Induction of Defense-Related Physiological and Antioxidant Enzyme Response against Powdery Mildew Disease in Okra (Abelmoschus esculentus L.) Plant by Using Chitosan and Potassium Salts

Mona H. Soliman1,2,* and Riad S. R. El-Mohamedy3

1Biology Department, Faculty of Science, Taibah University, Yanbu 464 29, Kingdom of Saudi Arabia
2Botany and Microbiology Department, Faculty of Science, Cairo University, 12613 Giza, Egypt
3Plant Pathology Department, National Research Centre, Cairo 12622, Egypt

Abstract Foliar sprays of three plant resistance inducers, including chitosan (CH), potassium sorbate (PS) (C6H7kO2), and potassium bicarbonates (PB) (KHCO3), were used for resistance inducing against Erysiphe cichoracearum DC (powdery mildew) infecting okra plants. Experiments under green house and field conditions showed that, the powdery mildew disease severity was significantly reduced with all tested treatments of CH, PS, and PB in comparison with untreated control. CH at 0.5% and 0.75% (w/v) plus PS at 1.0% and 2.0% and/or PB at 2.0% or 3.0% recorded as the most effective treatments. Moreover, the highest values of vegetative studies and yield were observed with such treatments. CH and potassium salts treatments reflected many compounds of defense singles which leading to the activation power defense system in okra plant. The highest records of reduction in powdery mildew were accompanied with increasing in total phenolic, protein content and increased the activity of polyphenol oxidase, peroxidase, chitinase, and β-1,3-glucanase in okra plants. Meanwhile, single treatments of CH, PS, and PB at high concentration (0.75%, 2.0%, and/or 3.0%) caused considerable effects. Therefore, application of CH and potassium salts as natural and chemical inducers by foliar methods can be used to control of powdery mildew disease at early stages of growth and led to a maximum fruit yield in okra plants.

Keywords Antioxidant enzymes, Induce resistance, Okra, Powdery mildew

Okra (Abelmoschus esculentus L.), is one of the most important vegetable crops belongs to the family Malvaceae, which grown in tropical, subtropical and warm temperate regions around the world. It is also popular vegetable for home garden and a good source to fulfill the energy needed to the body. Moreover, it provides vitamin A, B, C, protein, minerals, and iodine. Okra vegetable crop recently recorded a considerable yield losses range between 17% and 86.6%, when attacked by powdery mildew disease to all plant parts infected i.e., leaves, stem and fruits [1-3]. Reuveni et al. [4] tested the efficacy of various chemical inducers of systemic resistance against powdery mildew disease. In the same manner, an observed decrement in powdery mildew severity in pepper plants as exposed to foliage sprays of sodium or potassium bicarbonates (PB) was recorded [5]. Deliopoulos and colleagues [6, 7] reported that these salts were used as pesticide ingredients and horticultural fungicide, in the United States and United Kingdom, respectively. Several physiological mechanisms must be considered for the chemical compounds to be registered as fungicides, such as pH elevation on the leaf surface and collapse of fungal cell walls due to K+ imbalance or dehydration of fungal spores [8, 9].

Potassium bicarbonates (KHCO3) and PS (C6H7kO2) have been demonstrated to inhibit several fungal pathogens infecting vegetables and ornamental crops at the post-harvest stage [10-12]. Antimicrobial and antifungal activity of potassium sorbate (PS) as a weak acid are also studied [13, 14]. PS was the highly effective compound used to...
control orange molds caused by *Penicillium digitatum* and management of several food-borne fungal pathogens for plants infected with *P. italicum* [15, 16]. Furthermore, PB as chemical inducer is characterized by lower risk of fungal resistance development due to its multiple modes of action, lower cost and shorter application to harvest interval, as well as its potential role to reduce the environmental impact of synthetic fungicides [9]. Chitosan (CH) is a biopolymer, a chitin derivative, a compound that is eco-friendly and characterized by unique properties such as bioactivity and biocompatibility [17]. Several studies reported that CH could increase the yield, reduce transpiration, and induce more resistance to viral, bacterial, and fungal infections [18-20]. Regards to biochemical and physiological processes, CH can stimulate vital mechanism of plants at the single cells, tissues and molecular changes based on expression of genes [21-23].

Some of the unique properties of CH may specifically stimulate plant reactions and inhibit the microorganisms. Moreover, one of the most important factors affecting the biological activity of this biopolymer is the molecular weight [24-28]. In recent years, the importance of CH as plant growth promoter and pathogen control agent has been evaluated [29, 30]. CH as a biopolymer can stimulate the immune system in plant to pathogen infection resistance [31]. Faoro et al. [32] showed that the sprayed plants with CH recorded a decrement of systemically infection caused by powdery mildew pathogen *Blumeria graminis* f. sp. *hordei*. Enhancement levels of defense related antioxidant enzymes [such as protease inhibitors, β-1,3-glucanases, peroxidases (PODs), polyphenol oxidases (PPOs)] was demonstrated in leaves and also in some rhizomes grown under field condition and treated with CH as natural inducer [33].

Research works of certain plant resistance inducers (PRI) on growth, yield attributes, and fruit yield of okra is almost rare. Considering the above facts, our work was conducted to study the effect of CH and potassium salts, PS and PB as PRIs for inducing systemic protection in okra plants infected with powdery mildew and for improving the vegetative growth as well as final yield. Some biochemical mechanisms and antioxidant responses were also investigated.

**MATERIALS AND METHODS**

**Procurement of culture and seed.** The fungal pathogen inoculum was isolated from naturally infected okra plants, Okra seeds (cv. Baladi), obtained from Department of Vegetable Crop Research, Agricultural Research Centre, Giza, Egypt.

**Pathological studies.**

**Greenhouse experiments:** Okra seeds were sown in plastic pots (25-cm-diam.) containing loamy soil, four seeds/pot. Ten pots were used for each treatment. Irrigation were added as needed. The efficiency of PRI such as CH, PS, and PB in controlling okra powdery mildew were evaluated under artificial inoculation with conidia of *Erysiphe cichoracearum* for plants implanted in greenhouse conditions.

**Preparation of *E. cichoracearum* inoculum and pathogenicity:** The powdery mildew fungal inoculum was obtained from freshly infected leaves of naturally infected okra plants. Conidia were gently brushed into 100-mL distilled water with 5 mL of Tween-20 then counted by haemocytometer to give a mixture of $5 \times 10^{3}$ conidia/mL. For plant inoculation, the upper surfaces of all the leaves were sprayed with a conidial suspension delivered by a hand sprayer [34].

The following treatments of PRI by CH at concentrations of 0.25%, 0.5%, and 0.75% (w/v), PS at 0.5%, 1.0%, and 2% (w/v), and PB at 1%, 2%, and 3% (w/v) were applied alone and in combined treatments for evaluate their efficacy against powdery mildew. Plant inoculation was carried out by dusting of okra leaves with conidia of *E. cichoracearum*. All tested PRIs were applied as twice sprays on the okra plants four days after pathogenicity. Inoculated plants were covered with polyethylene bags overnight. Control plants were sprayed with distilled water under similar conditions. Observations were recorded on the development symptoms daily for a period of 15 days after inoculation.

**Disease assessment.** Powdery mildew disease severity was estimated into 5 categories according to Descalzo et al. [35] based on the following scale: 0 = no visual infection, 1 = 1–5% infections, 2 = 6–25%, 3 = 26–50%, and 4 = more than 50% of leaf area covered by fungal colonies. Final disease assessment was conducted at 11 days after each spray in each treatment.

Conidia production of *E. cichoracearum* on treated okra leaves were also evaluated. Leaves were detached gently at the early morning and immersed in screw cap jars containing 100 mL of distilled water. Conidia were released from lesions using a brush, and then counted using a haemocytometer. Fungal conidia/cm$^2$ of leaf area were counted in each treatment.

**Field experiments.** Based on data obtained from greenhouse experiments, the most effective treatments of the tested PRI were chosen and applied under field conditions to evaluate their efficiency in control of powdery mildew disease, and evaluate their effects on vegetative growth and yield parameters of okra. Fungicide, Tri-Miltox forte (2.5 g/L) was used as comparison treatment. Field experiment was carried out under condition of El-Qanater, El-Khairiya, Qualubiya Governorate, Egypt. Field experiments consisted of 36 plots designed in a randomly complete block design with four replicated plots for each particular treatment. Okra seeds (cv. Baladi) were planted in all plots.

The following treatments (T1, CH 0.75%; T2, PS 2.0%; T3, PB 3.0%; T4, CH 0.5% + PS 1.0%; T5, CH 0.75% + PS 2.0%; T6, CH 0.5% + PB 2.0%; T7, CH 0.75% + PB 3.0%; T8, Tri-Miltox forte 2.5 g/L; and T9, control) were applied as twice foliar spray at 7–8 leaf growth stage during 15 days intervals.
In addition, Tri-Miltox forte (2.5 g/L) was sprayed twice every 15 days started after 20 days of sowing date. Application with tap water was used for control. Assessment of powdery mildew disease severity was carried as mentioned before. Vegetative growth and yield parameters plant height (cm), number of branches per plant, pod length (cm), average weight of single pod (g), and total fresh yield of pods (ton/ fed) were estimated in 20 plants and 25 pod samples taken randomly from each treatments as well as control.

Biochemical studies. Determination of the activity of different enzymes responsible for diseases resistance in treated okra plant with resistance inducers grown under greenhouses was carried out. Protein content, catalase activity, total phenolic, the activity of POD, PPO, chitinase, and β-1,3-glucanase enzymes was estimated in sprayed and control plants as follows.

Leaves plant samples for biochemical analysis were taken after the second spray of each treatment. Extraction from okra leaves were prepared as follows: A representative samples, 10 g of each, were cut into small portions and immediately plunged into 95% boiling ethanol for ten minutes to kill the tissues. The extraction was resumed in a soxhlet apparatus by using 75% ethanol as an extractant until the percolate was colorless (8–10 hr). The combined ethanolic extracts were filtered and evaporated to near dryness on a mild water bath, 60°C. The dried residue was re-dissolved in 10 mL of 50% iso-propanol and used for chemical analysis as follows:

Estimation of total phenolic content. Total phenolic content in okra leaves of each treatment was determined according the methods described by Descalzo et al. [35] as follows: Okra leaves was immersed in liquid nitrogen, homogenized in 80% methanol (1 g plant material in 10 mL) and stored at ~20°C. Later, the homogenate was centrifuged at 15,000 xg for 30 min at 4°C. The pellet was discarded. After addition of ascorbic acid (0.1 g/5 mL), the homogenate was evaporated in rotary evaporator at 65°C. The dried residue was extracted in 5 mL of 80% methanol. For the determination, 0.02 mL methanol extract was incubated for 1 hr with 0.5 mL Folin-Ciocalteu reagent, 0.75 mL of Na2CO3 solution (20%) and 8-mL water. Total phenolic content was assayed spectrophotometrically at 767 nm. Gallic acid (0–5 mg) and methanol were used as a standard and blank, respectively. Total phenolic were estimated as gallic acid/g (mg) plant material. Activity of all enzymes was determined spectrophotometric as unit/g fresh weight (fw) by measuring the change in absorbance at different weave lengths refers to enzymes using Shimadzu UV-2401 PC UV-Vs recording spectrophotometer (Molecular Biology Lab., National Research Center NRC, Egypt).

Determination of POD activity. POD is routinely assayed by measuring the change in absorbance at 470 nm due to guaiacol oxidation in the presence of hydrogen peroxide and the enzyme was assayed every 30-sec intervals using Shimadzu UV-2401 PC UV-V is recording spectrophotometer in a 1-mL light path cuvettes. The reaction mixture (unless otherwise stated) contained in a volume of 3 mL, 8 μmoles hydrogen peroxide, 60 μmoles guaiacol, 50 μmoles sodium acetate buffer, pH 5.6 and POD at concentrations which gave a linear response over a period of 3 min. The reaction was initiated by introducing the enzyme and mixing, all assays were carried out at 25°C. A unit of POD activity was defined by monitoring the changes in the optical density (OD) values per minute due to amount of enzyme [36].

Determination of PPO activity. PPO activity was determined by measuring the initial rate of quinine formation, as indicated by an increase in absorbance at 420 nm [37]. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.001/min. The sample cuvette contained 2.95 mL of 20 mM catechol solution in 0.1 M phosphate buffer, pH 6.0 and 0.05 mL of the enzyme solution. The blank sample contained only 3 mL of substrate solution. Phenylalanine ammonialyase activity was determined according to Coseteng and Lee [38]. Two hundred microliters of sample crude extract was transferred to a 3-mL light path cuvettes containing 2.5 mL of 0.03 M L-phenylalanine dissolved in 0.05 M sodium borate buffer, pH 8.8. The reaction mixture was then incubated in water bath at 37°C for 1 hr, after cooling the unit of phenylalanine, ammonialyase activity was calculated as one OD change per minute at 290 nm.

Determination of chitinase activity. The chitinase activity was determined by colorimetric method [39] by using colloidal chitin as a substrate and dinitrosalicylic acid as a reagent to measure reducing sugars. The colloidal chitin substrate was prepared from chitin powder as the method described by Boller and Mauch [40], by milting 25 g of chitin and then suspended in 250 mL of 85% phosphoric acid and kept at 4°C for 24 hr. The mixture was blended in 2 L of distilled water and then centrifuged at 2,500 xg for 20 min. This washing step repeated twice and the colloidal chitin suspension in the final wash was adjusted to pH 7.0 and collected by centrifugation and stored at 4°C. Chitinase activity was assayed in test tube containing the reaction mixture consists of 1 mL of 1% colloidal chitin in 50 mM sodium acetate buffer pH 6.6 and 8-mL water. The mixture was incubated for 1 hr in controlled water bath at 37°C and centrifuged 2,500 xg for 20 min. The concentration as mM of N-acetyl-glucose amine (NAGA) in the supernatant was determined using absorbance at 540 nm and the unit of chitinase activity was calculated by the expressed NAGA equivalent released/g fresh weight tissue/60 min.

Determination of β-1,3-glucanase activity. The enzyme solution (100 μL) was mixed with 200 μL of 0.2% (w/v)
laminarin (Sigma, St. Louis, MO, USA) dissolved in 0.1 M sodium phosphate buffer, pH 6.0 and incubated at 30°C for 30 min. The reaction was determined by adding di-nitro-salicylic acid solution and boiling the reaction mixture for 5 min. The absorbance at 540 nm was measured and the unit was defined as the amount of the enzyme that released reducing sugar equivalent to 1 μg glucose per minute under the above conditions [41].

**Determination of catalase and total soluble protein.**

Okra leaves were collected 10 days after inducers application and were excised, weighed (100 mg) and ground with a pestle in an ice cold mortar with 2 mL of 0.05 M Na2HPO4/NaH2PO4 (pH 7.0) buffer. The homogenate was centrifuged at 4°C for 20 min at 15,000 × g. The supernatant was used for the assay of catalase activity and total soluble proteins. Catalase activity was measured according to Sun et al. [42]. Enzyme extract [20] was added to 3 mL of HP2-PO4 buffer (0.16 mL of HP2 to 100 mL phosphate buffer, pH 7.0) and the breakdown of HP2 was measured at 240 nm in a spectrophotometer. An equivalent amount of buffer containing H2O2 was used as reference. The enzyme activity was expressed in enzyme units/mg protein where one enzyme unit was defined as a change of 0.01 absorbance min caused by the enzyme aliquot. Soluble proteins in the supernatant were estimated according to Sun et al. [42] and expressed as mg/g fresh weight.

**Statistical analysis.** Data of all measurable characters were subjected to statistical analysis according to Chance and Maehly [43]. Duncan’s multiple range test was used for means separation [44]. The statistical analysis procedures were kindly carried out by Statistical Consulting Office, National Research Centre, Egypt.

### RESULTS

**Pathological studies.**

Control of powdery mildew on okra plants in green house: Different concentrations of PRIs i.e., CH, PS, PB were applied as single or in combined treatments (CH + PS and CH + PB) for controlling powdery mildew on okra plants under artificially infection with *E. cichoracearum* under greenhouse conditions. Data in Table 1 clearly expressed that all treatments of inducers were highly effective to reduce the powdery mildew disease severity in inoculated plants compared with untreated control. Reduction in disease severity was increased by increasing PRIs concentration. The most significant treatments were CH at 0.5% or 0.75% (w/v) plus PS at 1.0% or 2.0%, PB at 2.0% or 3.0%, which reduced powdery mildew disease severity by 64.7–75.0%. Meanwhile, single treatments of CH, PS, or PB at high concentration (0.75%, 2.0%, and 3.0%) caused considerable disease reductions (57.3%, 55.8%, and 58.8%) respectively. The lowest effects against powdery mildew disease severity observed with low concentrations of PS and CH respectively.

Data represented in (Table 2) also showed that the same trend of results in (Table 1) as all treatments have significantly reduced conidial production (conidia/mm² of lesion caused by *E. cichoracearum*). CH + PS or PB are reported as the inhibitory treatments; these treatments reduce conidial production from 82.8% to 94.2%, meanwhile, single treatments of CH, PS, or PB caused reduction range from 50.2% to 88.2% after the second spray.

**Control of powdery mildew on okra plants under field conditions:** The promised effective treatments of PRIs in controlling powdery mildew under greenhouse experiments were conducted and applied for controlling powdery mildew

### Table 1. Disease severity in okra plants in response to spraying with different plant resistance inducers under artificially inoculation with *Erysiphe cichoracearum* in green house

| Plant resistance inducer | Concentration (%) | 1st spray | 2nd spray | Mean | R (%) |
|--------------------------|-------------------|-----------|-----------|------|------|
| CH                       | 0.25              | 2.3b      | 32.2      | 1.9b | 41.4 | 2.10 |
| CH                       | 0.5               | 1.9b      | 42.2      | 1.6b | 52.8 | 1.75 |
| CH                       | 0.75              | 1.7b      | 47.8      | 1.2b | 64.4 | 1.45 |
| PS                       | 0.5               | 3.2a      | 30.2      | 1.8b | 46.4 | 2.50 |
| PS                       | 1.0               | 2.1b      | 38.4      | 1.6b | 52.8 | 1.85 |
| PS                       | 2.0               | 1.7b      | 48.2      | 1.3b | 60.6 | 1.50 |
| PB                       | 1.0               | 1.9b      | 42.4      | 1.6b | 52.8 | 1.75 |
| PB                       | 2.0               | 1.6b      | 51.8      | 1.5b | 55.8 | 1.55 |
| PB                       | 3.0               | 1.6b      | 52.8      | 1.2b | 64.2 | 1.40 |
| CH + PS                  | 0.5 + 1.0         | 1.5b      | 55.2      | 0.9bc | 72.2 | 1.20 |
| CH + PS                  | 0.75 + 2.0        | 1.3bc     | 60.2      | 0.8bc | 75.0 | 1.05 |
| CH + PB                  | 0.5 + 2.0         | 1.2bc     | 62.2      | 0.5bc | 84.4 | 0.85 |
| Control                  |                   | 3.4a      | 0.0       | 3.4a | 0.0  | 3.40 |

Values with the same letters in each column are not significantly differed (p ≤ 0.05). R, Reduction percent; CH, chitosan; PS, potassium sorbate; PB, potassium bicarbonates.
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disease on okra plants under field conditions during 2016. The effects of such treatments on vegetative growth and yield of okra plants were also investigated and comparative treatment was by fungicide, Tri-Miltox forte (2.5 g/L).

Table 3 indicated that all PRIs treatments have significantly reduced powdery mildew on treated plants compared with control. Integrative treatments of CH and PS or PB were the most effective treatments which reduce powdery mildew by 42.8–68.7% and 68.7–82.8% after the first and second spray respectively and the mean reduction after the two sprays was 64.2–76.6%. Meanwhile, single treatments of CH, PS, and PB showed considerable control of powdery mildew after the second spray, as the disease severity reduced by 57.2%, 60.9%, and 72.1%, respectively. PS 2.0% and CH 0.75% showed the lowest effect against powdery mildew (38.1% and 42.8%) after the first spray.

The efficacy of applied PRIs treatments on some vegetative growth and yield parameters of okra plants were also recorded. Table 4 clearly showed that all PRIs treatments improved both vegetative growth (plant height, number of branches) and yield parameters (number of pods/plant, pod length, pod weight, yield/ton fed) of treated okra plants compared with untreated control. The highest records of vegetative growth and yield parameter were observed with combined treatments of CH and PB. The highest effective treatment in okra yield/fed was CH + PB at 0.75% + 3.0% followed by concentration CH + PB at 0.5% + 2.0% and Tri-Miltox forte (2.5 g/L). They increased okra plants by 64.3%, 60.7%, and 60.7%, respectively. CH + PS at 0.5% + 1.0% or 0.75% + 2.0% and PB at 3.0% treatments show concealable yield increasing (53.6%, 57.1%, and 57.1%, respectively). Meanwhile, the least records of increase in okra yield were with the PS and CH treatments.

Biochemical studies. Effects of different concentrations of PS, PB, and CH separately and in combination as PRIs

Table 2. Reduction of produced conidia of Erysiphe cichoracearum in okra leaves in response to different plant resistance inducers under artificially inoculation in green house

| Plant resistance inducer | Concentration (%) | Reduction (%) of conidia/mm² of lesion |
|--------------------------|------------------|----------------------------------------|
|                          |                  | One spray | Two sprays | Mean |
| CH                       | 0.25             | 40.0      | 50.2       | 45.1 |
|                          | 0.5              | 55.4      | 70.4       | 62.9 |
|                          | 0.75             | 60.2      | 77.8       | 69.0 |
| PS                       | 0.5              | 44.2      | 64.4       | 54.3 |
|                          | 1.0              | 50.0      | 71.2       | 60.6 |
|                          | 2.0              | 57.2      | 77.2       | 67.2 |
| PB                       | 1.0              | 55.4      | 70.8       | 63.1 |
|                          | 2.0              | 60.2      | 76.4       | 68.3 |
|                          | 3.0              | 74.2      | 88.2       | 81.2 |
| CH + PS                  | 0.5 + 1.0        | 70.8      | 82.8       | 76.8 |
|                          | 0.75 + 2.0       | 75.0      | 91.0       | 85.5 |
| CH + PB                  | 0.5 + 2.0        | 72.2      | 90.0       | 81.1 |
|                          | 0.75 + 3.0       | 80.4      | 94.2       | 87.3 |
| Control                  |                  | 0.0       | 0.0        | 0.0 |

CH, chitosan; PS, potassium sorbate; PB, potassium bicarbonates.

Table 3. Disease severity of powdery mildew in okra leaves as affected by different treatments of plant resistance inducers under field conditions during 2016 season

| Plant resistance inducer | Concentration (%) | Powdery mildew disease severity |
|--------------------------|------------------|--------------------------------|
|                          |                  | 1st spray | R (%)  | 2nd spray | R (%)  | Mean   | R (%) |
| CH                       | 0.75             | 1.20b     | 42.8   | 1.37b     | 57.2   | 1.28   | 51.6  |
| PS                       | 2.0              | 1.29b     | 38.1   | 1.25b     | 60.9   | 1.27   | 52.1  |
| PB                       | 3.0              | 0.90c     | 57.1   | 0.89c     | 77.2   | 0.89   | 66.4  |
| CH + PS                  | 0.5 + 1.0        | 0.9bc     | 42.8   | 1.00c     | 68.7   | 0.95   | 64.2  |
|                          | 0.75 + 2.0       | 1.00c     | 52.3   | 0.88c     | 78.5   | 0.94   | 64.5  |
| CH + PB                  | 0.5 + 2.0        | 0.80c     | 61.9   | 0.83c     | 74.0   | 0.81   | 69.4  |
|                          | 0.75 + 3.0       | 0.66cd    | 68.7   | 0.55d     | 82.8   | 0.62   | 76.6  |
| Tri-Miltox forte (2.5 g/L)| 0.76cd         | 63.8      | 0.60d  | 73.2      | 0.63   | 76.3   |
| Control                  |                  | 2.1a      | 0.0    | 3.2a      | 0.0    | 2.65   | 0.0   |

Values with the same letters in each column are not significantly differed (p ≤ 0.05).
R, reduction percent; CH, chitosan; PS, potassium sorbate; PB, potassium bicarbonates.
on activity of antioxidant compounds (such as phenolic) as well as antioxidant enzymes in okra leaves during growth of okra plants under artificially inoculation with powdery mildew caused by *E. cichoracearum* were investigated.

**Total phenolic content:** Results in Table 5 reported that the total phenolic content (non-enzymatic antioxidant defense systems) was increased with 96.6% over control when treated with mixed chemical inducers CH + PB at 1.0% + 3.0% concentrations. Measuring phenolic content as mg per 100 g leaves showed gradual increase in amounts estimated in a similar trend by increasing concentration of treatments in individual manner for CH, PS, and PB, as data clear that phenolic content was (0.632, 0.623, and 0.688 mg/100 g leaves), respectively. On the other hand, the maximum values were 0.788 mg/100 g leaves and 0.810 mg/100 g leaves, obtained in okra leaves when sprayed with combined treatments, CH + PS at 0.75% + 2.0% and CH + PB at 1.0% + 3.0% mixed concentrations, in respective order.

**PPO activity:** Regards to the effect of the plant induce resistance agents on PPO activity, all different inducers produced an increasing in PPO in all inducers with different levels of treatments (Table 5). Data indicated the effects of CH + PB and CH + PS treatments on PPO were PPO was increased up to (3.05 and 2.90 unit/mL) in comparison with control (1.62 unit/mL). On the other hand, the lowest increase in PPO activities were induced by applied treatment (CH, PS, and PB) when data recorded (2.10, 2.32, and 2.44 unit/mL). The highest level of PPO activity was determined in treatment (CH + PB) at the concentrations (1.0% + 3.0%), at higher concentrations for combined inducers showed enhanced activities of PPO in comparison with other tested treatments. In contrast, the lowest activity of PPO as percentage change was represented at CH treatment (29.6%).

### Table 4. Vegetative growth and yield parameters of okra plants as affected by different treatments of plant resistance inducers under field conditions during 2016 season

| Plant resistance inducer | Concentration (%) | Vegetative growth | Yield attributes |
|--------------------------|-------------------|-------------------|-----------------|
|                          |                   | Plant height (cm) | No. of branches per plant | No. of pods per plant | Pod length (cm) | Pod weight (gm) | Yield per ton fed | Increasing (%) |
| CH                       | 0.75              | 104b              | 3.2b             | 26b              | 8.0c           | 13.2b           | 4.1c             | 46.4          |
| PS                       | 2.0               | 92a               | 2.2a             | 22a              | 7.2b           | 13.4b           | 3.1b             | 39.3          |
| PB                       | 3.0               | 112b              | 4.1c             | 23a              | 6.4a           | 14.0c           | 4.4c             | 57.1          |
| CH + PS                  | 0.75 + 1.0        | 100b              | 3.4b             | 25b              | 7.2b           | 13.2b           | 4.3c             | 53.6          |
|                          | 0.75 + 2.0        | 104b              | 3.2b             | 22a              | 9.0c           | 14.2c           | 4.4c             | 57.1          |
| CH + PB                  | 0.5 + 2.0         | 108b              | 3.8b             | 24b              | 9.4c           | 14.8c           | 4.5cd            | 60.7          |
|                          | 0.75 + 3.0        | 110b              | 4.0c             | 27b              | 8.8c           | 14.4            | 4.6cd            | 64.3          |
| Tri-Miltox forte (2.5 g/L)|                  | 105b              | 3.2b             | 24               | 9.2c           | 14.0c           | 4.5d             | 60.7          |
| Control                  |                   | 85a               | 2.1a             | 21a              | 6.4a           | 12.2a           | 2.8a             | 0.0           |

Values with the same letters in each column are not significantly differed (*p* ≤ 0.05).

CH, chitosan; PS, potassium sorbate; PB, potassium bicarbonates.

### Table 5. Accumulation of phenolic, activity of PPO and POD enzymes in okra leaves in response to spraying with different plant resistance inducers under artificially inoculation with *Erysiphe cichoracearum* in green house condition

| Plant resistance inducer | Concentration (%) | Phenolic | PPO | POD |
|--------------------------|-------------------|---------|-----|-----|
|                          |                   | mg/100 g leaves | PPO activity | Change (%) | POD activity | Change (%) |
| CH                       | 0.25              | 0.511bc | 24.0 | 1.79b | 10.4 | 1.3b | 18.1 |
|                          | 0.75              | 0.614c | 49.0 | 1.88b | 16.0 | 1.4b | 27.2 |
|                          | 1.0               | 0.632c | 53.3 | 2.10c | 29.6 | 1.8c | 63.6 |
| PS                       | 0.75              | 0.492b | 19.4 | 1.74b | 7.4  | 1.2b | 9.0  |
|                          | 1.0               | 0.612c | 48.5 | 1.98c | 22.2 | 1.4b | 32.4 |
|                          | 2.0               | 0.623c | 51.2 | 2.32d | 43.2 | 1.6b | 45.4 |
| PB                       | 1.0               | 0.592c | 43.6 | 1.95c | 20.3 | 1.5b | 36.3 |
|                          | 2.0               | 0.638c | 54.8 | 2.28d | 40.7 | 1.8c | 63.6 |
|                          | 3.0               | 0.688cd | 66.9 | 2.44d | 50.6 | 2.0c | 81.8 |
| CH + PS                  | 0.75 + 1.0        | 0.740d | 79.6 | 2.60d | 60.4 | 1.9c | 72.7 |
|                          | 0.75 + 2.0        | 0.788d | 91.2 | 2.90c | 79.0 | 2.1c | 90.9 |
| CH + PB                  | 0.75 + 2.0        | 0.772d | 87.3 | 2.78d | 71.6 | 2.0c | 81.8 |
|                          | 1.0 + 3.0         | 0.810d | 96.6 | 3.05e | 88.2 | 2.1c | 90.9 |
| Control (inoculated and untreated) | 0.412b | 0.00 | 0.00 | 1.62b | 0.00 | 1.1b | 0.00 |
| Control (noninoculated and untreated) | 0.323a | 0.00 | 0.00 | 1.40a | 0.00 | 0.8a | 0.00 |

Values with the same letters in each column are not significantly differed (*p* ≤ 0.05).
POD activity. POD activity was affected also by treatments; data in Table 5 showed maximum increasing in POD activity as percentage change (90.9%) at both treatments of CH + PS and CH + PB, when 0.75% + 2.0% and 1.0% + 3.0% combined concentration are applied. Moreover, it was obvious that, POD activity recorded the same results when okra leaves sprayed with CH + PS and CH + PB, as results showed (2.1 for both treatments). Data referred to effect of CH, PS, and PB treatments on POD activity indicated that POD activity was increased up to (1.8, 1.6, and 2.0 unit/mL). Results obtained in Table 5, it could be concluded that, individual treatments showed more pronounced enhancement for POD activity. In contrast, combined treatments have showed increasing in POD activity but with non-significant difference between them.

Illustrated data by Table 5 represented that PRI in respective order with applied treatments CH, PS, and PB as individual treatments and in gradual increase in concentration, showed an increment in both non-enzymatic and enzymatic antioxidant defense systems in okra leaves. The obtained results showed high defense when mixed chemical inducers applied as foliar spray against powdery mildews of okra plants.

Effect on chitinase and β-1,3-glucanase activity. Chitinase activity was affected by treatments as the data presented in Table 6 revealed that the different chemical inducers induced different levels of chitinase activities in okra leaves. The highest level was 106.6% and 89.1% as percentage change over control of chitinase activity by applied treatment (CH + PB and CH + PS), respectively. Moreover, the lowest chitinase activities were 43.3% over control (CH + PB and CH + PS), respectively.

CH + PB treatment and 106.6% for chitinase activity. Meanwhile, when the treatments used individually showed a higher significant increment in chitinase activity than β-1,3-glucanase activity for (CH and PS) treatments as results recorded (46.6% and 43.3% over control) and CH + 1,3-glucanase activity which recorded (41.8% and 37.5%). Opposite results obtained in okra leaves treated with PB indicated that, activity of β-1,3-glucanase increased with increasing the concentrations of chemical inducers (CH, PS, and PB) as single treatments. More increment was recorded at CH + PB (1.0% + 3.0%) and CH + PS (0.75% + 2.0%) treatments were 90.0% and 80.0%, respectively. On the other hand, lower value of β-1,3-glucanase activity (37.5% change over control) was observed in PS as single treatment. Whereas, the mixed lower concentrations of CH + PS (0.5% + 1.0%) and CH + PB (0.75% + 2.0%), indicated a similar or nearly results (63.7% and 68.7%), respectively.

Data in Table 6 revealed that there was noticeably difference in antioxidant enzymes activities among the employed chemical inducer treatments in okra leaves. Meanwhile, when the treatments used individually showed a higher significant increment in chitinase activity than β-1,3-glucanase activity for (CH and PS) treatments as results recorded (46.6% and 43.3% over control) and β-1,3-glucanase activity which recorded (41.8% and 37.5%). The best significant increase was obtained at (1.0% + 3.0%) for CH + PB treatment and 106.6% for chitinase activity.

**Table 6.** Chitinase and β-1,3-glucanase enzymes activity in okra leaves in response to spraying with different plant resistance inducers under artificially inoculation with Erysiphe cichoracearum in green house condition

| Plant resistance inducer | Concentration (%) | Chitinase Activity (mL) | Change (%) | β-1,3-Glucanase Activity (mL) | Change (%) |
|--------------------------|------------------|------------------------|-----------|-------------------------------|-----------|
| CH                       | 0.25             | 1.42d                  | 18.3      | 1.80b                         | 12.5      |
|                          | 0.5              | 1.57dc                 | 30.8      | 2.10c                         | 31.2      |
|                          | 0.75             | 1.76c                  | 46.6      | 2.27c                         | 41.8      |
| PS                       | 0.5              | 1.32d                  | 10.0      | 1.72b                         | 7.5       |
|                          | 1.0              | 1.56d                  | 30.0      | 2.02c                         | 26.2      |
|                          | 2.0              | 1.72c                  | 43.3      | 2.20c                         | 37.5      |
| PB                       | 1.0              | 1.62c                  | 35.0      | 2.08c                         | 30.0      |
|                          | 2.0              | 1.86c                  | 55.0      | 2.35c                         | 46.8      |
|                          | 3.0              | 1.94c                  | 61.6      | 2.56c                         | 65.6      |
| CH + PS                  | 0.5 + 1.0        | 2.01b                  | 67.5      | 2.62d                         | 63.7      |
| CH + PB                  | 0.75 + 2.0       | 2.27b                  | 89.1      | 2.88d                         | 80.0      |
|                          | 1.0 + 3.0        | 2.48b                  | 106.6     | 3.04e                         | 90.0      |
| Control (inoculated and untreated) | 1.20 | 0.0 | 1.60b | 0.0 |
| Control (noninoculated and untreated) | 2.30a | 0.0 | 2.10a | 0.0 |

Values with the same letters in each column are not significantly differed (p ≤ 0.05).

CH, chitosan; PS, potassium sorbate; PB, potassium bicarbonates.
okra leaves, maximum catalase activity was (457.3, 425.9, and 405.1 U/mg protein) at (0.5% PS, 0.25% CH, and 1.0% PB) concentrations as individual inducer, respectively (Table 7). Meanwhile, minimum value in catalase (175.3 U/mg protein) was mentioned at 1.0% + 3.0% of CH + PB treatment and then decreased over control, which recorded (220.4 U/mg protein). Moreover, data showed that catalase activity was (522.0 U/mg protein) for control, plants (inoculated and untreated). Recorded results revealed that, lower concentrations of different individual chemical inducers (CH, PS, and PB), caused significant increase in catalase activity. In contrast, treatments in combination at higher concentrations produced a significant decrease in comparison with individual treatments. The highest percentage change for catalase activity (66.4%) was recorded in CH + PB treatment, while PS treatment showed the lowest catalase activity as percentage change (46.4%).

**Protein content:** Our results clearly revealed that, foliar spraying of bio-stimulants "chitosan" and other chemical inducers significantly had influence on protein content accumulation in okra plant under artificial infection with powdery mildew. Results in Table 7 mentioned that by gradual increase in different concentrations of chemical inducer, there are increments in protein content either for treatments used individually or in mixtures. The highest value of protein content was (341.4 mg/g fw) at CH + PB (1.0% + 3.0%) mixed concentration compared with control plants (inoculated or non-inoculated) which recorded (225 and 276 mg/g fw), respectively.

Furthermore, the increase in PS concentration led to decreasing of protein content and rise in catalase activities in okra leaves (Table 7). PS treatment indicated the minimum increasing in protein content where recorded (302.2 mg/g fw) and percentage change (34.0%) in comparison with other treatments. The extent of increases under combined effects of CH + PB and CH + PS were 51.2% and 46.3%, respectively. In different way, the highest catalase activity (457.3 U/mg protein) was observed under PS treatments condition when used in a single treatment, whereas, the lowest catalase activity (175.3 U/mg protein) was recorded under combined treatments condition (CH + PB). The application of tested chemical inducer treatments induced significant stimulation effect on protein content in okra plant under artificial infection with powdery mildew. Results in Table 7 showed that by gradual increase in different concentrations of chemical inducer, there was increment in protein content either for treatments used as individually or as mixtures. The highest value for protein content was (341.4 mg/g fw) with CH + PB (1.0% + 3.0%) mixed concentration compared with control plants (inoculated or non-inoculated) which recorded (225 and 276 mg/g fw), respectively.

**DISCUSSION**

There is a worldwide trend to explore new alternatives approaches for controlling plant diseases, giving priority to methods that are efficient reliable and safe for environment. Induce of host resistance by using PRI such as CH and other safe chemicals such as potassium salts seems to be one of these new approaches to substitute or at least to decrease the usages of fungicides in plant disease control. In the present study, our data indicate that using CH as individually or mixed with potassium salts successfully controlled powdery mildew disease in okra plants either with *E. cichoracearum* under greenhouse condition or naturally infection under field condition. We use different levels of chemical inducer treatments as potassium salt (sorbate and bicarbonate) tested and as well as CH. The

### Table 7. Catalase activity and protein content in okra leaves in response to spraying with different plant resistance inducers under artificially inoculation with *Erysiphe cichoracearum* in greenhouse condition

| Plant resistance inducer | Concentration (%) | Catalase activity | Protein content |
|---------------------------|-------------------|-------------------|-----------------|
|                           | Unit/mg protein   | Change (%)        | mg/g fresh weight | Change (%) |
| CH                        | 0.25              | 425.9b            | 18.4            | 253.3b     | 12.2       |
|                           | 0.5               | 345.5c            | 33.8            | 277.2b     | 22.8       |
|                           | 0.75              | 248.5d            | 52.4            | 318.8c     | 41.2       |
| PS                        | 0.5               | 457.3b            | 12.4            | 246.5b     | 9.2        |
|                           | 1.0               | 374.7c            | 28.2            | 270.0b     | 19.6       |
|                           | 2.0               | 279.7d            | 46.4            | 302.2c     | 34.0       |
| PB                        | 1.0               | 405.1b            | 22.4            | 265.0b     | 17.4       |
|                           | 2.0               | 254.7d            | 51.2            | 319.7c     | 41.6       |
|                           | 3.0               | 233.8d            | 55.2            | 323.3c     | 43.2       |
| CH + PS                   | 0.5 + 1.0         | 228.6d            | 56.4            | 316.7c     | 40.3       |
|                           | 0.75 + 2.0        | 202.5d            | 61.2            | 330.3c     | 46.3       |
|                           | 1.0 + 3.0         | 175.3c            | 66.4            | 341.4d     | 51.2       |
| Control (inoculated and untreated) | 522.0a          | 0.0              | 225.8a         | 0.0        |
| Control (noninoculated and untreated) | 220.4d          | 0.0              | 276.0b         | 0.0        |

The specific activity of catalase activity was expressed in terms of unit of H2O2 decomposed per min per mg of protein.

CH, chitosan; PS, potassium sorbate; PB, potassium bicarbonates.
required concentration to control pathogen was differed from each other, either when used individually or in combination. Data cleared that, the application of CH combined with potassium bicarbonate and PS offers a commercially acceptable, economically viable, and effective alternative to control of powdery mildew in okra leaves.

Spraying okra plants after inoculation with *E. cichoracearum* by CH at 0.75% (w/v) reduce powdery mildew severity by 64.4%, and spore production by 77.8%. Moreover, mixed treatments such as CH + PB lead to increasing in phenolic content (53.3% to 96.6%), PPO (29.6% to 88.2%), POD (63.6% to 90.9%), chitinase (46.6% to 106.6%), β-1,3-glucanases (41.8% to 90.0%), catalase (52.4% to 66.4%), and protein content (41.2% to 51.2%) in leaves of treated plants. These results are in consistent with other studies demonstrating that suppression of microbial strains varies in response to different organic and inorganic salts. In addition, results indicated that the degree of fungal inhibition was concentration dependent and the maximum value for decrement in mycelia growth was positively correlated with the concentration of salt tested [10, 45]. Our results indicated that application of CH as foliar spray method significantly reduced the severity of powdery mildew disease in okra leaves where increased by 20% in yield and an enhanced improvement in controlling powdery mildew disease of tomatoes when applied with CH and significant reduction in local and systemic infection caused by *Blumeria graminis f. sp. hordei*, respectively [32, 46].

Physiologically CH is an effective elicitor of systemic acquired resistance, improved controlling pathogens, as well as stimulate the immune system for plant resistance to broad infection [31, 47]. Barley plants infected with *Blumeria graminis f. sp. hordei* showed a reduced locally and systemically infection by powdery mildew pathogen when sprayed with CH [32]. Meanwhile, activation of protective mechanisms in plant tissues and inhibition of the growth of taxonomically different pathogens can be recorded in previous studies [48]. Moreover, physical barriers are formed around the penetration sites of pathogens, preventing them from infecting other healthy tissues. These bioactive derivatives can activate H−ATPases, depolarizing biological membranes and inducing other series of events. In addition, on the molecular changes, some defensive genes can be activated by CH through octadecanoid signaling pathways and oligouronides [49]. In the same trend, in bacterial cells permeability of the outer and inner membrane was increased and ultimately causing release of cellular contents [50].

Treatments with different concentrations of potassium salts and CH as antimicrobial compound showed an elevation in PPO and POD activity as compared to untreated plants during the present work. Same trend of results obtained with [49] who mentioned that CH can induce elicitation of the POD and PPO activity in palm roots. Protease inhibitors, β-1,3-glucanases, POD, PPO as an antioxidant defense enzymes were elevated in leaves and also in rhizomes in plants grown in field condition and treated with CH [33]. Chitinases and β-1,3-glucanase are members of a group of plant pathogenesis-related (PR) proteins showed a close relationship with resistance against pathogens that can be induced by CH [51-53]. These PR proteins may play a role in host plant defense systems by degrading the cell walls of some phytopathogens [54-56]. High-level expression of defense-related proteins such as POD and PPO in CH treated plants might involve in the formation of lignin to control the entry and movement of fungal pathogens in the plant system. Antioxidant enzyme activity as (POD and PPO) can be increased and associated with decreases in disease severity involved in plant pathogenic fungi, bacteria and viruses in a number of resistant interactions [57-60]. Seed germination, yield enhancement was stimulated in many crop species such as faba bean under effect of CH [61], cucumber [62], corn [63], green bean [64], barley [32], and curcumin [33].

The application of CH as elicitors with chemical inducers may be considered as a promising tool in production of biologically active phytoalexins and other secondary metabolites such as phenolic that have a structural function in lignification and an antibiotic activity [65, 66]. In this study, the CH mixed with PB and PS treatments increased the activity of phenolic in infected okra leaves. These results indicate that the response mechanisms induced by combined CH treatments are positive in controlling disease attack. In lines with the present results, caffeoylshimick acids (sinapic, p-coumaric, and feluric derivatives), were reported as the major phenolic constitutive compounds in date palm roots as influence of CH biopolymer [51]. Phenolic concentration increment considered as a suitable substrate for the oxidative reactions catalyzed by PPO that produce fungitoxic polymerisation of quinones, consuming oxygen and so creating an incompatible environment for fungal growth [67, 68]. Both PPO and POD could generate reactive oxygen species molecules such as hydrogen peroxide (H₂O₂); that inhibit pathogens directly or generate other free radicals with antimicrobial effects; which can scavenged with POD [69-71]. Catalase activity (CAT), which catalyses conversion of hydrogen peroxide into water and oxygen, is the major H₂O₂-scavenging enzyme in all aerobic organisms. Results reported in the present work, suggested that CAT activities was stimulated in production over control.

Conversely, our results indicated that, sprayed inoculated okra plants with PS (2.0%) and/or PB (3.0%) twice applications lead to decreasing of powdery mildew severity by 60.6% and 64.2%, spore production by the pathogen by 77.2% and 88.2%. Moreover, such treatments enhancing defense resistance proteins and increasing in phenol continent by (51.2% and 66.9%) and enhancement of PPO (43.2% and 50.6%), POD (45.4% and 81.8%), chitinase (43.3% and 61.6%), β-1,3-glucanases (37.5% and 65.6%), catalase (46.4% and 55.2%), and protein content (34.0% and 43.2%) in leaves of treated plants.

In addition to inhibiting effects of CH, other potassium salts showed effects on growth inhibition of powdery
mildew in okra leaves. Many investigators [72] have tested the efficacy of various chemical inducers of systemic resistance against powdery mildew disease. Pepper plants showed inhibition of powdery mildew severity as influenced by foliage sprays of sodium bicarbonate or PB. These salts were registered as pesticide ingredients and horticultural fungicide in the United States and United Kingdom [6, 7]. Physiological mechanism of these inhibiting effects may be due to changes in pH medium, which became more alkaline as bicarbonate concentration increased. Besides changes to pH that affect fungal growth, bicarbonates may also affect membrane permeability and alter physiological pathways such as oxidative phosphorylation [10]. In our study, the best controlling effects for pathogen was obtained at elevated concentrations of CH + PB at (1.0% + 3.0%) or CH + PS at (0.75% + 2.0%). Moreover, some chemical inducers such as K2HPO4, dichloroisocitonic acid, and ethephone (0.75% + 2.0%).

In treated plants. Among the concentrations, 0.75% CH, 1.0% PB and 2.0% PS had superiority for controlling plant disease. Pepper plants resistance against powdery mildew disease. Pepper plants might have collectively contributed to the induced resistance β-1,3-glucanase, catalase and protein content in plant leaves, improve photosynthetic rate, and increase the synthesis recorded a significant increase (51.2% and 46.3%) over control was in combined treatments CH + PB or CH + PS, respectively. These results could be attributed to the stimulator effects of CH application when mixed with potassium salts. Higher protein content in okra plants treated with CH, may reflect the degree of assimilation of inorganic nitrogen and promoting protein synthesis in leaves, improve photosynthetic rate, and increase the synthesis as well as accumulation of plant photosynthetic products. CH also can increase soluble sugar content and enhance the activity of protease conversion to protein and increasing free amino acid content, which has obvious inhibiting effect for many plant pathogenic fungi. All pervasive explanation are in agreement with [74, 75]. The present findings demonstrate that PRI in future can be used on a commercial scale for controlling such diseases especially under system used for protective cultivation. With results such as those reported here, foliar applications of natural and inorganic chemical inducers when used in combination, could become effective components of disease management system in okra plants.

The foliar applications of CH and potassium salts at vegetative stage could inhibit the powdery mildew caused by Erysiphe cichoracearum DC and improve the biochemical pathways during growth of okra plants, which resulted from increased of defense enzymatic systems. Hence, the high-level accumulation of PR-protein, POD, PPO, chitinase and β-1,3-glucanase, catalase and protein content in plant leaves might have collectively contributed to the induced resistance in treated plants. Among the concentrations, 0.75% CH, 3.0% PB and 2.0% PS had superiority for controlling plant pathogen and accelerate metabolic profile in tested okra plant over control treatments. Therefore, application of CH at concentration of 0.75% or 1.0% in combination with inorganic inducers may be recommended for okra plant. However, more experiments should be conducted in different locations and seasons to valid conclusion regarding the CH foliar application for yield improvement of okra.

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