index of disease severity was expressed as a percentage of the maximum possible level. In detail, this was calculated according to the following equation:

$$I = \{\sum (d \times f)/ (N \times D)\} \times 100$$

Where d is the category of disease intensity scored for the grapevine, f is the disease frequency, N is the total number of organs examined (healthy and rotted), and D is the highest category of disease intensity.

Evaluations of grape yield were recorded as the average of yield/plant (kg).

Chemical analyses

After one, five and ten days after chitosan application, 50 lower leaves and 50 upper leaves were collected from each treatment for chemical analyses. After being detached, each leaf was cut into small pieces about one square cm and thoroughly mixed before analysis samples were taken.

SA analysis

Leaf tissue (0.5 g) from each replication was randomly sampled, frozen with liquid nitrogen and macerated in a cold mortar with 1 ml of extraction solution (90:9:1 volume of absolute methanol, glacial acetic acid, and distilled water). The extract was subsequently centrifuged at 12000 rpm at 4 °C for 15 min, and the supernatant was collected for the analysis. To determine the SA content, 500 µl of the supernatant was mixed with an equal volume of 0.02 M ferric ammonium sulfate, incubated at 30 °C for 5 min and the absorbance at 530 nm was read by a spectrophotometer. The read absorbance was subsequently compared to that of the reference standard to obtain the actual amount of SA in the sample (Raskin *et al.*, 1989).

Defence enzyme analyses

Two grams of leaf tissue was homogenized with 0.2M Tris HCL buffer (pH 7.8) containing 14 mM of β-mercaptoethanol at the rate of 1/3 (w/v). The homogenate was centrifuged at 3000 rpm for 15 min; the supernatant was used to determine enzyme activities (Tuzun *et al.,* 1989).

The method of Abeles and Forrence (1970) was used to determine β-1.3-glucanase and assayed using a spectrophotometer at 500 nm β-1.3-glucanase activity is expressed as mM glucose equivalent released/gram fresh weight/ 60 min. Chitinase activity was determined according to Monreal and Reese (1969) and assayed using a spectrophotometer at 540 nm. Chitinase activity is expressed as mM N-acetyl glucose amine equivalent released/gram fresh weight/ 60 min.

Two grams of leaf tissue were cut into small portions and grounded in a mortar in the presence of purified sand and 4 ml of 0.1 M sodium phosphate buffer pH 7.1 as described by Goldschmidt *et al.* (1968), then the supernatant fluids (enzyme extracts) were used for assaying the oxidative enzymes peroxidase (PO) and polyphenol oxidase (PPO) using a spectrophotometer at 425 and 495 mm, respectively according to Allam and Hollis (1972); Matta and Dimond (1963), The reaction substrate of each oxidative enzyme was pyrogallol, and catechol, respectively. Peroxidase activity was expressed as the change in absorbance/minute/1.0 g fresh weight, and polyphenol oxidase activity was expressed as the change in absorbency/ minute/1 g fresh weight.

Phenolic compounds analyses

Two grams of leaf tissue were cut into small portions and extracted in Soxhlet units using 75% ethanol for 10-12 h, then used to determine phenolic compounds as described by Snell and Snell (1953). The phenol contents were also calculated as milligrams equivalent of catechol /g fresh weight.

Statistical analysis

The data were statistically analyzed by analysis of variance (ANOVA) using the Statistical Analysis software "CoStat 6.4". Means were separated by the Duncan test at $P < 0.05$.

Results

Effect of chitosan as inducer resistance on GDM compared with Amistar fungicide

Diseases severity (D.S)

The effectiveness of four tested concentrations of chitosan, besides Amistar fungicide, as check control on controlling GDM at Nobaria and Somesta during seasons 2022 and 2023, shown in Tables 2, 3, 4, 5. The Assessment of Disease Severity Using the Adopted Scale "The disease severity scale, consisting of 11 levels ranging from 0 (no symptoms) to 10 (more than 90 , treatments demonstrated varying efficacy in mitigating GDM severity and exhibited a significant effect compared to the untreated control group. Chitosan at 8 mM, followed by 4 mM, demonstrated the highest efficacy in mitigating GDM severity in both cultivars across two seasons and locations, compared to other chitosan treatments. In contrast, Amistar fungicide (control) exhibited the most pronounced effect in reducing GDM severity; however, its impact was not statistically significant when compared to chitosan at 8 mM across all disease assessments, seasons, and locations.

Data indicated that certain concentrations of chitosan inducers exhibited efficacy comparable to the fungicide Amistar in mitigating GDM severity, with effectiveness increasing over time. Chitosan at a concentration of 8 mM demonstrated the closest efficacy to fungicides over the two consecutive seasons, 2022 and 2023 (Tables 2, 3, 4, 5).

Data also indicated that, in both locations, all treatments had a more significant effect on reducing GDM severity during season 2022. Moreover**,** there was a positive relationship between increasing the chitosan concentration and its effect on reducing GDM severity at both locations during seasons 2022 and 2023 (Tables 2, 3, 4, 5), confirming that induction of systemic resistance had occurred.

Table 2. Effect of chitosan as inducer resistance on GDM severity compared to Amistar fungicide at Nobaria, Behera, Governorate during season 2022

Treatment	June		Efficacy to fungicides	July		Efficacy to fungicides	August		Efficacy to fungicides
	D.S	*Efficacy%		D.S	*Efficacy%		D.S	*Efficacy%	
Chitosan 1 mM	14.78^{b}±1.45	38.20	49.53	17.06^{b}±1.40	36.84	50.14	19.72^{b}±1.56	34.27	51.83
Chitosan 2 mM	11.80^{c}±1.15	50.67	65.69	13.76^{c}±1.34	49.07	66.79	16.16^{c}±1.33	46.15	69.80
Chitosan 4 mM	9.64^{c}±0.94	59.72	77.43	12.03^{c}±1.30	55.48	75.51	14.36^{d}±1.81	52.15	78.87
Chitosan 8 mM	7.22^{d}±0.68	69.81	90.51	8.96^{d}±1.31	66.84	90.98	12.00^{e}±1.60	60.03	90.79
Amistar	5.47^{d}±0.57	77.13	100	7.17^{e}±1.34	73.47	100	10.17^{f}±1.37	66.12	100
Control	23.92^{a}±1.91	0.00	0.00	27.01^{a}±1.49	0.00	0.00	30.01^{a}±1.26	0.00	0.00

* % Treatment efficiency = ((Control-treatment)/Control) × 100

**Efficacy to fungicides= (treatment efficacy/fungicides efficacy) × 100

Table 3. Effect of chitosan as inducer resistance on GDM severity compared to Amistar fungicide at Nobaria, Behera, Governorate during season 2023

Treatment	June		Efficacy to fungicides	July		Efficacy to fungicides	August		Efficacy to fungicides
	D.S	*Efficacy%		D.S	*Efficacy%		D.S	*Efficacy%	
Chitosan 1 mM	17.63^{b}±1.62	32.37	44.34	19.32^{b}±1.94	31.62	45.32	21.98^{b}±1.26	33.08	50.87
Chitosan 2 mM	13.54^{c}±1.33	48.07	65.85	15.42^{c}±1.18	45.43	65.11	18.08^{c}±1.74	44.95	69.12
Chitosan 4 mM	11.36^{d}±1.11	56.41	77.27	13.32^{d}±1.11	52.85	75.75	15.74^{d}±1.91	52.09	80.10
Chitosan 8 mM	8.87^{dc}±1.26	65.98	90.38	10.18^{c}±1.28	63.97	91.69	13.18^{c}±1.74	59.88	92.08

Amistar	7.04 ^{e} ±1.19	73.00	100	8.54 ^{f} ±1.16	69.77	100	11.49 ^{f} ±1.90	65.03	100
Control	26.07 ^{a} ±1.88	0.00	0.00	28.25 ^{a} ±1.33	0.00	0.00	32.85 ^{a} ±1.69	0.00	0.00

* % Treatment efficiency = ((Control-treatment)/Control) × 100

**Efficacy to fungicides= (treatment efficacy/fungicides efficacy) × 100

Table 4. Table 4. Effect of chitosan as inducer resistance on GDM severity compared to Amistar fungicide at Somesta, Beni-Suef, Governorate during season 2022

Treatment	June		Efficacy to fungicides	July		Efficacy to fungicides	August		Efficacy to fungicides
	D.S	*Efficacy%		D.S	*Efficacy%		D.S	*Efficacy%	
Chitosan 1 mM	12.67 ^{b} ±1.22	35.99	46.30	14.27 ^{b} ±1.19	35.36	47.94	17.59 ^{b} ±1.55	29.09	45.45
Chitosan 2 mM	9.88 ^{c} ±1.39	50.09	64.43	11.78 ^{c} ±1.27	46.64	63.23	14.20 ^{c} ±1.33	42.76	66.80
Chitosan 4 mM	7.62 ^{d} ±1.01	61.50	79.11	8.83 ^{d} ±1.31	60.01	81.36	11.83 ^{d} ±1.49	52.31	81.72
Chitosan 8 mM	6.31 ^{de} ±1.22	68.16	87.68	7.57 ^{de} ±1.21	65.70	89.07	10.83 ^{de} ±1.18	56.37	88.06
Amistar	4.41 ^{e} ±1.26	77.74	100	5.79 ^{e} ±1.25	73.76	100	8.93 ^{e} ±1.94	64.01	100
Control	19.80 ^{a} ±1.74	0.00	0.00	22.08 ^{a} ±1.32	0.00	0.00	24.81 ^{a} ±1.06	0.00	0.00

* % Treatment efficiency = ((Control-treatment)/Control) × 100

**Efficacy to fungicides= (treatment efficacy/fungicides efficacy) × 100

Table 5. Effect of chitosan as inducer resistance on GDM severity compared to Amistar fungicide at Somesta, Beni-Suef, Governorate during season 2023

Treatment	June		Efficacy to fungicides	July		Efficacy to fungicides	August		Efficacy to fungicides
	D.S	*Efficacy%		D.S	*Efficacy%		D.S	*Efficacy%	
Chitosan 1 mM	14.75 ^{b} ±1.36	34.35	46.20	16.76 ^{b} ±1.30	34.29	47.68	19.87^{b} +1.61	29.46	46.34
Chitosan 2 mM	11.92 ^{c} ±1.31	46.95	63.15	13.88 ^{c} ±1.51	45.58	63.38	17.91 ^{b} ±1.49	36.42	57.28
Chitosan 4 mM	10.16 ^{c} ±1.39	54.76	73.65	12.03 ^{d} ±1.41	52.81	73.44	15.14 ^{c} ±1.43	46.25	72.74
Chitosan 8 mM	7.89 ^{d} ±1.29	64.88	87.26	9.46 ^{e} ±1.69	62.90	87.47	12.13 ^{d} ±1.51	56.94	89.56
Amistar	5.76 ^{d} ±1.13	74.35	100	7.16 ^{f} ±1.05	71.91	100	10.26 ^{d} ±1.77	63.58	100
Control	22.46 ^{a} ±1.42	0.00	0.00	25.50 ^{a} ±1.36	0.00	0.00	28.17 ^{a} ±1.09	0.00	0.00

* % Treatment efficiency = ((Control-treatment)/Control) ×100

**Efficacy to fungicides= (treatment efficacy/fungicides efficacy) × 100

Yield

Data presented in Table 6 showed that applying tested treatments significantly increased the grape yield of 'Crimson' and 'Flame' compared with untreated control at both locations during the two seasons, 2022 and 2023. In this respect, chitosan at 8 mM and 4 mM showed the highest increase compared to other chitosan treatments. In comparison, Amstar fungicide treatment (check control) recorded the best grape yield ever in the two locations without any significant difference with most chitosan treatments at 8 mM during the two seasons, 2022 and 2023.

Data also showed a positive relationship between increasing the concentration of chitosan and increasing grape yield, whether in 'Crimson' or 'Flame' during the two seasons 2022 and 2023.

Table 6. Effect of chitosan on the yield of grape cultivars at two locations during 2022 and 2023

Treatment	'Crimson'						'Flame'					
	2022			2023			2022			2023		
	Yield/plant (kg)		*Increases (%)	Yield/plant (kg)		*Increases (%)	Yield/plant (kg)		*Increases (%)	Yield/plant (kg)		*Increases (%)
Chitosan 1 mM	7.12^{cd}	±1.08	11.61	5.88^{d}	±1.10	14.67	6.83^{e}	±1.10	11.44	5.51^{cd}	±1.10	22.62
Chitosan 2 mM	8.30^{c}	±1.12	30.04	7.53^{c}	±1.13	46.81	8.25^{d}	±1.13	38.61	6.89^{c}	±1.18	60.12
Chitosan 4 mM	10.26^{b}	±1.20	60.88	9.64^{b}	±1.24	87.87	10.39^{c}	±1.15	79.59	9.64^{b}	±1.16	134.70
Chitosan 8 mM	13.99^{a}	±1.23	119.20	12.97^{a}	±1.33	152.79	13.18^{b}	±1.29	132.93	11.87^{a}	±1.15	195.31
Amistar	15.31^{a}	±1.29	139.93	13.71^{a}	±1.21	167.34	15.13^{a}	±1.26	170.12	13.64^{a}	±1.20	243.41
Control	6.88^{d}	±1.19	0.00	5.73^{d}	±1.08	0.00	5.23^{e}	±1.18	0.00	4.68^{d}	±1.21	0.00

*Increases related to the control

Biochemical changes associated with chitosan inducers

This study evaluated chitosan's ability to induce defence responses and related chemicals to protect grapevine from GDM infection. Grapevine treatment with chitosan triggered increased accumulation of biochemical markers associated with induced resistance *i.e.* SA contents, phenol content and activity of β-1.3-glucanase, chitinase, peroxidase (PO), and polyphenol oxidase (PPO).

Effect of Chitosan as inducer resistance on SA contents in the leaf of grape cultivars at two locations during seasons 2022 and 2023

Table 7 showed that SA was obviously higher in plants treated with either chitosan than the untreated control. Four tested concentrations of chitosan affected SA contents; the highest contents were induced by chitosan at 8 mM. Data indicated that increasing the chitosan concentration significantly increased SA content in both CVs during the two seasons.

On the other hand, the data in Figure 2 showed a relationship between the time of treatment with chitosan and the rate of SA content in plant leaf tissue. The data indicated that the slope of the curve increased noticeably after 5 days, and the increase was high after 10 days of treatment with chitosan.

The data also showed a big difference between the treatment with chitosan and the treatment with the fungicide on the content of SA in plant leaf tissue, in which the curve was in tangency with the control curve. In addition, the effect of the timing of sampling after treatment was slight.

Table 7. Effect of chitosan as inducer resistance on SA contents in leaf of grape cultivars compared to Amistar fungicide at locations during seasons 2022 and 2023

Location	Behera ('Crimson')						Beni-Suef ('Flame')					
Season	2022			2023			2022			2023		
Date after trt	1 day	5 days	10 days	1 day	5 days	10 day	1 day	5 days	10 days	1 day	5 days	10 days
Chitosan 1 mM	1.56^{c}	1.95^{c}	10.43^{d}	0.97^{c}	1.30^{d}	8.05^{d}	1.68^{c}	2.44^{d}	11.98^{d}	1.43^{c}	2.23^{d}	11.23^{d}
Chitosan 2 mM	1.65^{bc}	2.14^{c}	11.45^{c}	1.03^{bc}	2.00^{c}	10.04^{c}	1.95^{bc}	3.03^{c}	12.84^{c}	1.58^{bc}	2.96^{c}	11.98^{c}
Chitosan 4 mM	1.88^{b}	3.04^{b}	12.10^{b}	1.12^{b}	2.94^{b}	11.04^{b}	2.03^{b}	3.98^{b}	13.19^{b}	1.77^{b}	3.72^{b}	12.87^{b}
Chitosan 8 mM	2.19^{a}	3.96^{a}	13.11^{a}	1.87^{a}	3.25^{a}	11.89^{a}	2.65^{a}	4.86^{a}	14.11^{a}	1.98^{a}	4.12^{a}	13.92^{a}
Amistar	0.58^{d}	1.07^{d}	2.96^{e}	0.44^{d}	0.97^{e}	1.86^{e}	0.88^{d}	1.33^{e}	3.94^{e}	0.44^{d}	1.22^{e}	3.12^{e}
Control	0.31^{d}	0.45^{e}	2.64^{e}	0.21^{e}	0.30^{f}	1.74^{e}	0.35^{e}	0.63^{f}	3.03^{f}	0.32^{d}	0.48^{f}	2.89^{f}

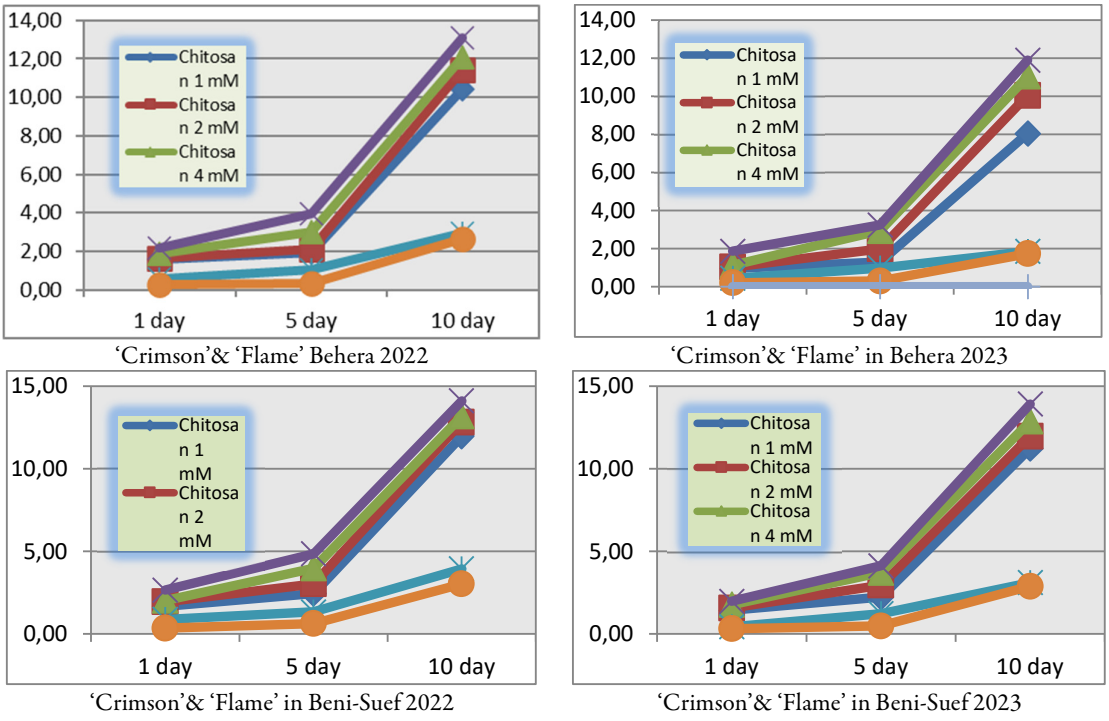


Figure 2. Relationship between different concentrations of chitosan and content of SA in plant leaf tissue during different periods after treatment

Effect of chitosan on total phenols contents in the leaf of grape cultivars at two locations during seasons 2022 and 2023

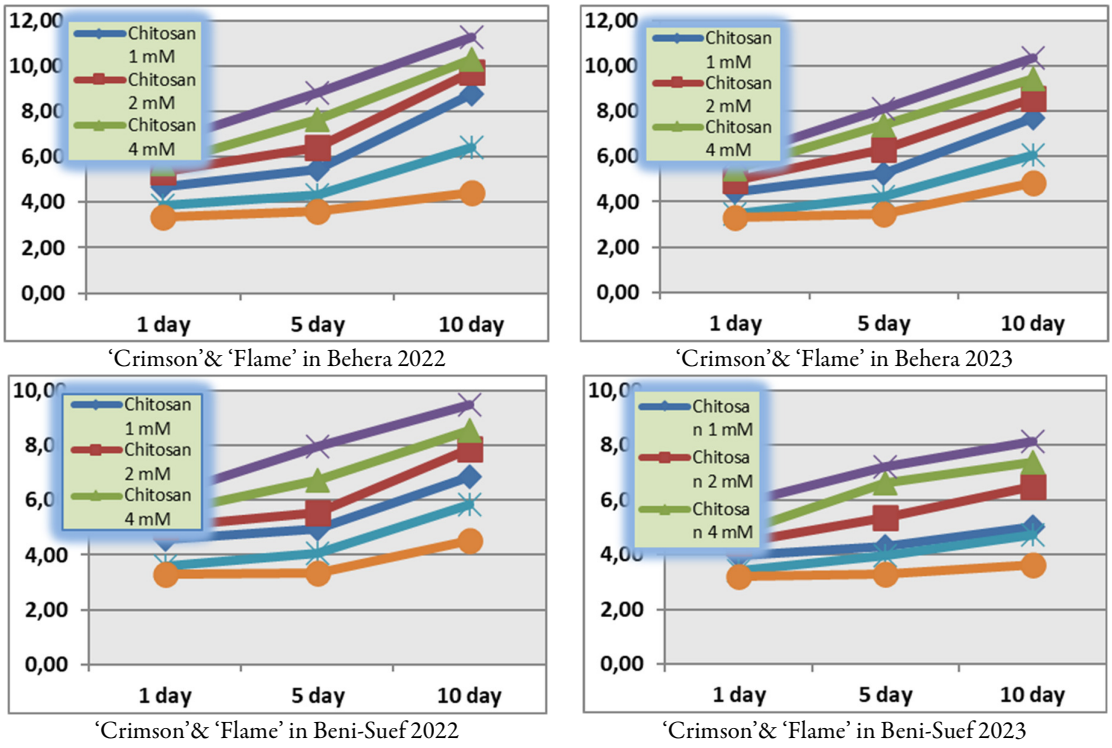
The data presented in Table 8 showed that total phenols in plant leaves were significantly affected by chitosan treatment compared to Amistar and the control (untreated treatment). In this respect, the highest contents were induced by chitosan at 8 mM. Data also clearly indicated that increasing the chitosan concentration significantly increased total phenol content in both CVs during the two seasons, 2022 and 2023.

Data in Figure 3 showed a relation between the time of treatment with chitosan and the rate of total phenols in plant leaf tissue, which was clearly shown in the slope of the curve, which increased after 5 days and increased more than 10 days.

The data also showed a big difference in the content of total phenols in plant leaf tissue between the treatment with chitosan and the treatment with the fungicide. This is clear because the sampling timing after treatment slightly affected the fungicide treatment (check control) and the control (untreated). In contrast, the difference in the case of chitosan treatments was noticeable.

Table 8. Effect of chitosan as inducer resistance on content of total phenols in leaf of grape cultivars compared to Amistar fungicide at two locations during seasons 2022 and 2023

Cultivar	'Crimson'						'Flame'					
Season	2022			2023			2022			2023		
Date after treatment	1 day	5 days	10 days	1 day	5 days	10 days	1 day	5 days	10 days	1 day	5 days	10 days
Chitosan 1 mM	6.68^{c}	7.44^{d}	10.76^{d}	6.43^{c}	7.23^{d}	9.70^{c}	6.56^{c}	6.95^{cd}	8.86^{d}	5.97^{c}	6.30^{d}	7.01^{d}
Chitosan 2 mM	7.33^{bc}	8.41^{c}	11.76^{c}	6.96^{b}	8.34^{c}	10.55^{bc}	7.03^{bc}	7.52^{c}	9.86^{c}	6.41^{bc}	7.38^{c}	8.51^{c}
Chitosan 4 mM	7.71^{b}	9.66^{b}	12.31^{b}	7.45^{ab}	9.40^{b}	11.42^{b}	7.56^{b}	8.72^{b}	10.55^{b}	6.80^{b}	8.62^{b}	9.37^{b}
Chitosan 8 mM	8.63^{a}	10.84^{a}	13.27^{a}	7.96^{a}	10.10^{a}	12.38^{a}	8.17^{a}	9.94^{a}	11.47^{a}	7.85^{a}	9.23^{a}	10.15^{a}
Amistar	5.88^{d}	6.33^{e}	8.43^{e}	5.44^{d}	6.22^{e}	8.05^{d}	5.58^{d}	6.07^{d}	7.85^{e}	5.44^{d}	5.97^{de}	6.73^{de}
Control	5.35^{de}	5.63^{ef}	6.42^{f}	5.32^{de}	5.48^{ef}	6.82^{e}	5.29^{de}	5.33^{de}	6.53^{f}	5.21d^{e}	5.30^{f}	5.61^{f}



'Crimson'& 'Flame' in Behera 2022 'Crimson'& 'Flame' in Behera 2023

'Crimson'& 'Flame' in Beni-Suef 2022 'Crimson'& 'Flame' in Beni-Suef 2023

Figure 3. Relationship between different concentrations of chitosan and content of total phenols in leaf tissue during different periods after treatment

Effect of chitosan as inducer resistance on enzyme activity in the leaf of grape cultivars at two locations during seasons 2022 and 2023

Data in Tables 9, 10, 11, and 12 clearly show that all tested concentrations of chitosan increased the activity of hydrolytic and oxidative enzymes, *i.e.,* 1.3-glucanase, chitinase, peroxidase (PO) and polyphenol oxidase (PPO) in plant leaf tissue compared to fungicides treatment (Amistar) and untreated plant (control). In this respect, the activities of those enzymes showed the highest increase when chitosan was used at 8 mM either than hydrolytic enzymes (1.3-glucanase and chitinase) or oxidative enzymes (peroxidase and polyphenol oxidase) during the two seasons, 2023 and 2024.

Table 9. Effect of chitosan as inducer resistance on 1.3-glucanase activity in leaf of grape cultivars compared to Amistar fungicide at two locations during seasons 2022 and 2023

Governorate	Behera ('Crimson')						Beni-Suef ('Flame')					
Season	2022			2023			2022			2023		
Date after trt	1 day	5 days	10 days	1 day	5 days	10 days	1 day	5 days	10 days	1 day	5 days	10 days
Chitosan 1 mM	0.335 ^{d}	0.540 ^{c}	0.710 ^{c}	0.300 ^{d}	0.465 ^{d}	0.530 ^{d}	0.210 ^{c}	0.390 ^{d}	0.540 ^{d}	0.196 ^{c}	0.265 ^{d}	0.380 ^{d}
Chitosan 2 mM	0.397 ^{c}	0.571 ^{bc}	0.730 ^{bc}	0.355 ^{c}	0.506 ^{c}	0.616 ^{c}	0.293 ^{b}	0.417 ^{c}	0.690 ^{c}	0.253 ^{b}	0.308 ^{c}	0.462 ^{c}
Chitosan 4 mM	0.427 ^{b}	0.596 ^{b}	0.759 ^{b}	0.402 ^{b}	0.584 ^{b}	0.680 ^{b}	0.318 ^{b}	0.442 ^{b}	0.725 ^{b}	0.293 ^{a}	0.387 ^{b}	0.531 ^{b}
Chitosan 8 mM	0.501 ^{a}	0.675 ^{a}	0.792 ^{a}	0.459 ^{a}	0.605 ^{a}	0.730 ^{a}	0.362 ^{a}	0.516 ^{a}	0.754 ^{a}	0.308 ^{a}	0.476 ^{a}	0.578 ^{a}
Amistar	0.139 ^{e}	0.248 ^{d}	0.318 ^{d}	0.135 ^{e}	0.240 ^{e}	0.298 ^{e}	0.137 ^{d}	0.233 ^{e}	0.310 ^{e}	0.132 ^{d}	0.228 ^{e}	0.286 ^{e}
Control	0.134 ^{e}	0.240 ^{d}	0.303 ^{d}	0.129 ^{e}	0.233 ^{e}	0.286 ^{e}	0.131 ^{d}	0.223 ^{e}	0.296 ^{e}	0.127 ^{d}	0.218 ^{e}	0.268 ^{e}

Data also showed that increasing the chitosan concentration significantly increased the activity of enzymes in both CVs during the two seasons compared to the untreated treatment and the Amistar fungicide treatment (check control).

Table 10. Effect of chitosan as inducer resistance on chitinase activity in leaf of grape cultivars compared to Amistar fungicide at two locations during seasons 2022 and 2023

Governorate	Behera ('Crimson')						Beni-Suef ('Flame')					
Season	2022			2023			2022			2023		
Date after trt	1 day	5 days	10 days	1 day	5 days	10 days	1 day	5 days	10 days	1 day	5 days	10 days
Chitosan 1 mM	0.442^{b}	0.498^{c}	0.521^{d}	0.384^{d}	0.449^{c}	0.482^{d}	0.306^{c}	0.377^{c}	0.447^{c}	0.269^{d}	0.322^{c}	0.418^{d}
Chitosan 2 mM	0.462^{b}	0.508^{c}	0.569^{c}	0.415^{c}	0.494^{b}	0.514^{c}	0.444^{b}	0.466^{b}	0.508^{b}	0.327^{c}	0.466^{b}	0.491^{c}
Chitosan 4 mM	0.487^{a}	0.534^{b}	0.613^{b}	0.460^{b}	0.515^{ab}	0.581^{b}	0.465^{ab}	0.497^{a}	0.584^{a}	0.435^{b}	0.494^{ab}	0.521^{b}
Chitosan 8 mM	0.504^{a}	0.562^{a}	0.668^{a}	0.492^{a}	0.538^{a}	0.613^{a}	0.495^{a}	0.519^{a}	0.605^{a}	0.473^{a}	0.507^{a}	0.583^{a}
Amistar	0.224^{c}	0.264^{d}	0.323^{c}	0.203^{c}	0.242^{d}	0.299^{c}	0.211^{d}	0.242^{d}	0.299^{d}	0.197^{c}	0.223^{d}	0.276^{e}
Control	0.201^{c}	0.253^{d}	0.296^{c}	0.196^{c}	0.236^{d}	0.273^{c}	0.194^{d}	0.230^{d}	0.283^{d}	0.185^{c}	0.203^{d}	0.237^{f}

Table 11. Effect of chitosan as inducer resistance on peroxidase (PO) activity in leaf of grape cultivars compared to Amistar fungicide at two locations during seasons 2022 and 2023

Governorate	Behera ('Crimson')						Beni-Suef ('Flame')					
Season	2022			2023			2022			2023		
Date after trt	1 day	5 days	10 days	1 day	5 days	10 days	1 day	5 days	10 days	1 day	5 days	10 days
Chitosan 1 mM	1.757^{d}	2.113^{c}	2.498^{d}	1.566^{c}	1.885^{d}	2.199^{d}	1.631^{c}	1.894^{d}	2.212^{c}	1.455^{c}	1.718^{d}	1.934^{c}
Chitosan 2 mM	2.155^{c}	2.637^{b}	2.965^{c}	1.977^{b}	2.095^{c}	2.586^{c}	1.973^{b}	2.103^{c}	2.470^{bc}	1.719^{b}	1.973^{c}	2.171^{bc}
Chitosan 4 mM	2.495^{b}	2.840^{ab}	3.358^{b}	2.106^{b}	2.324^{b}	2.922^{b}	2.112^{ab}	2.364^{b}	2.648^{b}	1.959^{ab}	2.211^{b}	2.472^{b}
Chitosan 8 mM	2.965^{a}	3.038^{a}	4.023^{a}	2.569^{a}	2.778^{a}	3.672^{a}	2.596^{a}	2.798^{a}	3.363^{a}	2.107^{a}	2.667^{a}	3.088^{a}
Amistar	1.393^{e}	1.412^{d}	1.474^{e}	1.234^{cd}	1.266^{e}	1.308^{e}	1.229^{d}	1.257^{e}	1.290^{d}	1.139^{d}	1.206^{e}	1.249^{d}
Control	1.192^{e}	1.243^{d}	1.278^{e}	1.114^{d}	1.175^{e}	1.213^{e}	1.103^{d}	1.136^{e}	1.212^{d}	0.994^{e}	1.132^{e}	1.199^{d}

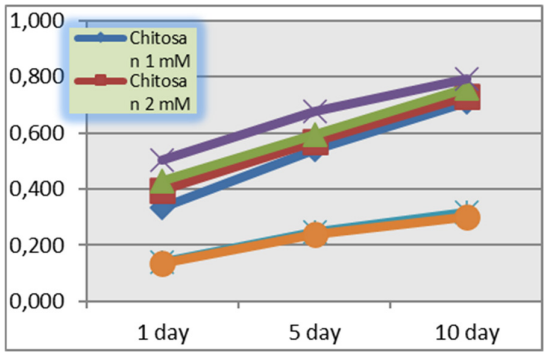
Table 12. Effect of chitosan as inducer resistance on polyphenol oxidase (PPO) activity in the leaf of grape cultivars compared to Amistar fungicide at two locations during seasons 2022 and 2023

Governorate	Behera ('Crimson')						Beni-Suef ('Flame')					
Season	2022			2023			2022			2023		
Date after trt	1 day	5 days	10 days	1 day	5 days	10 days	1 day	5 days	10 days	1 day	5 days	10 days
Chitosan 1 mM	0.729^{c}	0.765^{b}	0.789^{c}	0.702^{c}	0.727^{d}	0.751^{c}	0.706^{c}	0.738^{c}	0.758^{c}	0.676^{b}	0.709^{c}	0.738^{d}
Chitosan 2 mM	0.773^{b}	0.793^{b}	0.832^{b}	0.742^{b}	0.779^{c}	0.801^{b}	0.751^{b}	0.791^{b}	0.807^{b}	0.686^{b}	0.733^{c}	0.792^{c}
Chitosan 4 mM	0.792^{ab}	0.827^{ab}	0.859^{b}	0.775^{ab}	0.802^{b}	0.842^{ab}	0.776^{ab}	0.804^{ab}	0.842^{ab}	0.712^{a}	0.782^{b}	0.822^{b}
Chitosan 8 mM	0.832^{a}	0.866^{a}	0.907^{a}	0.805^{a}	0.848^{a}	0.890^{a}	0.813^{a}	0.842^{a}	0.886^{a}	0.741^{a}	0.805^{a}	0.862^{a}
Amistar	0.653^{d}	0.672^{c}	0.687^{d}	0.617^{d}	0.657^{e}	0.678^{d}	0.618^{d}	0.655^{d}	0.676^{d}	0.606^{c}	0.628^{d}	0.653^{e}
Control	0.607^{e}	0.633^{d}	0.668^{d}	0.577^{e}	0.605^{f}	0.630^{e}	0.577^{e}	0.612^{d}	0.650^{d}	0.573^{d}	0.594^{e}	0.622^{e}

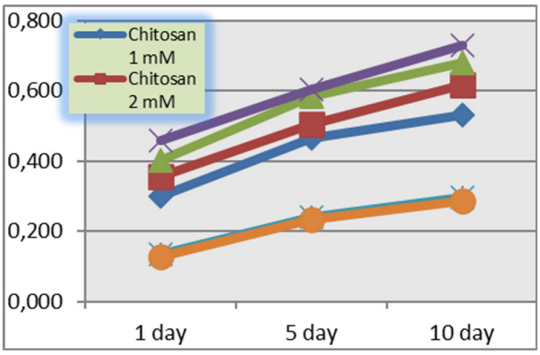
Moreover, the data in Figures 4, 5, 6, and 7 revealed a relationship between the timing of treatment with chitosan and the enzyme activity in plant leaf tissue. The increase in the curve slope matched the sampling timing after being treated with chitosan from 1 day to 10 days.

The data also showed that there are positive, significant differences between the effect of chitosan treatments and fungicide treatment on the efficiency of the activity of enzymes, whether hydrolytic or oxidative, in plant leaf tissues in both cvs 'Crimson' and 'Flame' during the two seasons 2022 and 2023 compared to untreated treatment and Amistar fungicide treatment (check control).

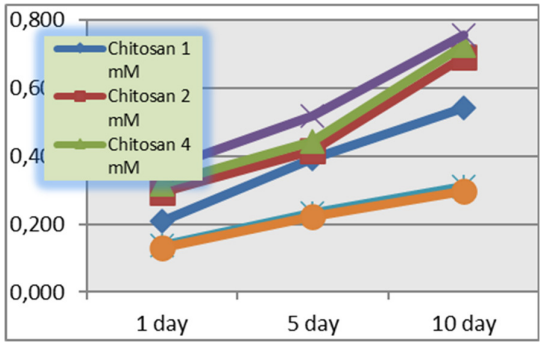
At the same time, the Amistar fungicide curve was tangent to the control curve in many cases; in addition to that, the timing of sampling after treatment had a slight effect on the fungicide treatment (check control) and the control (untreated), while in case of chitosan treatments were noticeable and should be clarified that, the effect of sampling timing had more effect on hydrolytic enzymes than oxidative enzymes which their curve slop high compared to hydrolytic enzymes from I day to 10 days after treatment in both cvs during the successive seasons 2022 and 2023 (Figures 4, 5, 6 and 7).



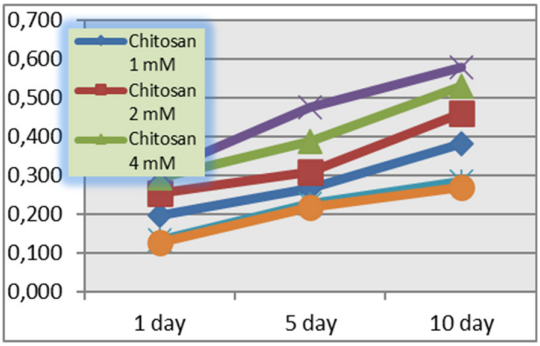
'Crimson'& 'Flame' in Behera 2022



'Crimson'& 'Flame' in Behera 2023

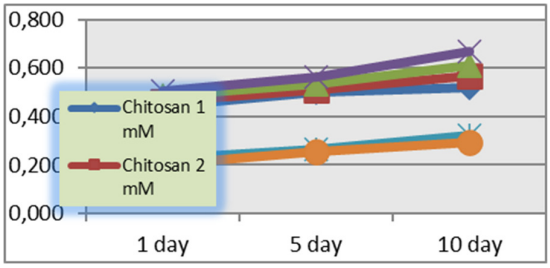


'Crimson'& 'Flame' in Beni-Suef 2022

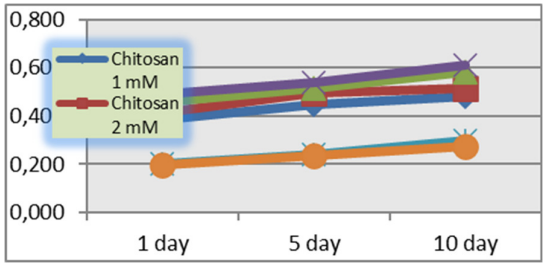


'Crimson'& 'Flame' in Beni-Suef 2023

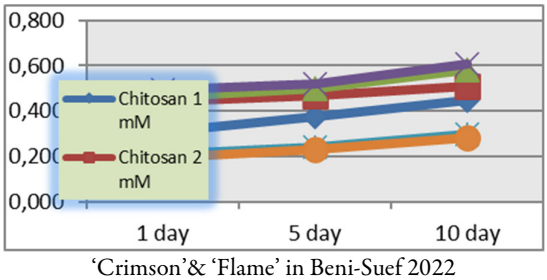
Figure 4. Relationship between different concentrations of chitosan and activity of chitinase in plant leaf tissue during different periods after treatment



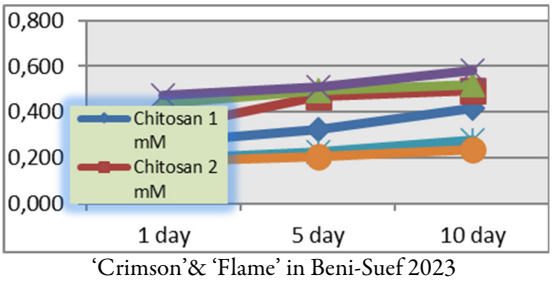
'Crimson'& 'Flame' in Behera 2022



'Crimson'& 'Flame' in Behera 2023



'Crimson'& 'Flame' in Beni-Suef 2022



'Crimson'& 'Flame' in Beni-Suef 2023

Figure 5. Relationship between different concentrations of chitosan and activity of β-1,3-glucanase in plant leaf tissue during different periods after treatment

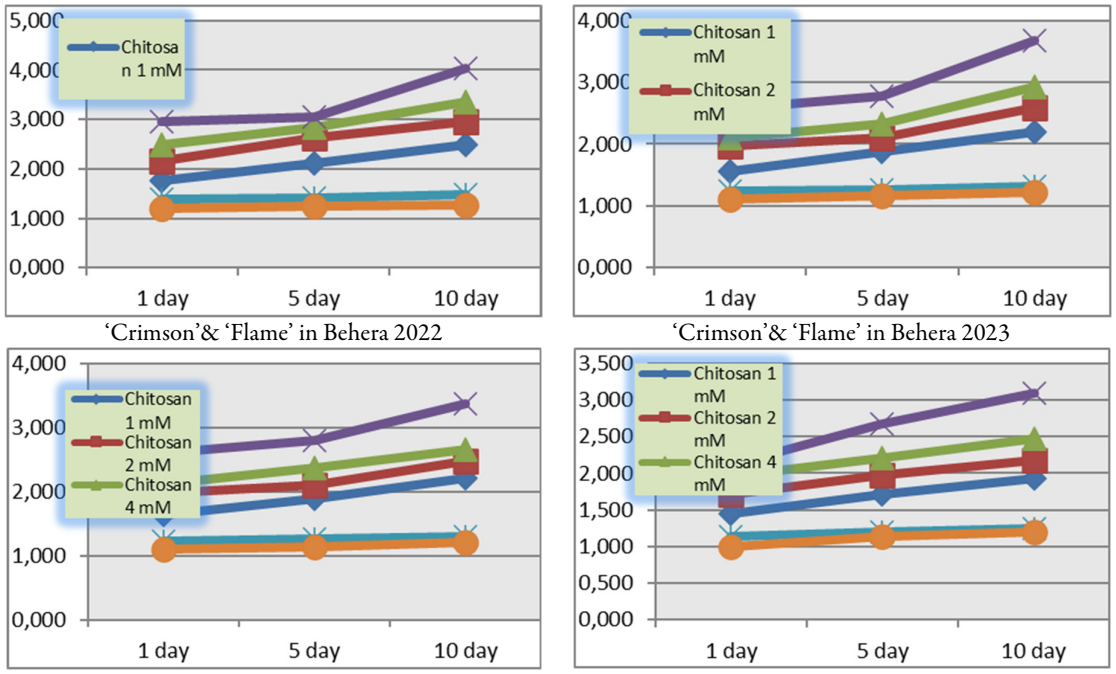


Figure 6. Relationship between different concentrations of chitosan and activity of PPO in plant leaf tissue during different periods after treatment

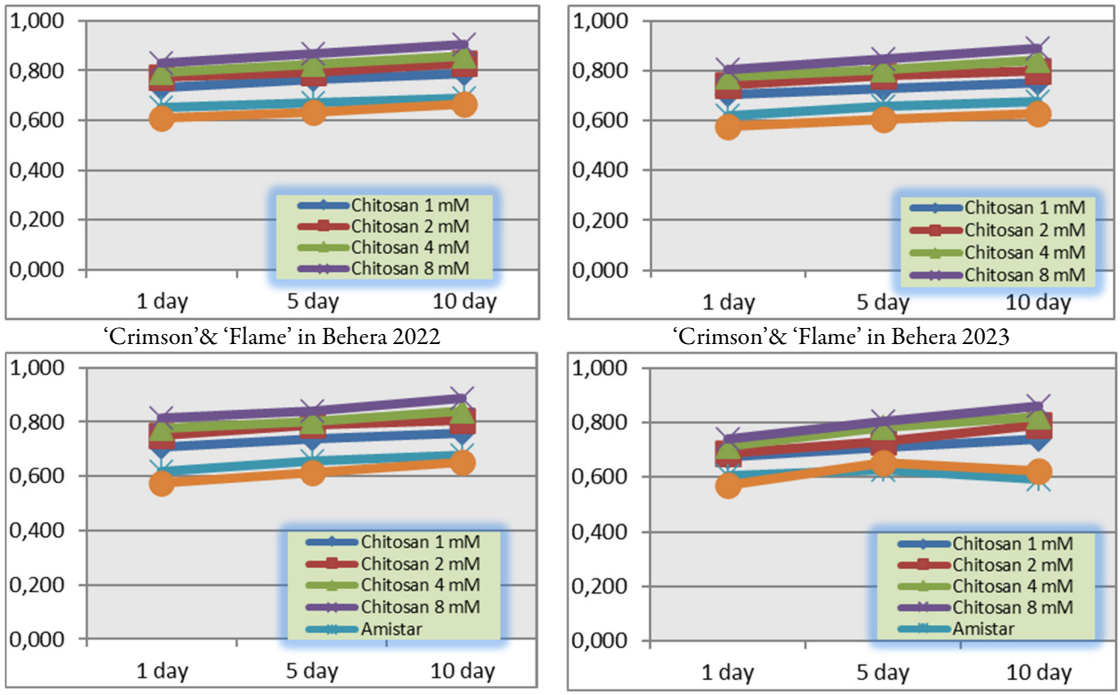


Figure 7. Relationship between different concentrations of chitosan and activity of PO in plant leaf tissue during different periods after treatment

Discussion

The present work shows that applying chitosan through a foliar spray can induce resistance in the grapevine against GDM caused by *Plasmopara viticola*. The induction of resistance was directly influenced by the concentration of chitosan, as demonstrated by the clear relationship between higher chitosan concentrations and reduced GDM severity. This finding aligns with similar observations reported in other pathosystems by Aziz *et al*. (2006), Faoro *et al*. (2008), Prakongkha *et al*. (2013), and Orzali *et al*. (2017). Moreover, many studies show that chitosan is a powerful elicitor and inducer of systemically acquired resistance of plants against a wide array of pathogens. Chitosan induces hosts to generate protein, enzymes, and secondary metabolites linked to protection from pathogens (Hadwiger, 2013; Goy *et al.,* 2009; Xing *et al.,* 2015; Mahmoud *et al.,* 2021).

The presented data demonstrate that this resistance is associated with an increase in salicylic content, and there was a significant accumulation in SA content by chitosan-treated grapevine compared to the control.

SA regulates biological processes in plants, including defence (Kumar *et al.,* 2015). It acts on the accumulation of superoxide and hydrogen peroxide in the apoplast, causing cell death at the infection site, promoting lignin synthesis in the cell wall, and acting in the establishment of systemic acquired resistance (Gao *et al.,* 2015; Moreau *et al.,* 2020). Systemic acquired resistance is a powerful mechanism based on the salicylic acid (SA) signalling pathway, which allows plants to resist a wide range of pathogens. Moreover, plays a key role in plant tolerance to abiotic stress. Several authors have shown that this occurs whether SA is added or SA production is stimulated endogenously, and this is what was done in this work, where the salicylic formation was stimulated endogenously as a result of treatment with chitosan (Gao *et al.,* 2015; Kumar *et al.,* 2015; Tripathi *et al.,* 2019; Liu *et al.,* 2020; Miao *et al.,* 2020).In this respect, De Bona *et al.,* 2021 stated that chitosan induced grapevine defence response: three days after the treatment, repression of the salicylic acid-mediated signalling and a transient accumulation of trans-resveratrol were registered. Moreover, they stated that chitosan can protect the grapevine against *Botrytis cinerea* infection when used in the preventive application.

The obtained results found that total phenols in plant leaves were significantly affected by chitosan treated, and increasing the concentration of chitosan led to a significant increase of total phenols that confirms the role of chitosan in stimulating the systematic induce resistance in plants (SIR) considers that, phenols one of the responses of SIR (Mahmoud *et al.,* 2021). While Zhang *et al*. (2015), found that the impacts of chitosan on the plant-fungal interaction of Botrytis cinerea in tomatoes have been linked with phenolic increment and phytoalexin precursors elicitation, increased production of chitinases and other plant defence factors.

The presented results also showed that there is a clear relationship between treatment with chitosan and increasing the effectiveness and activity of defence enzymes such as 1.3-glucanase, chitinase, peroxidase, and polyphenol oxidase, which are common plant biochemical responses associated to induced resistance (Zhang *et al.,* 2015; Mahmoud *et al.,* 2021). This is in agreement with several studies that showed that chitosan and its derivatives enhance the activity of chitinase, peroxidase, phenylalanine ammonia-lyase, polyphenol oxidase, superoxide dismutase and catalase in wheat, cucumber, tomato, sweet cherries, table grapes, pears, orange, strawberries, and ginseng (Romanazzi*,* 2010; Orzali *et al.,* 2014; Xing *et al.,* 2015; Li *et al.,* 2016).

The data also indicated that applying chitosan significantly increased the grape yield of 'Crimson' and 'Flame' compared with untreated control during the two seasons, 2022 and 2023, there is a relationship between increasing the concentration of chitosan and increasing grape yield. This may be due to the reduction of GDM severity, as well as the role of chitosan in the enhancement of the growth of plants. This agrees with many authors who stated that chitosan functions as a plant growth promoter in crops such as beans, potatoes, radishes, gerbera, soybeans, and cabbage (Hadwiger, 2013). It also enhances yield (Chandrkrachang, 2002; Chakraborty *et al.,* 2020). That is due to the role of chitosan in reducing stress damage in plant cells by decreasing water content and accelerating several biological macromolecule activities (Nahar *et al.,* 2012).

The results of the present work highlighted the high potential of chitosan as an efficient resistance inducer against grapevine downy mildew disease caused by Plasmopara viticola. Resistance induction was closely related to chitosan concentration, as reflected by increased salicylic acid content, total phenolics, and enhanced activity of key defense enzymes such as 1,3-glucanase, chitinase, peroxidase, and polyphenol oxidase. These results are in agreement with previous works on the ability of chitosan to induce systemic resistance in plants. For example, De Bona *et al*. (2021) presented the ability of chitosan to delay the defense response of grapevine to Botrytis cinerea, increasing the production of critical secondary metabolites like trans-resveratrol. This further confirms that chitosan acts as an elicitor for the induction of defense-related responses in grapevine varieties.

Xing *et al*. (2015) also reported the applicability of chitosan as an elicitor for various crops, aimed at enhancing enzymatic activities related to plant defense. Such findings are in agreement with the results of this study, where similar induction was reported in grapevine. Such an increase in enzyme activity upon application of chitosan confirms its potential role in biochemical changes in developing resistance against diseases.

Besides, Mahmoud *et al*. (2021) reported the efficiency of chitosan to induce synthesis, one of the most crucial responses in the development of systemic induced resistance: that of phenolic compounds. The profound rise in total phenolics found within this work also agrees with this, showing how efficient chitosan is in the activation of plant defense pathways.

SA's role in systemic acquired resistance has also been well chronicled, an example being seen in works like Kumar *et al*. (2015) and Gao *et al*. (2015), where the involvement of SA in signaling pathways related to enhancing pathogen resistance is described. The high level of SA accumulated in grapevine leaves subjected to chitosan treatment constitutes evidence of the ability developed by the latter compound to trigger mechanisms of SAR. The fact is further supported by the results of Prakongkha *et al*. (2013) and Orzali *et al*. (2017), who have reported similar findings with respect to inducing related defense enzymes and secondary metabolites in chitosan-treated plants.

Finally, the positive relationship of chitosan concentration with grape yield in this study is supported by Chakraborty *et al*. (2020), who showed that chitosan exerts its positive effect on plant growth and yield through the mitigation of stress and improvement of cellular functions. The fact that chitosan acts as both a resistance inducer and a growth promoter reinforce its potential as an environmentally friendly alternative to traditional chemical fungicides in viticulture.

Conclusions

Foliar application of chitosan significantly induces systemic resistance in grapevines against grape downy mildew caused by *Plasmopara viticola*. Higher concentrations of chitosan effectively reduce disease severity and enhance grape yield, approaching or matching results obtained with conventional fungicides. Chitosan treatment increases levels of critical biochemical markers, including salicylic acid, phenolic compounds, and defense-related enzymes such as chitinase, β-1,3-glucanase, peroxidase, and polyphenol oxidase. These biochemical changes confirm chitosan's role as a potent elicitor of plant defenses, reinforcing its potential as an eco-friendly alternative to traditional chemical fungicides in sustainable viticulture practices.

Authors' Contributions

Conceptualization: EM, ZNH, AS, MK; Methodology: EM, ZNH, AS, MK; Investigation and Data Collection: EM, ZNH, AS, MK; Data analysis: EM, ZNH, AS, MK; Validation and Interpretation: EM, MA, MK; Formal Analysis: EM, ZNH, AS, MK; Writing – Original Draft: EM, ZNH, AS, MA, MK; Writing –

Review & Editing: EM, ZNH, AS, NM, EA, TA, MA, MK; Supervision: EM, AS, MK; Conceptualization and Methodology Supervision: EM, AZM, AS, MK; All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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