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Research Article

Evaluation of Nano Zinc Oxide and Fungicide Applications against Chocolate Spot Disease in Faba Bean

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Abstract:

Faba bean is a legume that is economically important throughout the world. One of the global diseases, chocolate spot disease, causes losses in crops that range too modest to catastrophic. *Botrytis cineria* is one of the main causes of this disease. To control the effects of the biotic stress brought on by the occurrence of disease, certain phenolic acids were sprayed topically. Some chemical catalysts such as Nano zinc-oxide (ZnONPs) with concentration (10, 50 and 100 ppm), also the fungicide (Diacin) with the recommended dose (100L/fed) and control, were applied in this study to evaluate its effectiveness in controlling chocolate spot disease. The severity of the disease was substantially reduced by every chemical inducer that was tested. Additionally, they improved the treated plants' defense mechanism by raising the activity of antioxidant enzymes (catalase in comparison to the corresponding control. Comparing plants infected with Botrytis fabae to healthy leaves, the latter showed the highest values of antioxidant activity ($p < 0.05$). According to the results, ZnONPs could be used to reduce the effects of the chocolate disease.

1. Introduction

As a major source of protein for both human and animal use, the faba bean (*Vicia faba* L.) is cultivated all over the world especially across the Mediterranean region (Crépon et al., 2010). The nutritional benefit of faba beans has been attributed to its high protein content, which varies from 25 to 35 percent. In addition, the seeds include significant levels of calcium and iron and are rich in tocopherols, niacin, thiamin and folic acid. (Hassanein et al., 2012). Concurrently, the soil's nitrogen content rises with its cultivation. (Hungria and Vargas, 2000).

In Egypt, the faba bean (*Vicia faba*) is regarded as the most significant legume crop. In 2020, there were around 117309 feddan under cultivation, producing 168437 tons. It is one of the most important dietary legumes because of its high nutritional value.

Globally, the most common faba bean disease is called chocolate spot. *Botrytis cineria* as well as *B. fabae* can cause this illness. This disease may easily spread under low temperature and high relative humidity conditions (El-Banoby et al., 2013). Significant yield losses of 60–80% can be caused by the disease in cultivars that are sensitive to it (Mbazia *et al*., 2016). The amount of nitrogen, protein, nucleic acid, and total carbohydrates in the produces seeds is reduced (Aldesuquy et al., 2015). Oxidative stress in plants is caused by an increase in reactive oxygen species (ROS) generation, which is recognized for its role as signaling intermediates in both biotic and abiotic stress periods (Latef and Chaoxing, 2014). Indeed, reactive oxygen species (ROS) may cause distraction of chlorophyll, DNA, and proteins through the process of lipid peroxidation, which affects cellular membranes (Mittova et al., 2003). Many important antioxidant enzymes are produced by plants, such as superoxide dismutase (SOD), which is crucial for scavenging reactive oxygen species (ROS) from the cell's mitochondria, chloroplasts, as well as cytosol (Lu and Finkel, 2008). A major fungal disease affecting faba bean in Egypt, is *B. cinerea* chocolate spot disease, which may result up to 50% losses in faba bean yield (Omar, 2021). Small, distinct reddish-brown lesions on leaves, stems, and also pods are the known symptoms of the faba bean disease known as "chocolate spot" (Baka and El-Zahed, 2022).

The disease can affect any part of the plant. In ideal circumstances, it manifests as reddish-brown dots on leaves and, in certain cases, on stems, flowers, and pods. These patches then enlarge and can even combine to form a single, dark mass. Crop lodging may occur in warm, humid circumstances, and the disease causes severe early defoliation. There is usually a significant decrease in yield due to the negative impact on plant development and most physiological processes (Sahile et al., 2011). According to Estayih (2018), chocolate spot resulted in yield losses of faba beans that ranged from 34% on a tolerant genotype to 61% on a susceptible genotype, as well as total crop failure because of persistently favorable climatic conditions for the diseases (Mitiku, 2017). When climatic conditions are favorable, a crop may lose all of its yield (Mitiku, 2017).

There has been a recent change in scientific theory and practice around the management of faba bean illnesses, with a focus on location-specific identification, assessment, and integration of integrated disease management (Denekea et al., 2009).

Due to the high costs of the fungicides, detrimental impacts on the environment and human health, and ability to destroy important soil microbiota, fungicide usage is not recommended (Arora et al., 2018). This makes it essential to propose innovative approaches and adapt sustainable practices. An amazing dedication to alleviating these issues is provided by nanotechnology, especially green innovation. It has spurred modifications and breakthroughs in a wide range of technologies and can support the growth of several agricultural industries, including composting, fungicides, fertilizers, and other industrial products.

Nanomaterials were employed in pathogen detection, disease control, and agricultural loss prevention via nanotechnology, a relatively new technique (Baka and El-Zahed, 2022). Nanomaterials can be synthesized through labor-intensive and expensive chemical and physical processes. However, Biological approaches can provide safer, faster, more affordable, simpler, and more environmental friendly ways to synthesize nanoscaled platforms than alternative chemical or physical processes (El Messaoudi et al., 2022). In terms of managing their release rate and mechanism, nanomaterials were safer and more effective antifungal agents than chemical fungicides, herbicides, and fertilizers (Li *et al*., 2007). Nanomaterials were employed in pathogen detection, disease control, and agricultural loss prevention via nanotechnology, a relatively new technique (Baka and El-Zahed, 2022). Nanomaterials can be synthesized through biological approaches provide a safer, faster, more affordable, simpler, and more environmentally friendly way compared with alternative chemicals or physical processes (El Messaoudi et al., 2022). ZnO NPs were administered at varying doses to tomato, alfalfa, and cucumber seeds; the results showed that only cucumber seed germination was improved (Raliya and Tarafdar, 2013) found that ZnONPs significantly increased the biomass of *Cyamopsis tetragonoloba* plants, as well as the shoot and root development, root area, chlorophyll and protein synthesis, rhizospheric microbial population, and activity of the enzymes acid phosphatase, alkaline phosphatase, and phytase in the rhizosphere of cluster beans. The plantlets' ability to withstand biotic stress was improved by increased nano ZnO somatic embryogenesis, shooting, and regeneration when supported by MS medium. Additionally, proline synthesis, the activities of catalase, peroxidase, and superoxide dismutase were all stimulated (Helaly et al., 2014). (Sofy et al., 2021) detected that plants had a variety of defense mechanisms against and solutions for viral stress. The non-enzymatic and enzymatic defense antioxidant system is one such mechanism that labor-intensive and expensive chemical and physical processes. Faba bean plants treated with nanoparticles, growth indicators increased along with biochemical features (antioxidant enzymes and markers of oxidative stress), this was discovered by (Sofy et al., 2020c).

2. Materials and Methods

The experiments were conducted during the 2021/2022 and 2022/2023 growing seasons. Experiments in the laboratory and greenhouse were carried out at the Faculty of Agriculture, Tanta University and at Giza Agricultural Research Centre (ARC). To assess faba bean (Giza716)' resistance or vulnerability to chocolate spot disease caused by *Botrytis fabae*, this experiment was performed under greenhouse conditions. This study incorporated nano zinc-oxide (ZnONPs) at concentrate of 10, 50 and 100ppm, fungicide (Diacin) and control as key materials.

2.1. Experimental treatments and artificial pathogen inoculation

Based on (Dere et al., 1998), fresh leaf samples (0.5gm) were sliced into smaller fragments, left to soak for 48 h at 4°c in 15 ml methanol (80%) followed this,the sample was filtered through Whatman GF/C filter paper (47 mm).

Each filterate's absorbance was measured at wavelengths 666 nm and 653nm for chlorophyll a&b, and at 470nm for carotenoids, with an 80% methanol blank as the baseline.

The obtained values were expressed in mg g^{-1} fresh weight (FW) and computed utilizing the following equations:

Chlorophyll (chl.) – a=(15.65A666-7.34A653)

Chlorophyll (chl.) - b=(27.05A653-11.21A666)

Carotenoids= {((1000A470) – (2.86Chl. a + 129.2 Chl. b))\245}

2.2. Total soluble phenolics (TSP)

To 0.2 ml of methanolic extract (80%) of dried faba bean leaves, 1.0 ml of 10% FCR was added, followed by vortexing. Following a 3-minute incubation, 0.8ml of 7.5 % w/v sodium carbonate solution was carefully incorporated into the reaction mixture. After being Shaked, the mixture was set aside to incubate at room temperature for a duration of 30 minutes. At 765nm, absorbance was recorded and the phenolic content was reported as mg Gallic Acid Equivalents (GAE) per gram of dry weight (g DW) (Kähkönen et al., 1999).

2.3. Antioxidant enzymes

In 10ml of ice-cold 50mM phosphate buffer (7.8) within 1mM EDTA and also 2% (w/v) polyvinlpyrrolidone (PVP) fresh leaves (0.5g) were crushed and put. A through four layers cheese-cloth and then centrifuged at 5000 for 10 min at 4°c (Zhang et al., 2007) was strained the homogenated. The supernatant was gathered and used for spectrophotometric assessment of catalase (CAT) activity. Spectrophotometric measurements were taken every 30sec. Except that the substrate-solution was substituted with the extraction buffer, the reference cuvette in the spectrophotometer contained identical component concentrations as the sample cuvette.

2.3.1. Catalase (CAT; EC.1.11.1.6) activity

CAT activity was evaluated according to (Kato and Shimizu, 1987) by monitoring the initial rate of hydrogen peroxide degradation of H_2O_2. For the assay a sample of 3ml of reaction-mixture encompassing 2mM H_2O_2, 100mM sodium phosphate buffer (pH7), and 100micro ml enzyme extract was assembled. CAT activity was determined by recording reaction-mixture

absorbance changes at 240nm tissue (the unit of measurement of the catalase enzyme).min-1 g^{-1} with fresh 240 $min^{-1}g^{-1}$ FW.

2.4. Lipid peroxidation

Indicator was quantified by measuring the Malondialdehyde (MDA) concentration, following the method outlined by Bao *et al*. (2009). A 2 ml supernatant was combined with 2 ml of 0.67% (w/v) thiobarbituric acid (TBA), and the mixture was incubated in boiling water for 30 minutes before being cooled. The absorbance was recorded at 450, 532, and 600 nm. MDA concentration was calculated using theformula:

MDA (nmol g−1 FW) = ((6.45 × (A532 − A600) − (0.56 × A450)) × V (ml) / W(g)."

2.5. Statistical analysis

The data collected were analyzed utilizing analysis of variance as outlined by Steel and Torrie (1960). Additionally, Mean comparisons of several parameters were conducted using the procedures of SPSS statistical analysis software version 16. Mean separation was estimated using one-way ANOVA and Duncan's multiple range test and Differences were deemed statistically remarkably ($P<0.05$) (Duncan, 1955).

3. Results

3.1. Impact of chemical inducers on chlorophyll in faba bean plants both prior to and following inoculation with *Botrytis fabae* under controlled greenhouse conditions

Table (1) shows that The chlorophyll (Chl) a and b concentrations in faba bean leaves were assessed both before and after inoculation, revealing different responses to foliar applications of ZnONPs at varying concentrations (10, 50, and 100 mg $^{-1}$), applied before artificial inoculation with *B. fabae*. A significant increase in Chl a concentration was observed in all exogenous treatments compared to the control, both before and after inoculation (Table 1). The concentration of (Chl a) markedly increased over control. The control plants had the lowest concentration of chlorophyll a, whereas the plants treated with fungicide (Diacin) and ZnONPs 3at (100 mg l^{-1}) had the highest concentration. Consequently, foliar treatment with ZnONPs 3 at 100 mg l^{-1} boosted the chlorophyll levels in *B. fabae*-infected faba bean plants. Moreover, before inoculation chlorophyll b level in faba leaves increased in ZnONPs at varying concentrations (10, 50, and 100 mg $^{-1}$) compared with control plants.

3.2. Impact of chemical inducers on chlorophyll b in faba bean plants both prior to and following inoculation with *Botrytis fabae* under controlled greenhouse conditions

The greatest increase in chlorophyll b content over control was observed by in BI plants treated with ZnONPs 3. Furthermore, AI showed a lower Chl b concentration than BI. In addition, after inoculation chlorophyll b increased in all the treatments over control.

3.3. Impact of chemical inducers on total soluble phenolic (TSP) in faba bean plants both prior to and following inoculation with *Botrytis fabae* under controlled greenhouse conditions

All treatments led to an increase in the Total soluble Phenolic in the plants, and this effect was observed both before and after inoculation. Infected (AI) plants had lower phenolic levels than healthy (BI) plants. Moreover, plants treated with ZnONPs 3 exhibited the most substantial growth compared to the control group. Following inoculation (AI), total soluble phenolic levels in ZnONPs 2 and ZnONPs 1-treated plants rose significantly in comparison to the control group. Additionally, all treated plants, excluding the control, had notably higher total soluble phenolic levels prior to treatment. All treatments markedly decreased slightly in response to the occurrence of infection except for the control (Table 2).

3.4. Impact the influence of chemical inducers on CAT activity (Δ240 min^{-1} g^{-1} FW) in faba bean plants both prior to and following inoculation to *Botrytis fabae* under controlled greenhouse conditions

Overall, CAT activity was higher in AI plants compared to BI plants. Furthermore, all treatments notably enhanced CAT activity in comparison to the control group prior to inoculation. ZnONPs 3 and Fungicide (Diacin)-treated plants exhibited the highest MDA activity relative to the control (Table 3). Likewise, post-inoculation, all exogenous treatments considerably increased CAT activity, with ZnONPs 3-treated plants showing the highest activity. All exogenous treatments notably boosted CAT activity upon inoculation, resembling the pattern observed in the control group. The significance that exogenous therapies—fungicide, Sio_2-NPs_2 and ZnONPs 2 treatments, in particular—play in fostering resistance against oxidative damage brought on by Chocolate spot disease is indicated by the overexpression of CAT in both healthy and infected faba bean plants.

3.5. Impact of chemical inducers on MDA in faba bean plants both prior to and following inoculation with *Botrytis fabae* under controlled greenhouse conditions

In comparison to the control, the MDA concentration was significantly reduced by fungicide and ZnONPs 3, prior to inoculation. Over control, the plants sprayed with fungicide (Diacin) and ZnONPs 3, had the lowest MDA concentration. The MDA concentration was also significantly reduced by all treatments following inoculation, when compared to the control. Respectively, plants sprayed with fungicide (Diacin) and ZnONPs 3 showed the greatest drop in MDA content (Table 4).

Table 1. Impact of chemical inducers on cholorophyll a in faba bean plants both prior to and following inoculation to *Botrytis fabae* under controlled greenhouse conditions

Chl a (Pre-inoculation)		**Chl a (Post-inoculation)**		Mean
C1	0.8±0.44	C1	0.7±0.048	3.45±0.244
F	1.7±0.36	F	1.3±0.36	1.5±0.36
ZnoNPs1	2.29±0.19	ZnoNPs1	2.7±0.14	2.495±0.165
ZnoNPs2	2.66±0.077	ZnoNPs2	2.45±0.063	2.555±0.07
ZnoNPs3	2.98±0.033	ZnoNPs3	2.77±0.097	2.875±0.065

Table 2. Impact of chemical inducers on cholorophyll b in faba bean plants both prior to and following inoculation to *Botrytis fabae* under controlled greenhouse conditions

Chl b (Pre-inoculation)		**Chl b (Post-inoculation)**		Mean
C1	0.4±0.01	C1	0.244±0.04	0.222±0.025
F	0.62±0.0165	F	0.36±0.06	0.49±0.038
ZnoNPs1	0.7±0.026	ZnoNPs1	0.63±0.045	0.665±0.036
ZnoNPs2	0.78±0.06	ZnoNPs2	0.77±0.03	0.775±0.045
ZnoNPs3	0.8±0.02	ZnoNPs3	0.8±0.02	0.8±0.02

Table 3. Impact of chemical inducers on Carotenoids (CAR) in faba bean plants both prior to and following inoculation to *Botrytis fabae* under controlled greenhouse conditions.

CAR (Pre-inoculation)		**CAR (Post-inoculation)**		Mean
C1	1.24±0.022	C1	1.14±0.067	1.19±0.0445
F	1.265±0.012	F	1.2±0.04	1.233±0.026
ZnoNPs1	1.34±0.05	ZnoNPs1	1.28±0.07	1.31±0.06
ZnoNPs2	1.1±0.01	ZnoNPs2	1.02±0.024	1.06±0.017
ZnoNPs3	1.98±0.023	ZnoNPs3	1.54±0.056	1.76±0.04

Table 4. Impact of chemical inducers on total soluble phenolic (TSP) in faba bean plants both prior to and following inoculation to *Botrytis fabae* under controlled greenhouse conditions.

TSP (Pre-inoculation)		**TSP (Post-inoculation)**		Mean
C1	0.06±0.0003	C1	0.05±0.0003	0.0695±0.0003
F	0.0843±0.0006	F	0.067±0.0013	0.0757±0.00095
ZnoNPs1	0.129±0.0009	ZnoNPs1	0.115±0.0009	0.122±0.0009
ZnoNPs2	0.136±0.016	ZnoNPs2	0.121±0.0006	0.129±0.0083
ZnoNPs3	0.185±0.001	ZnoNPs3	0.162±0.0006	0.092±0.0008

Table 5. Impact of chemical inducers on CAT activity (Δ_{240} min^{-1} g^{-1} FW) in faba bean plants both prior to and following inoculation to *Botrytis fabae* under controlled greenhouse conditions

CAT (Pre-inoculation)		**CAT (Post-inoculation)**		Mean
C1	0.45±0.0006	C1	0.523±0.0003	0.45±0.0006
F	1.535±0.00058	F	1.85±0.00058	1.535±0.00058
ZnoNPs1	1.465±0.0009	ZnoNPs1	1.51±0.0015	1.465±0.0009
ZnoNPs2	1.515±0.0008	ZnoNPs2	1.55±0.00058	1.515±0.0008
ZnoNPs3	1.635±0.00075	ZnoNPs3	1.88±0.0006	1.635±0.00075

Table 6.Impact of chemical inducers on MDA in faba bean plants both prior to and following inoculation to *Botrytis fabae* under controlled greenhouse conditions.

MDA(Pre-inoculation)		**(Post-inoculation) MDA**		Mean
C1	3.71±0.115	C1	4.24±0.115	3.71±0.115
F	1.8±0.0495	F	1.83±0.056	1.8±0.0495
ZnoNPs1	2.99±0.0485	ZnoNPs1	3.092±0.04	2.99±0.0485
ZnoNPs2	2.712±0.0635	ZnoNPs2	2.96±0.063	2.712±0.0635
ZnoNPs3	2.227±0.0475	ZnoNPs3	2.43±0.035	2.227±0.0475

C, control; F, fungicide Diacin; ; ZnO NPs1, nano-zinc oxide at 10 mg l^{-1}; ZnONPs 2, nano-zinc oxide at 50 mg l^{-1}; ZnONPs 3, nano-zinc oxide at 100 mg l^{-1}.

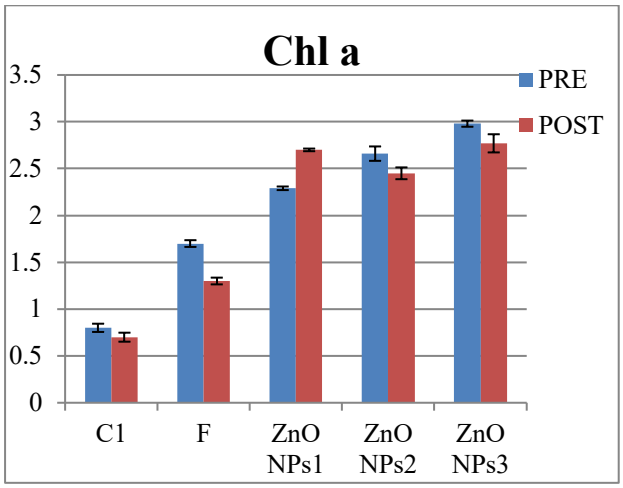

Figure 1. Impact the influence of chemical inducers on cholorophyll a activity in faba bean plants both prior to and following inoculation to *Botrytis fabae* under controlled greenhouse conditions

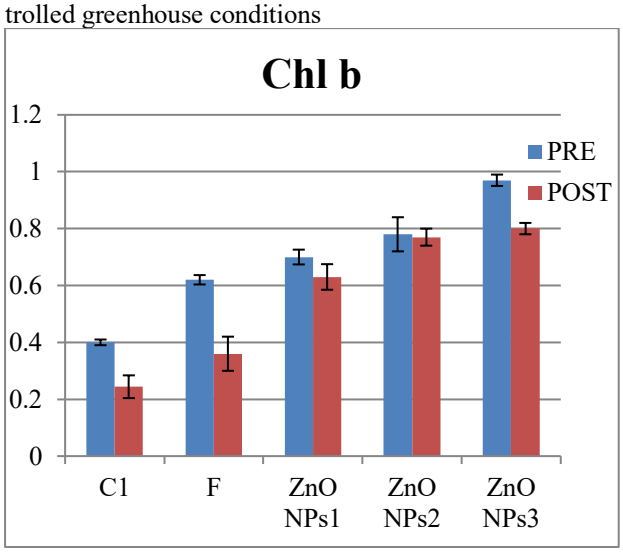

Figure 2. Impact the influence of chemical inducers on cholorophyll b activity in faba bean plants both prior to and following inoculation to *Botrytis fabae* under controlled greenhouse conditions

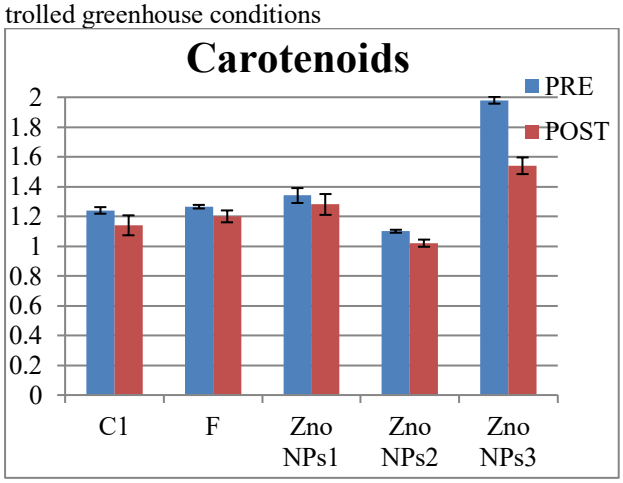

Figure 3. Impact the influence of chemical inducers on Carotenoids activity in faba bean plants both prior to and following inoculation to *Botrytis fabae* under controlled greenhouse conditions.

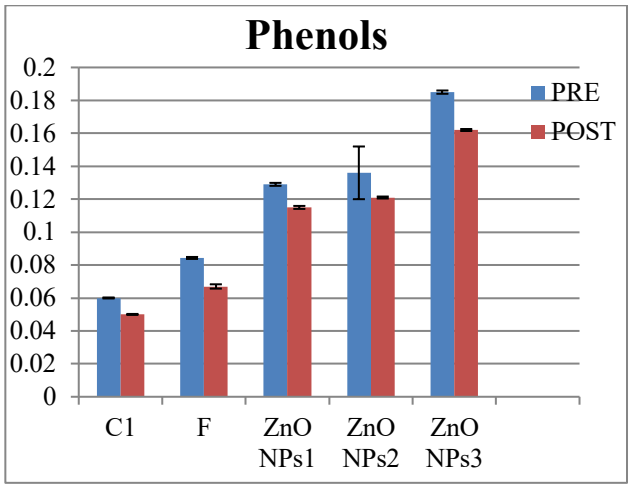

Figure 4. Impact the influence of chemical inducers on total soluble phenolic activity (Δ_{240} min^{-1} g^{-1} FW) in faba bean plants both prior to and following inoculation to *Botrytis fabae* under controlled greenhouse conditions.

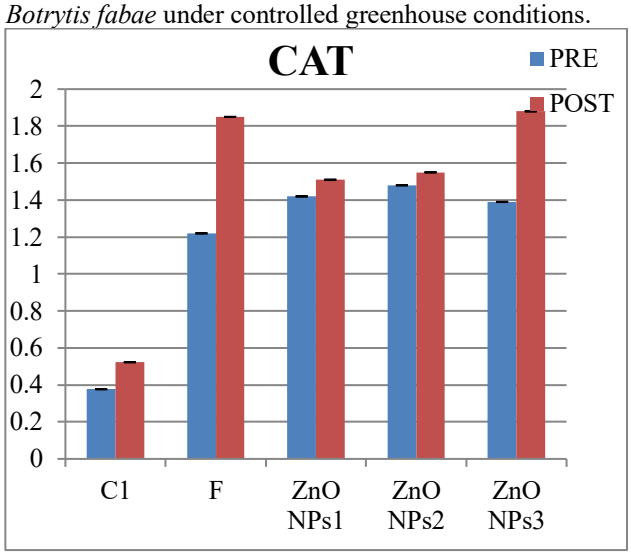

Figure 5. Impact the influence of chemical inducers on CAT activity (Δ_{240} min^{-1} g^{-1} FW) in faba bean plants both prior to and following inoculation to *Botrytis fabae* under controlled greenhouse conditions.

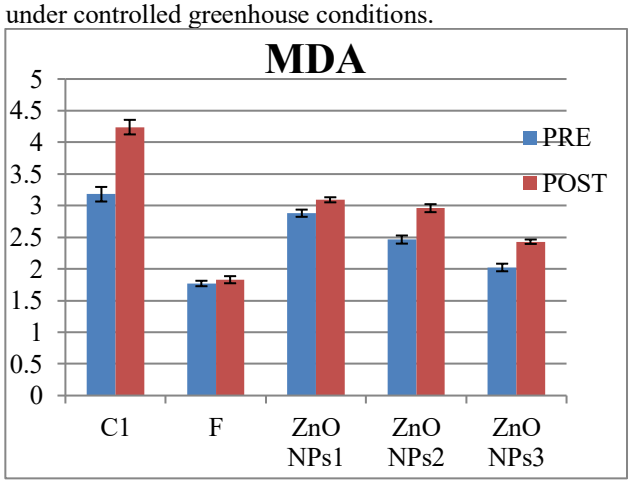

Figure 6. Impact the influence of chemical inducers on MDA activity (Δ_{240} min^{-1} g^{-1} FW) in faba bean plants both prior to and following inoculation to *Botrytis fabae* under controlled greenhouse condition.

4. Discussion

Faba bean (*Vicia faba* L.) is primarily cultivated in Egypt for its dried seeds and green pods, which are rich in protein (18.5 to 37.8%) and other nutrients that can serve as alternatives to animal protein (El Hendawy et al., 2010; Sahile et al., 2011). The objective of this study was to assess whether specific chemical inducers could alleviate the detrimental effects of *B. fabae* on faba bean plants and stimulate resistance to this virulent pathogen. Zinc oxide (ZnO) is essential for protecting and maintaining the structural stability of cell membranes (Welch et al., 1982). Consequently, seed priming and foliar application of ZnO nanoparticles (ZnO NPs) contribute to enhanced plant growth, as well as increased chlorophyll and carotenoid levels. ZnO NPs also play a vital role in protein synthesis, maintaining membrane integrity, promoting cell elongation, and enhancing stress tolerance (Cakmak, 2000), all of which can help reduce disease occurrence and further support plant growth and pigment accumulation. Chaudhary et al. (2017) highlighted that zinc is crucial for plant metabolism and the synthesis of proteins. Moreover, applying zinc increases the amount of leaf area, chlorophyll content, and other photosynthetic pigments in plants, which improves growth and production (Karim et al., 2012).

According to Sasan and Seyedeh (2017), spraying safflower with zinc boosted chlorophyll content, emphasizing zinc's important role in nitrogen metabolism and chlorophyll production. Khan et al. (2019) highlighted various mechanisms through which NPs enhance photosynthesis. NPs stimulate different steps and enzymes involved in photosynthesis, leading to increased photosynthetic efficiency. Ebrahim and Helmy (2016) showed that higher total phenol levels effectively boost the plants' defense mechanisms against disease infection and development. (Hafez et al., 2018) demonstrated that the decrease of phenolic compounds in response to exogenous treatments may be explained by stating that phenolics provide an adequate substrate to oxidative reactions catalyzed by peroxidase and/or polyphenol oxidase, Consuming oxygen and producing fungitoxic quinones, make the medium unfavorable to the further development of pathogens. Sofy et al. (2021) stated that treatment with ZnO-NPs significantly enhanced the activity of antioxidant enzymes (CAT, SOD, POX, APX, GR, LOX, CA, and NR). Additionally, it has been mentioned that metal-NPs promote seed germination in tomatoes and strengthen antioxidant systems under stress conditions.

As for MDA (Hafez et al., 2018) supported these findings, demonstrating that squash plants infected with powdery mildew exhibited-higher MDA concentrations, indicating increased lipid peroxidation, compared to healthy plants. Singh et al. (2016) found that the greatest increase occurred in seedlings treated with the highest concentrations of NPs.

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