

Original Research

The Impact of Hydrogen Peroxide Against Cucumber Green Mottle Mosaic Virus Infection in Watermelon Plants

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Abstract

The aim of this work was to study the effects of hydrogen peroxide (H_2O_2) application against cucumber green mottle mosaic virus (CGMMV) infection in watermelon (*Citrullus lanatus*) plants. From the obtained results, the induced resistance using H_2O_2 treatments caused a delay in the appearance of CGMMV symptoms in watermelon plants. The viral infection showed abnormal morphological symptoms such as mosaics, yellow blisters and reduction in size. Pretreatment with H_2O_2 before infection was beneficial in increasing the contents of pigments, total proteins, total free amino acids and proline. Consequently, plants appeared morphologically similar to healthy controls. Signaling the effect of the H_2O_2 treatment could induce partial resistance or delay the appearance of symptoms and decreased virus concentration. The induced mechanism of resistance was suggested to be through alterations of plant antioxidant status – both enzymatic and non-enzymatic. All analyzed antioxidant enzymes were induced in response to H_2O_2 ±CGMMV. Due to the H_2O_2 application prior to infection, malondialdehyde (MDA) content was reduced, indicating a lowering in lipid peroxidation caused by virus infection. On the other hand, internal H_2O_2 and phenolics contents were induced in H_2O_2 + CGMMV-treated leaves. To confirm: total antioxidant activity was increased to be double the value (80.67%) of that recorded in healthy plants (47.18%), indicating changes in antioxidant status as a response to H_2O_2 and/or CGMMV infection. This work provided evidence of the signaling role of exogenous H_2O_2 , which led to systemic acquired resistance (SAR) induction acting against CGMMV infection in watermelon plants. From the present findings, a suggestion of spraying of H_2O_2 might be helpful in avoiding the appearance of CGMMV severe symptoms throughout the plants' life.

Keywords: cucumber green mottle mosaic virus, hydrogen peroxide, systemic acquired resistance, antioxidant enzymes, phenolics, malondialdehyde, antioxidant activity

Introduction

Cucumber green mottle mosaic virus (CGMMV) is one of the major cucurbit viral diseases resulting in economic losses in cucurbit production worldwide. CGMMV belongs to the *Tobamovirus* genus that systemically infects many cucurbitaceous plants such as watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), pumpkin (*Cucurbita pepo*), squash (*Cucurbita moschata*) and bottle gourd (*Lagenaria siceraria*) [1, 2]. CGMMV is spread and transferred easily by many ways, including mechanical transfer, foliage contact, soil contamination, propagation materials, pollen grains and infected seeds [3-6]. Although there is no insect vector that transmits CGMMV, the widespread nature of the virus is due to the stability of virus particles for a long period under extreme conditions. CGMMV disease causes severe symptoms, including mottling, mosaics, distortion, blistering of leaves and discoloration, and deterioration of the internal part of watermelon fruits – making them undesirable for marketing [1].

Less is known about induced resistance to CGMMV in cucurbit plants, as there are no commercial melon cultivars resistant to CGMMV. Moreover, there are no chemicals used to avoid the spread of CGMMV; only methyl bromide has been used for soil disinfection of CGMMV and has been restricted since 2005. A kind of resistance against CGMMV-SH strain was reported by Crespo et al., [7] in *C. sativus* accessions through restriction of movement of the virus within the plant. Induction of systemic acquired resistance (SAR) provides immunity against viral infection. SAR induction is a good way to control virus spread. Chemical elicitors of SAR (such as salicylic acid, benzoic acid, aminobenzoic acid, oligomers of chitosan, etc.) provide resistance against different virus classes [8]. The mechanism of SAR defense might be through callose deposition and hydrogen peroxide (H_2O_2) production, which acts as a signal molecule in a plant that activates pathogenesis-related gene expression.

The signaling effect of hydrogen peroxide is well reported as a fast response of plant defense mechanism [9]. Hydrogen peroxide is important for physiological processes of plants. It can control stress responses and systemic signaling [10, 11]. Production of hydrogen peroxide is an early response to environmental stressors due to alterations in biochemical processes [12, 13]. Under stress, hydrogen peroxide is produced in response to oxidative stress. It is a safe form of reactive oxygen species (ROS), where cells can be controlled by an antioxidant enzymatic system. In plant-pathogen interaction, cellular signaling via ROS is generated. Hydrogen peroxide is a non-radical type of reactive oxygen species (ROS). Due to its diffusibility it can move across membranes and reach numerous biomolecules and affect the activity of proteins and act as a signaling factor. H_2O_2 operates as an oxidant or reductant in many cellular reactions. It can transfer

across membranes passively or through water channels [14]. Hydrogen peroxide is less toxic than other ROSs. It is scavenged by H_2O_2 scavenging enzymes such as catalase, peroxidase and ascorbate peroxidase. As an enzymatic antioxidant, catalase plays a key role in preventing cellular oxidative damage by degrading hydrogen peroxide (H_2O_2) into water and oxygen with high efficiency [15]. Pan et al. [16] reported exogenous application of H_2O_2 in *Arabidopsis thaliana*, which can activate signaling pathways able to produce antioxidants [10]. Recently, it was reported that H_2O_2 can induce gene expression and enzymatic defense responses in pepper plants [17, 18].

This work was to prove that a protective role of hydrogen peroxide was generated through the induction of SAR in watermelon plants against CGMMV. This role was explained in this article through alterations of both enzymatic and non-enzymatic antioxidant status of plants.

Materials and Methods

Plant Material and Treatments

A six-week experiment was carried out to test the effects of hydrogen peroxide (Sigma Aldrich, USA. 50 wt. % in H_2O) in induction of resistance against CGMMV-SH infection in watermelon plants. Seeds of *Citrullus lanatus* (cv. Malali) were germinated in soil (sand and clay 3:1) in 3 L pots in a greenhouse. Completely randomized design was used in this study. Three weeks after planting, plants were grouped into 6 groups (each group consists of 4 pots containing 3-4 plants, sampling from 4 independent plants). Groups were treated according to the following:

1. Control: Plants were sprayed with tap water.
2. Infected: Plants were treated with tap water one day prior CGMMV inoculation.
3. 10 mM H_2O_2 : Plants were sprayed with 10 mM solution of H_2O_2 .
4. 5 mM H_2O_2 +V: Plants were sprayed with 5 mM solution of H_2O_2 one day prior inoculation.
5. 10 mM H_2O_2 +V: Plants were sprayed with 10 mM solution of H_2O_2 one day prior inoculation.
6. 20 mM H_2O_2 +V: Plants were sprayed with 20 mM solution of H_2O_2 one day prior inoculation.

For inoculation, freshly infected leaves found to be positive for CGMMV were ground in a mortar containing 0.1 M phosphate buffer of pH = 7.0 (1:2 w/v). The homogenate was then filtrated through two layers of muslin. Plants were dusted with 600-mesh carborundum and rubbed gently with a cotton swap previously dipped into the suspension of virus inoculum [1]. Plants were inoculated once throughout the experimental period. Control plants were treated with carborundum and phosphate buffer only. Natural infections were avoided by keeping plants in isolated controlled conditions (growth chamber). Plants were

kept at 20±2°C and 65% humidity in a growth chamber. Symptoms appeared two weeks after inoculation. Leaf samples were photographed and taken for analyses after three weeks of virus inoculation (i.e., at the end of this experiment).

DAS- ELISA for CGMMV Detection

100 Healthy and infected plants were subjected to a DAS-ELISA test (double antibody sandwiched-enzyme linked immunosorbent assay) [19]. A ready for use kits (Agdia Inc., Madison, USA) were used to analyze the samples. All the buffers, coating antibodies and conjugated antibodies were diluted as the protocol provided by the manufacturer. Absorbance at 405 nm was determined with a plate reader for all samples at the same time.

Percentage of Infection and Severity of Symptoms

Three weeks after inoculation the percentage of infected plants and the severity of symptoms were examined using the following rating scale: 0 = no symptoms; 1 = chlorotic local lesions and mild mosaic; 2 = severe mosaic and 3 = blisters and malformation. Disease severity values were calculated using the following equation [20]:

$$\text{Disease severity (DS)} = \frac{\sum(\text{disease grade} \times \text{number of plants in each grade})}{(\text{Total number of plants} \times \text{highest disease grade})}$$

Three weeks after inoculation the youngest fully developed leaves from both control and treated plants were sampled for biochemical analyses.

Photosynthetic Pigments Content

The analysis of Chlorophyll A (Chl A), Chlorophyll B (Chl B) and Carotenoids (Cars) was carried out using the Lichtenthaler and Buschmann Method [21]. Fresh leaves (0.1 g) of control and treated fresh leaves were extracted by grinding in 10 ml acetone (85%) in a mortar. The extract was centrifuged at 5000 rpm. Supernatant was separated in a test tube for analysis. Pigment extract was then analyzed by colorimeter (T80 UV-VIS spectrophotometer, PG Instruments, UK) using three wavelengths: 663, 647 and 470 nm.

The absorbance of the extracts was then used to calculate pigment concentrations through the following formulas:

$$\begin{aligned} \text{Chlorophyll } a &= 12.25 A_{663} - 2.79 A_{647} \\ \text{Chlorophyll } b &= 21.50 A_{647} - 5.10 A_{663} \\ \text{Carotenoids} &= (1000 A_{470} - 1.82 * \text{Chl } a \\ &\quad - 95.151 * \text{Chl } b) / 225 \end{aligned}$$

The contents of pigments were calculated as mg g⁻¹ FW.

Antioxidant Enzyme Analyses

Fresh leaves (0.5 g) of control and treated plants were extracted in 10 ml of phosphate buffer (pH = 7.0) by grinding in a mortar. The extract was then centrifuged at 14000 rpm in cool conditions 4°C for 20 min. The supernatant was separated and prepared for analysis of antioxidant enzymes (peroxidase; POD, catalase; CAT, ascorbate peroxidase; APX and superoxide dismutase; SOD).

For analysis of POD activity we used the Zhang [22] method. The extract was added to the analyzing medium containing 5 mM guaiacol, 0.3 mM hydrogen peroxide, and 0.1 mM EDTA in 40 mM phosphate buffer (pH = 7.2). The increase in oxidation of guaiacol was analyzed at 470nm using a UV-VIS spectrophotometer (T80, PG Instruments, UK). Using the extinction factor = 26.2 mM cm⁻¹, the POD activity was calculated as μmol of guaiacol oxidized min⁻¹ g⁻¹ FW.

In the case of CAT activity we used the Chandless and Scandalios method [23]. The enzyme extract was mixed with the assay medium that contained 10mM H₂O₂ in 25 mM potassium phosphate buffer (pH 7.0). The rate of decomposition of H₂O₂ was then detected at 240 nm, expressing CAT activity as μmol min⁻¹ H₂O₂ (Extinction factor = 0.036mM⁻¹ cm⁻¹).

For APX activity analysis we used the method of Nakano and Asada [24]. The assay medium consisted of 0.3 mM ascorbate, 0.1 mM EDTA, and 0.06 mM H₂O₂ in 50 mM phosphate buffer (pH 7.0). The analysis was based on the decrease of ascorbate by determination of the decline in absorbance at 290 nm (extinction factor = 2.8 mM⁻¹ cm⁻¹).

SOD activity was determined using the Beauchamp and Fridovich [25] method. The extract was added to the assay medium, which contained 9.9 mM L-methionine, 0.025% (w/v) nitroblue tetrazolium (NBT), and 0.0044% (w/v) riboflavin in 50 mM phosphate buffer (pH 7.8). Photo-reduction of NBT (purple color) was analyzed at 560nm. One unit of SOD enzyme extract caused 50% inhibition of photo-reduction of NBT.

Antioxidant Metabolites MDA and H₂O₂

Malondialdehyde (MDA) content was determined in leaves by the Zhang method [22]. Fresh leaves were extracted in 5% TCA (trichloroacetic acid), followed by 10 min centrifugation at 3000 rpm. The supernatant was then mixed with 0.03 mM of 2-thiobarbituric acid (TBA) and incubated at 94°C for 15 min. After cooling, the developed color was then analyzed by spectrophotometer at 532 nm. Using extinction factor ($E = 155 \text{ mM cm}^{-1}$), the MDA concentration was calculated as nmol MDA g⁻¹ FW.

Hydrogen peroxide (H₂O₂) content was determined by using the method of Jana and Choudhuri [26]. Leaves were extracted in 50 mM phosphate buffer (pH = 6.5) and centrifuged at 6000g for 25 min. The supernatant

was mixed with 1 ml of 0.1% titanium sulfate in 20% H₂SO₄ after the ppt formation; the mixture was centrifuged at 6000g for 15 min. The pellet was then dissolved in 5 ml H₂SO₄ (2M) and the absorbance was then measured by spectrophotometer at 410 nm. Using the extinction coefficient ($E = 0.28 \mu\text{mol}^{-1} \text{cm}^{-1}$), H₂O₂ content was calculated as nmol g⁻¹ FW.

Phenolics Content

A folin- Ciocalteu reagent was used to determine the content of phenolics in control, infected and treated leaves. The method of Singleton and Rossi [27] was used. Extracts were prepared in methanol (80%) and mixed with 1.8 ml of Folin-Ciocalteu reagent (diluted 1:10) and stand for 5 min at room temperature. 1.2 ml of NaHCO₃ (7.5% w/v) was added. Color was analyzed at 765 nm after standing for 60 min at room temperature. Phenolic contents were presented as $\mu\text{g GAEg}^{-1} \text{DW}$, where GAE is the equivalent of gallic acid.

Assay of Total Antioxidant Activity

DPPH (1,1-diphenyl-2-picrylhydrazyl, Sigma Aldrich) free radical scavenging assay test was used to analyze the antioxidant status of control and treated leaves [28]. Fresh leaves were extracted in methanol and mixed with a similar amount of freshly prepared DPPH solution (80 ppm in methanol). After mixing thoroughly, tubes were kept in the dark for 30 min. The color was then determined by spectrophotometer at 517nm. Using the following equation, total antioxidant activity was calculated as a percentage of DPPH scavenging activity:

$$\text{Antioxidant activity} = [1 - (A_i - A_j) / A_c] * 100$$

...where A_i is absorbance of extract+ DPPH, A_j is the absorbance of extract + methanol and A_c is absorbance of DPPH + methanol.

Proteins Content

Total proteins content of control, infected and H₂O₂-treated leaves were analyzed using the Lowry method [29]. Samples were extracted in NaOH (0.1N) in water bath (100°C) for 1 hr. The extracts were centrifuged at 4000 rpm and the supernatant was taken for analysis. Alkaline-Folin reagent was used. One ml of extract was mixed with 5 ml of alkaline reagent prepared as follows (reagent A: 2% Na₂CO₃ in 0.1N NaOH and reagent B: 0.5% CuSO₄ in Sod. Pot. Tartrate). After standing 20 min the folin reagent 0.5 ml was added and mixed thoroughly and left to stand for 20 min. Absorption was determined at 750 nm. Total protein contents were expressed as mg /g dry weight of leaves.

Total Free Amino Acids

The Moore and Stein [30] method was used to analyze the free amino acids of different leaves. Tissue samples were extracted in distilled water by heating in a water bath at 90°C for 2 hrs. The extracts were then centrifuged and the supernatants were collected. Supernatant was added to 1 mL of ninhydrin solution with stannous chloride. The mix was heated in a boiling water bath for 20min.; a purple color developed. Diluents (5 mL) were added and contents were mixed. Fifteen minutes later, the developed color was read at 570 nm against blank. The free amino acid concentrations were calculated as mg/g dry matter.

Proline Content

The method of Bates et al. [31] was used to determine the proline content control, infected and H₂O₂-treated leaves. The extract was prepared using 0.1 g of dried powdered leaves in 10 ml of 3% sulfosalicylic acid for 12h. Centrifuging was carried out for 10 min at 1500 rpm. For analysis, supernatants were mixed with acid ninhydrin reagent +2 ml glacial acetic acid and heated in a water bath at 100°C for 1 h. Cooling of the mixture using ice bath then 4 ml toluene were added for extraction of the pink color. The absorbance was measured at 520 nm for toluene phase containing the color. Proline content was calculated as $\mu\text{g g}^{-1} \text{DW}$ using a pre-analyzed standard curve using proline amino acid.

Statistical Analysis

The results were reported as mean±SD of four independent replicates. Statistical analyses of data were carried out by computer using SPSS ver. 23.0 software. One-way ANOVA and least significant differences test (LSD) for multiple comparisons were used to evaluate the differences among the means.

Results

Morphological Changes and Growth Analysis

Fifteen days after inoculation, leaves became mosaic with green and yellow blisters and were reduced in size compared to the control (Fig. 1). The leaf was mostly yellow with heavy hairy surface – especially in the petiole and leaf edges. Infected leaf area was reduced to 79% less than control. Treatment with H₂O₂ without infection increased leaf area to 12% more than control. The application of 20 mM H₂O₂ prior to infection made it able to keep the leaf area similar to control. Analysis of shoot length revealed a severe decrease by 55% compared to control. H₂O₂±infection treatment was able to increase shoot length. In detail, shoot lengths were increased by 78% more than control with 20 mM H₂O₂,

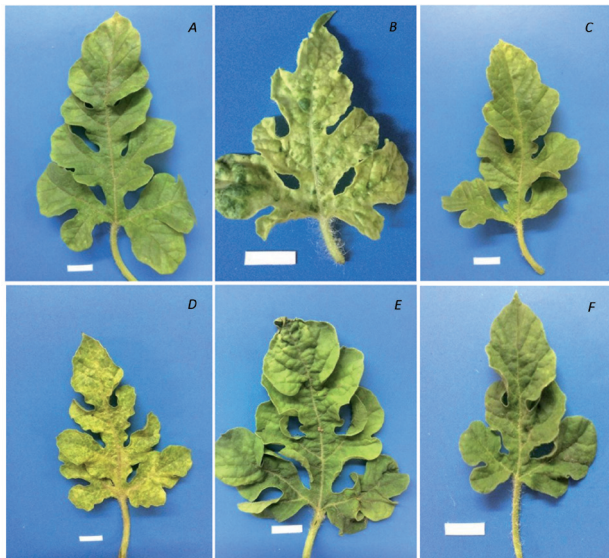


Fig. 1. Effects of CGMMV infection and H₂O₂ treatments on leaf morphology and severity of symptoms of watermelon leaves. Control (A) shows healthy appearance; infected (B) shows mosaics and blisters; 10 mM H₂O₂ (C) shows healthy appearance; 5 mM H₂O₂ + inf (D) shows mild symptoms; 10 mM H₂O₂ + inf (E) shows no symptoms; and 20 mM H₂O₂ + inf (F) shows no symptoms. Scale bar (1 cm).

followed by GCMMV infection. A decrease in leaf area and shoot length of the infected plants led to an obvious decrease in dry matter. Lowered dry matter of watermelon plants by 49% was recorded with infection. In contrast, a gradual increase in dry matter was noticed with H₂O₂ treatment followed by infection. Spraying 20 mM H₂O₂ before infection was able to increase dry matter by up to 60% more than control (Table 1). Changes in water content of infected and H₂O₂-treated plants was observed. A highly significant decrease of water content in both infected and (5 mM H₂O₂ + inf) plants was noticed, while 20mM H₂O₂ followed by infection was almost similar to the control (Table 1).

Both percentage of infection and disease severity showed a noticeable decrease with H₂O₂ pretreatment in inoculated plants. Although all concentrations of H₂O₂ (5-20) were able to decrease the infection percentage, the most effective concentration was the highest one (20mM H₂O₂). This concentration was able to minimize the percentage of infection and lowered the severity of disease (Table 2). The concentration of CGMMV virus using the ELISA test showed positive results in infected and (H₂O₂ +virus) treated leaves. This means the appearance of symptoms in plants with different degrees according to the detected concentration of the virus. It was noticed that the concentration declined gradually by increasing the applied H₂O₂ dose (Table 2).

Photosynthetic Pigments Content

The analyzed photosynthetic pigment contents in infected leaves showed a highly significant reduction in Chl A, Chl B and Carotenoids (Fig. 2). This reduction reached 50% of control in Chl A and carotenoids due to virus infection. The use of H₂O₂ without a virus showed a highly significant increase of photosynthetic pigment contents (55, 39 and 25% more than control in case of Chl A, Chl B and cars, respectively). On the other hand, H₂O₂ treatments followed by virus infection could pronouncedly increase the contents of photosynthetic pigments. It was noticeable that the increase in pigments was concomitant with H₂O₂ concentrations – especially in Chl A and Chl B. It is noticeable that 10 and 20 mM H₂O₂ + infections were more effective in pigment content improvement. The most obvious result was obtained with the treatment 20 mM H₂O₂ + virus infection, which led to increasing Chl A, Chl B and carotenoids to be 69, 94 and 42% more than their corresponding controls (Fig. 2).

Table 1. Effect of CGMMV infection and H₂O₂ treatments on Leaf area (cm² plant⁻¹), Shoot length (cm plant⁻¹), Water content (%) and Dry matter (g plant⁻¹) of watermelon plants. The values are means (M) of four replicates±standard deviation (SD). The values are means (M) of four replicates±standard deviation (SD).

Treatments	Leaf area (cm ² plant ⁻¹)		Shoot length (cm plant ⁻¹)		Water content (%)	Dry matter (g plant ⁻¹)	
	M±SD	%	M±SD	%	M±SD	M±SD	%
Control	105.92±9.87	100	43.64±5.38	100	87.34±0.49	3.73±0.09	100
Infected	22.15**±12.67	20.91	19.87**±4.39	45.53	76.48**±1.94	1.93**±0.04	51.74
10 mM H ₂ O ₂	111.37±11.65	105.15	45.65±6.34	104.61	89.95±0.72	3.99±0.02	106.97
5 mM H ₂ O ₂ + inf	78.29**±8.23	73.91	62.59**±2.85	143.42	77.13**±0.81	4.32**±0.06	115.82
10 mM H ₂ O ₂ + inf	96.17*±15.74	90.79	69.13**±3.46	158.41	83.85*±0.95	5.72**±0.03	153.35
20 mM H ₂ O ₂ + inf	100.33±10.63	94.72	77.62**±4.64	177.86	87.54±0.37	5.98**±0.05	160.32

Statistical significance of differences compared to control: *, significant at $P < 0.05$; **, significant at $P < 0.01$.

Table 2. Effect of CGMMV infection and H₂O₂ treatments on Percentage of infection (%), Disease severity (%) and Virus concentration using ELISA test of watermelon plants. The values are means (M) of four replicates±standard deviation (SD).

Treatments	Percentage of infection (%)	Disease severity (%)	Virus concentration*
	<i>M</i> ± <i>SD</i>	<i>M</i>	<i>M</i> ± <i>SD</i>
Infected	97.54±3.27	74.65	1.273±0.175
5 mM H ₂ O ₂ + inf	66.18**±2.65	33.89	0.932±0.065
10 mM H ₂ O ₂ + inf	13.87**±0.64	12.7	0.645±0.028
20 mM H ₂ O ₂ + inf	4.83**±0.45	1.97	0.193±0.056

Statistical significance of differences compared to control: *, significant at $P<0.05$; **, significant at $P<0.01$. *ELISA test for virus concentration, the positive and negative controls are 1.497 and 0.105 respectively; Positive control means infected leaves showed symptoms typically. And negative control means infected leaves showed no symptoms

Antioxidant Enzyme Activities POD and CAT Activities

Activities of POD and CAT were induced significantly in the infected leaf samples (Table 3). The activities were almost doubled in response to infection. With all H₂O₂ treatments, POD and CAT showed enhanced activities compared to control. Leaves treated with 10 mM H₂O₂ induced POD and CAT activities to be 86% and 80% more than those of control, respectively. A highly significant increase in POD and CAT activities was noticed with the increase of H₂O₂ treatment followed by infection. This increase reached 182% (POD) and 118% (CAT) more than

control in the case of spraying 20 mM H₂O₂ prior to infection.

APX and SOD Activities

It was noticeable that APX activity in infected leaves was highly induced (Table 3). Compared to control, APX activity was 3-fold higher (220% increase) in response to GCMMV infection. SOD activity increased by 25% more than control due to virus infection. Inductions of APX and SOD activities were recorded in leaves treated with 10mM H₂O₂ without infection. The application of H₂O₂ (5-20 mM) followed by infection caused a concomitant decrease in APX activity. On the

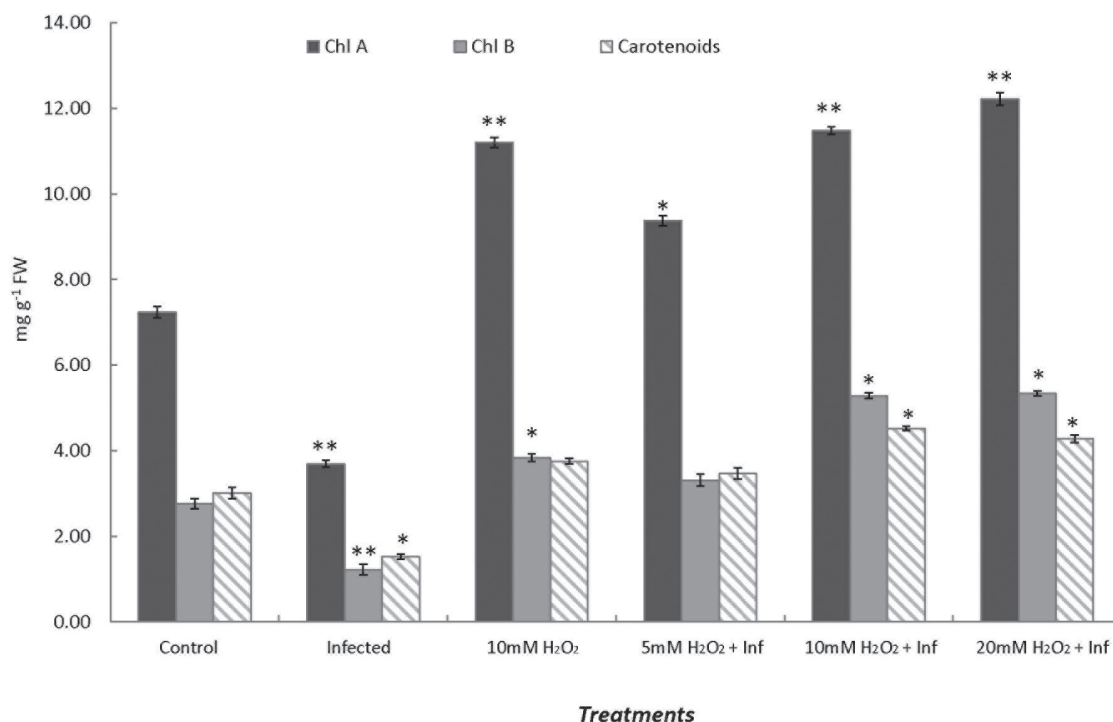


Fig. 2. Effects of CGMMV infection and H₂O₂ treatment of pigment contents (Chl a, Chl b and Carotenoids) (mg g⁻¹ FW) of watermelon leaves. The values are means of four replicates±standard deviation. Statistical significance of differences compared to control: *, significant at $P<0.05$; **, significant at $P<0.01$.

Table 3. Effect of CGMMV infection and H₂O₂ treatments on Peroxidase, Catalase, Ascorbate Peroxidase and Superoxide dismutase activities (Unit g⁻¹FW) of watermelon plants. The values are means (M) of four replicates±standard deviation (SD). The values are means (M) of four replicates±standard deviation (SD).

Treatments	POD (Unit g ⁻¹ FW)		CAT (Unit g ⁻¹ FW)		APX (Unit g ⁻¹ FW)		SOD (Unit g ⁻¹ FW)	
	M±SD	%	M±SD	%	M±SD	%	M±SD	%
Control	94.83±3.27	100	43.64±2.94	100.00	48.86±1.75	100	54.64±0.71	100
Infected	187.07**±6.34	197.27	88.41**±0.57	202.59	156.34**±0.58	319.98	68.74**±0.20	125.81
10 mM H ₂ O ₂	176.42**±4.83	186.04	78.60**±0.22	180.11	86.45**±0.49	176.93	83.39**±0.54	152.62
5 mM H ₂ O ₂ + inf	234.53**±3.62	247.32	86.37**±0.84	197.91	75.83**±1.22	155.20	95.84**±1.03	175.40
10 mM H ₂ O ₂ + inf	274.36**±8.75	289.32	93.76**±0.74	214.85	66.90*±0.37	136.92	101.45**±2.62	185.67
20 mM H ₂ O ₂ + inf	266.95**±5.83	281.50	95.04**±1.53	217.78	83.54±0.45	170.98	131.64**±1.66	240.92

Statistical significance of differences compared to control: *, significant at $P<0.05$; **, significant at $P<0.01$.

other hand, leaves treated with H₂O₂ only showed higher SOD activities compared to the control. Moreover, an increase of (141%) more than control was recorded in SOD activity in watermelon leaves sprayed with 20 mM H₂O₂ plus infection.

Antioxidant Metabolites MDA and H₂O₂

The amounts of both MDA and H₂O₂ were analyzed in control and treated leaves (Table 4). MDA was commonly used as an indicator for lipid peroxidation and oxidative stress. An increase in MDA content was noticed with infection (about 37% more than control). The use of H₂O₂ prior to infection showed variable results. H₂O₂ without infection was able to lower the MDA content by 5%, but with infection MDA contents increased even if pretreated with H₂O₂. On the other side, H₂O₂ content increased as a response to infection (65% more). Pretreatments with H₂O₂±infection were able to accumulate H₂O₂ and the amounts were dose-dependent. The highest amount of H₂O₂ was recorded in leaves treated with 20 mM H₂O₂ followed by infection. Compared with the amounts present in infected leaves, H₂O₂ couldn't

record the same ratio of increase with the applied H₂O₂ concentrations.

Total Phenolics and Antioxidant Activity

Analysis of phenolics content in control and CGMMV-infected leaves revealed the accumulation of amounts of phenolics in infected leaves by 45% (Table 4). Treatment with 20 mM H₂O₂ without virus infection was able to increase phenolics slightly (9%), while 20 mM H₂O₂ plus infection increased phenolics content by 23% more than control. With all concentrations of H₂O₂ followed by infection, the phenolics content recorded a highly significant increase that reached 22-30% more than that of control. The antioxidant activity of the tested leaves showed significant differences. In general, all treatments could increase the antioxidant activity of leaves. Infection with CGMMV induced AOA to be 66% while control leaves recorded AOA of about 47%. In the case of H₂O₂ treatments, the increase in AOA was concentration dependent. Moreover, the highest improvement of AOA was noticed in leaves treated with 20 mM H₂O₂±infection, which reached 81-85%.

Table 4. Effect of CGMMV infection and H₂O₂ treatments on MDA (μmol MDA g⁻¹FW), cellular H₂O₂ (nmol H₂O₂ g⁻¹FW), phenolics (μg g⁻¹FW) and antioxidant activity (%) of watermelon plants. The values are means (M) of four replicates±standard deviation (SD).

Treatments	MDA (μmol MDA g ⁻¹ FW)		H ₂ O ₂ (nmol H ₂ O ₂ g ⁻¹ FW)		Total Phenolics (μg g ⁻¹ FW)		AOA (%)
	M±SD	%	M±SD	%	M±SD	%	M±SD
Control	15.64±0.93	100.00	19.72±0.38	100.00	53.89±1.38	100.00	47.18±1.94
Infected	26.37**±1.43	136.64	32.54**±1.56	165.01	78.34**±2.91	145.37	65.92**±3.51
10 mM H ₂ O ₂	14.82±0.56	94.76	21.32±0.52	108.11	58.74*±3.09	109.00	85.10**±2.75
5 mM H ₂ O ₂ + inf	21.34*±0.71	136.45	23.96*±0.45	121.50	67.61**±2.80	125.46	69.27**±2.06
10 mM H ₂ O ₂ + inf	21.95*±0.65	140.35	25.63*±0.71	129.97	69.92**±2.81	129.75	71.85**±1.83
20 mM H ₂ O ₂ + inf	19.84*±1.06	126.85	26.45*±0.87	134.13	65.74**±3.63	121.99	80.67**±2.90

Statistical significance of differences compared to control: *, significant at $P<0.05$; **, significant at $P<0.01$.

Table 5. Effect of CGMMV infection and H₂O₂ treatments on Total proteins (mg g⁻¹FW), Total free amino acids (mg g⁻¹DW) and Proline (mg g⁻¹DW) contents of watermelon plants. The values are means (M) of four replicates±standard deviation (SD).

Treatments	Total proteins (mg g ⁻¹ DW)		Total free amino acids (mg g ⁻¹ DW)		Proline content (mg g ⁻¹ DW)	
	M±SD	%	M±SD	%	M±SD	%
Control	218.78±12.54	100.00	38.17±0.30	100.00	1.18±0.01	100.00
Infected	276.81*±11.65	126.52	55.95**±0.64	146.58	3.27**±0.03	277.12
10 mM H ₂ O ₂	332.16**±17.83	152.82	47.29*±0.48	123.89	2.48*±0.02	210.17
5 mM H ₂ O ₂ + inf	302.53**±32.45	138.28	48.37*±0.25	126.72	2.76**±0.04	233.90
10 mM H ₂ O ₂ + inf	354.34**±18.38	161.96	56.68**±0.63	148.49	2.68*±0.03	227.12
20 mM H ₂ O ₂ + inf	365.45**±19.59	167.04	60.86**±0.55	159.44	2.37*±0.04	200.85

Statistical significance of differences compared to control: *, significant at $P<0.05$; **, significant at $P<0.01$.

Total Proteins, Total Free Amino Acids and Proline Contents

The amounts of total proteins are presented in Table 5. Infected leaves showed significant accumulation of proteins due to infection. In infected leaves, the increase in proteins reached 27% more than control. H₂O₂ treatment prior to infection was able to raise the protein content. In detail, the application of 5 mM H₂O₂ + infection caused a 38% increase and 20 mM H₂O₂ + infection caused a 67% increase compared to control. Similar to proteins, free amino acid contents followed the same behavior of increase in treated samples. The free amino acid of infected leaves reached 47% more than the control. All H₂O₂ concentrations±infection caused the accumulation of more amounts of free amino acids. The accumulation was 59% more than the content of control in infected leaves pretreated with 20 mM H₂O₂. As proline accumulation is considered an indication of the presence of stress conditions, the analysis of proline in infected leaves showed a highly significant increase (177% more than control). Plants responded to H₂O₂ treatments (with or without infection) by increasing the proline content. For example, treatment with 20 mM H₂O₂ alone was able to increase proline content to be 110% more than that of control. On the other hand, treatment with 20 mM H₂O₂ followed by infection was able to increase the proline content to 101% compared to that of control.

Discussion

Induction of systemic acquired resistance (SAR) in plants is an efficient way to resist pathogen infection. SAR occurs in many plant species in response to pathogen infection and the application of certain chemicals. SAR is associated with the expression of plant defense genes [32]. Chemical elicitors can generate systemic signals that lead to SAR induction. Hydrogen peroxide is one of the chemical inducers of SAR, which

in turn signals the formation of pathogenesis-related proteins, and phytoalexins [33]. Hydrogen peroxide and superoxide act directly as second messengers in the regulation and expression of the genes encoding proteins responsible for oxidative stress response [13]. Hydrogen peroxide is a versatile molecule that acts as a non-radical reactive oxygen species (ROS). Harmful and beneficial consequences of ROS have been recorded [34, 35]. Hydrogen peroxide is safe and can be easily detoxified by an antioxidative enzyme system; catalase, peroxidase, ascorbate peroxidases. It is well reported that H₂O₂ plays a signaling role in plants under stress and pathogen defense [36-38] – especially against virus infection, as reported by Mejía-Teniente and Durán-Flores et al. [18]. In this work, treatment with H₂O₂ improves the growth of watermelon plants and protects against CGMMV infection. This improvement was noticed in increased growth, shoot lengths, dry matter, leaf area, minimized percentage of infection and lowered severity of disease and lowered virus concentrations. This is the first report about induced resistance using H₂O₂ treatment against CGMMV in watermelon plants.

Due to leaf CGMMV symptoms and mosaics in infected samples, the photosynthetic pigments content was significantly altered compared with control. Treatment with H₂O₂±infection was able to increase amounts of chl a, chl b and carotenoids of leaves significantly. The increase was concomitant with the concentration of H₂O₂ [36], treatments with which increased PSII photochemical efficiency. Moreover, H₂O₂ treatments increased the activity of Rubisco. Increased content of pigments reflects more efficiency of photosynthesis process in plants treated with H₂O₂. In support, Ashfaq et al. [39] reported the promotion of photosynthesis in wheat plants after exogenous application of 100 nM H₂O₂. Moreover, treatment with H₂O₂ exogenously to quinoa plants was able to improve the photosynthesis rate by 42% and increase chlorophyll content by 36% more than the control.

In this work, a resistance mechanism against CGMMV using an exogenous application of H₂O₂

in watermelon plants was suggested. This induced resistance occurred through alterations of an antioxidant (enzymatic and non-enzymatic) system due to the signaling effect of H_2O_2 . A significant increase of activity of POD, CAT and APX in response to 10mM H_2O_2 without infection was observed. The increase reached 86%, 80% and 77% more than control for POD, CAT and APX, respectively. On the other hand, the increase in POD, CAT, and APX with infection was 182%, 118%, and 70% more than control. These are H_2O_2 scavenging enzymes, and increasing their activities indicates the formation or presence of more amounts of H_2O_2 [40, 41]. More increase in POD and CAT activities with H_2O_2 treatment and CGMMV was because of the double source of induction; the pathogen and exogenous H_2O_2 application. Moreover, SOD activity was induced by infection, and H_2O_2 was able to induce SOD with or without the presence of infection. SOD is induced under biotic and abiotic stress and functions in dismutation of $O_2^{\cdot-}$ to produce H_2O_2 [42]. In this work, a 26% increase in SOD activity with infection might be to avoid oxidative stress produced by infection. On the other hand, spraying H_2O_2 caused highly significant induction of SOD (141% more than control). This is in accordance with the induced activity of H_2O_2 scavenging enzymes (POD, CAT and APX). In support are previous studies done by Clarke et al., [43] who reported changes in antioxidant activities as a response to WCIMV in *Phaseolus vulgaris* leaves. Moreover, changes in antioxidant enzymes were detected in biotic stress (ZYMV infection to pumpkin plants) [44, 45] and abiotic stress (herbicides application to peanut plants) by Radwan et al. [46]. Internal production of H_2O_2 or receiving exogenous help increased the signaling effect of H_2O_2 and induction of SAR against virus infection. Consequent to antioxidant enzymes changes, an increase in H_2O_2 levels or accumulation was noticed. This accumulation of H_2O_2 with infection and other treatments indicates oxidative stress as a response to CGMMV and H_2O_2 signaling cascade. As a defense to pathogen infection, radical and non-radical ROS are accumulated, triggering the oxidative burst that leads to recognition of the pathogen. H_2O_2 serves as a defensive barrier against pathogen infection [47].

MDA is a final product of fatty acid peroxidation caused by ROS accumulation [48]. MDA is used as an indicator for lipid peroxidation and oxidative stress due to the overproduction of ROS because of virus infection [49, 50]. In the present, CGMMV infection caused the accumulation of MDA, indicating lipid peroxidation and oxidative stress. The MDA amount was lowered by exogenous spraying of H_2O_2 to the plants prior to infection. The signaling effect of H_2O_2 induces some preparatory defense mechanism prior to infection. This defense might be through the formation of special membrane proteins, preventing cell membrane peroxidation or lowering MDA amount. These proteins were induced in advance to be used further against

infection. In the present study, analysis of total proteins showed the accumulation of more proteins as a response to infection and H_2O_2 spraying, i.e., protective action against infection was obtained. They are pathogenesis-related proteins or SAR proteins. The higher the amount of H_2O_2 level, the higher the proteins accumulated. 20mM H_2O_2 application followed by infection caused a 67% increase in proteins than control because of the double source of induction: treatment and infection. Proteins are among the main types of anti-plant viral substances [51]. Plants form certain proteins upon infection (pathogenesis-related proteins) to restrict pathogen growth and multiplication [52]. Pathogenesis-related proteins are molecular markers of SAR responses [53] that can be induced both by infection through activation of PR genes and by the exogenous application of chemical elicitors. They are plant defenses in the form of SAR, which is a broad level of resistance [8]. In this work, increasing free amino acids and proline contents upon infection and/or H_2O_2 application was noticed. Li et al. [54] reported that the accumulation of amino acids is among the pathways by which different genes are responsible for interactions between CGMMV and watermelon. For most compounds, sulfur and nitrogen are mainly synthesized from amino acids [55]. In this experiment, proline amino acid analysis revealed accumulation in response to CGMMV and H_2O_2 treatments. Proline amino acid is considered an antioxidant and ROS scavenger and functions in maintaining membrane integrity and stabilizer of both antioxidant enzymes and subcellular structures [56]. It is used in activating detoxification mechanisms, and functions in the signaling process. As previously reported, proline protects against H_2O_2 -induced oxidative stress of wild almond plants by increasing antioxidant enzyme activities and by decreasing MDA content and membrane damage [57].

Phenolics are among the secondary metabolites that are involved in plant protection [58]. In this study, the accumulation of phenolics content was noticed, accompanied with infection and/or H_2O_2 treatments. This is in accordance with the results obtained by Li and An et al. [54], who reported significant increases in phenolic compounds due to CGMMV infection. Phenolic compounds were previously reported to have antioxidant activity providing a self-defense role under stressful conditions [59, 60]. Phenolics have the ability to alter peroxidation properties by lowering the peroxidative reaction of membranes [61]. In this experiment, the increase in phenolics related to H_2O_2 treatments can be explained as their capability of being either proton or electron donors, hence they can participate in scavenging free radicals [62]. Scavenging oxygen radicals depends on the ability of the antioxidant system of the plant. This ability to scavenge oxygen radicals is known to be inversely proportional with the levels of lipid peroxidation [63]. To confirm the effect of H_2O_2 treatment on the antioxidant status and its protective action, the total antioxidant activity was

analyzed. It was found that the AOA was doubled to be 85% with 20 mM H₂O₂ + infection while the control records AOA of 47%. This increase in antioxidant activity might be due to the signaling effect of H₂O₂ sprayed prior to infection, which could induce SAR through the accumulation of proteins, phenolics, proline, and free amino acids contents and alteration of antioxidant enzyme activities.

Conclusions

This work investigated the impacts of H₂O₂ treatment against CGMMV infection in watermelon plants. The treatment delayed infection and the appearance of severe CGMMV symptoms in watermelon plants. Plants treated with H₂O₂+ CGMMV showed normal appearance as healthy plants during the experimental period. This can be discussed through alteration of both the enzymatic and non-enzymatic antioxidant status due to H₂O₂ treatment prior to infection. Moreover, H₂O₂ treatments lowered MDA content but accumulated proteins, free amino acids, cellular H₂O₂, proline and phenolics contents. These alterations in antioxidant status and contents of biochemical constituents due to H₂O₂ treatments can suggest a certain role of H₂O₂ in delay or resisting CGMMV infection in watermelon plants.

Conflict of Interest

The authors declare no conflict of interest.

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