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The effect of salicylic and jasmonic acids on tomato physiology and tolerance to *Cucumber mosaic virus* (CMV)

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Abstract *Cucumber mosaic virus* (CMV) is one of the most important plant viruses responsible for sharp reductions in the production of many cultivated plants. Activities of antioxidant enzymes, photosynthetic capacity, proline and total soluble carbohydrates (TSC) content were measured in the leaves of tomato (*Solanum lycopercicum* cv. Falat) plants treated with phytohormones (salicylic and jasmonic acids and their combination) and inoculated with CMV at 0, 1, 2, 4, 6, 8, and 15 days after the treatments. Based on the results, catalase (CAT) activity decreased in the healthy and infected plants, but peroxidase (POD) activity increased in the CMV-infected plants signifying that POD is more active in H_2O_2 scavenging in tomato. Because the hormone treatments inhibited the reduction in the enzyme activity, it may be considered as a controlling method against CMV. Superoxide dismutase (SOD) activity was lower in the control until 6 days post inoculation (dpi), but increased after 8 dpi. The infected plants and the hormone-treated plants showed an increased SOD activity from 0 to 15 dpi. Phenylalanine ammonia lyase (PAL) activity also increased in all the treatments over the time period (0-15 dpi). Net photosynthesis (NP) rate and chlorophyll content decreased under the virus infection and hormone treatment, whereas control plants had the highest NP and chlorophyll content. Proline accumulation occurred in the infected and hormone-treated plants, but TSC content decreased in comparison to the control. Reduction of TSC content was not significant in the hormone and virus-treated plants. Expression of CMV coat protein gene (CMV-*CP*) was decreased by approximately 34% in SA+JA+CMV treatment in comparison to the CMV-infected plants. In conclusion, CMV had harmful effect on physiological traits of tomato plants, but hormone application induced resistance. This resistance may be accomplished through the combination of both hormone-related signaling pathways which likely established a strong resistance network together.

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Introduction

Tomato (*Solanum lycopercicum*) is one of the most economically important plants that belongs to the family Solanaceae along with pepper, eggplant and potato (Prohens and Nuez 2008). Among the diseases that affect tomato globally and decrease its production, perhaps one of the most important is *Cucumber mosaic virus* (CMV) from the genus *Cucumovirus* and in the family *Bromoviridae*. CMV is a widespread virus that infects a total of 1287 vegetable plant species (Zitikaitė and Urbanavičienė 2010). It is transmitted through sap by aphids (Yang et al. 2016).

Plants exhibit several defense mechanisms against invading pathogens such as systemic acquired resistance (SAR), induced systemic resistance (ISR) and local acquired resistance (LAR) (Duan et al. 2014). An induced resistance response to pathogens, characterized by the translocation of a long-distance signal from induced leaves to distant tissues to prime them for increased resistance to future infection is defined as SAR (Champigny et al. 2011). LAR is a type of resistance characterized by localization of the pathogen at the site of infection (Ross 1961). Salicylic acid (SA) is an important defensive signal in plants required for elicitor-triggered immunity and establishment of local and systemic resistance (Loake and Grant 2007; Falcioni et al. 2014). It is required by restricting pathogens at the site of infection during hypersensitive reaction (HR) and its methylated form (Me-SA) is a volatile molecule that can diffuse into the air and induce SAR in neighboring healthy plants (Palukaitis and Carr 2008). SAR and LAR are associated with the accumulation of SA and the transcriptional upregulation of a number of genes including those encoding pathogenesis-related (PR) proteins such as PR1, PR2, and PR5 in both local and systemic tissues (Durrant and Dong 2004). SA synthesized through phenylalanine ammonia lyase (PAL) pathway is required for local defense and lesion formation around the site of infection. Development of a local resistance under SA influence is connected with the strengthening of lignification processes in places of pathogen localization (Hayat and Ahmad 2007). Tobacco mosaic virus (TMV) infection leads to the accumulation of SA in infected tissues that is, to activate local and systemic expression of pathogenesis-related proteins in the cells (Enyedi et al. 1992).

Beneficial soil-borne microorganisms such as mycorrhizal fungi and plant growth-promoting rhizobacteria,

can induce a phenotypically similar form of systemic immunity called ISR (Pieterse et al. 2009). ISR is dependent on jasmonic acid (JA) and ethylene (ET) pathways and is not associated with PR expression (Pieterse et al. 2009). The signaling pathways influence each other through a complex network of synergistic and antagonistic interactions (Koornneef and Pieterse 2008). Numerous researchers have shown that SA application strongly antagonized the JA pathway by suppressing JA-responsive gene expression (Koornneef et al. 2008; Leon-Reyes et al. 2009, 2010; Spoel et al. 2003; Zander et al. 2010). Van Wees et al. (2000) reported that the SA-dependent SAR pathway and the JA dependent ISR pathway could occur concurrently in the same plant without any antagonism. Some recent evidences also indicated that certain biotrophic pathogens can trigger the JA signaling pathway (Spoel et al. 2003; Ellis et al. 2002). Zhu et al. (2014) reported that SA and JA are essential for systemic resistance against *Tobacco mosaic virus* (TMV) in *Nicotiana benthamiana* and foliar application of JA followed by SA triggered the strongest systemic resistance against TMV.

Generation of reactive oxygen species (ROS) in plants is an important way by which they confront various stresses (Sedghi et al. 2012; Baxter et al. 2014). Previously, ROS have been considered as harmful agents which lead to cell death in high levels (Rao and Davis 1999), but recently, it has been proven that free radicals specially H_2O_2 have positive effect in response to low level stresses (Deng et al. 2016; Yang et al. 2016). For example, susceptibility of zucchini plants to CMV decreased in the presence of H_2O_2 (Tao et al. 2015). High levels of ROS cause oxidative stress in plants and plants have enzymatic and non-enzymatic defense mechanisms for scavenging free radicals. In the case of enzymes, superoxide dismutase (SOD) is the front line enzyme in ROS attack since it rapidly scavenges superoxide and dismutates it to oxygen and H_2O_2 (Cruz de Carvalho 2008). Subsequently, H_2O_2 is catalyzed to oxygen and water by the action of catalase (CAT) and peroxidase (POD) under biotic and abiotic stresses (Sedghi et al. 2012).

Many physiological responses occur in plants infected with viruses and hormones contribute to these changes. SA is a phenolic compound which is directly involved in plant growth, flower induction, ion uptake (Pacheco et al. 2013), and other physiological processes. Also, enhancing chlorophyll pigmentation, changing photosynthetic rate and modifying the activity of some

important enzymes have been attributed to salicylic acid (Boatwright and Mukhtar 2013). SA is involved in the signaling pathway in plants under biotic and abiotic stresses (Abd El-Gawad and Bondok 2015) and plays a role in defense and pathogen resistance by maintaining cellular redox hemostasis and regulation of antioxidant enzyme activities (Rivas-San Vicente and Plasencia 2011). SA treatment on tomato plants infected with *Potato virus X* (PVX) enhanced the plant resistance via changing in the physiological parameters, activation of secondary metabolism and expression of some antioxidant and PR genes (Falcioni et al. 2014). Infection of pumpkin plants with *Zucchini yellow mosaic virus* (ZYMV) decreased the amount of pigments, protein and carbohydrates, but SA application remarkably increased protein and carbohydrate levels (Radwan et al. 2007). *Brome mosaic virus* (BMV)-infected rice and CMV-infected beet plants have shown an increase on some osmoprotectants such as proline and some enzymes suggesting that virus infection can improve plant tolerance to abiotic stress (Xue et al. 2008).

Using technologies for reducing pesticides or introducing environmental-friendly techniques for disease control is the main goal in sustainable agriculture (Herlihy et al. 2003). Application of plant hormones such as SA and JA which naturally occur in plants seems to be a good choice for enhancing plant resistance to pathogens (Robert-Seilaniantz et al. 2011). It appears that plant response to hormones under infection with viruses differs in various conditions and is related to virus, plant, internal and external factors. When working with CMV, results maybe different due to these reasons. Therefore, more studies are required to confirm the real effects of SA and JA application on the control of CMV in tomato plants.

The objectives of this study were to determine the effect of CMV on tomato antioxidants systems, photosynthetic status, proline and total carbohydrate content and the influence of SA and JA on the amelioration of these traits against virus infection.

Materials and methods

Plant material and treatments

Tomato (*Solanum lycopersicum*) seeds were sterilized using 5% sodium hypochlorite and planted in sterilized clay loamy soil in paper pots. Four weeks after planting,

seedlings were transplanted to the controlled insect-proof greenhouse with 25/18 °C day/night temperature and 16/8 hours day/night photoperiod. The tomato cultivar used in this experiment was Falat provided by the Department of Horticulture, University of Tabriz, Iran. Treatments included *Cucumber mosaic virus* (CMV), salicylic acid (SA), jasmonic acid (JA) and their combinations as follows:

1) Foliar application with distilled water as the control treatment
2) Inoculation with CMV
3) Foliar application with 1 mM SA
4) Foliar application with 0.5 mM JA
5) Foliar application with 1 mM SA and 0.5 mM JA
6) Foliar application with 1 mM SA and CMV inoculation
7) Foliar application with 0.5 mM JA and CMV inoculation
8) Foliar application with 1 mM SA and 0.5 mM JA and CMV inoculation

All chemicals were purchased from Sigma Aldrich, Germany.

The experiment was conducted as a factorial based on a completely randomized design with three replicates. When transplants reached the four-leaf stage (10 to 12 days after transplanting), all plants were treated as follows:

SA and JA with the concentrations of 1 and 0.5 mM, respectively were sprayed on plants 24 hours before inoculation with CMV. CMV was propagated on cucurbit (*Cucurbita pepo* cv. Styriaca). A 0.1 g of cucurbit plant tissue was ground in 1 ml inoculation buffer (50 mM potassium phosphate buffer, pH 7.0) and the extract was used for inoculation of the tomato leaves by gently rubbing in the presence of carborundum (silicon carbite) according to Sudhakar et al. (2006).

Samples were collected at 0, 1, 2, 4, 6, 8 and 15 days post inoculation (dpi) from inoculated leaves and frozen immediately in liquid nitrogen and then kept at -70 °C until processing.

Enzyme extraction and activity measurement

Tomato leaves (1 g) were homogenized in a mortar in 5 ml potassium phosphate buffer (50 mM, pH 7.5) containing 1 mM EDTA, 1 mM dithiotreitol and 2% polyvinyl pyrrolidone (PVP). This homogenate was

centrifuged at 15000 g for 25 min and the supernatant was used for assaying antioxidant enzymes (Kar and Mishra 1976).

Superoxide dismutase (SOD) assay

SOD (EC 1.15.1.1) activity was determined according to the procedure proposed by Giannopolitis and Ries (1977). A 20 μl of the supernatant was added to 50 mM potassium phosphate buffer (pH 8), 9.9 mM L-Methionine, 57 mM NBT (nitro blue tetrazolium) and 0.025% triton X-100. Reaction was initiated with the addition of 10 μl riboflavin under fluorescent lamp for 10 min. Absorbance was measured for both blank and control at 560 nm.

Catalase (CAT) Assay

Measurement of CAT (EC 1.11.1.6) activity was performed according to Aebi (1984). A 20 μl of the supernatant from the enzyme extraction stage was added to 1.5 ml of reaction mixture containing 30 μl water, 50 μl of 1 M Tris-HCl buffer (pH 8.0), 5 mM EDTA and 900 μl of 10 mM H_2O_2. The light absorbance was recorded at 240 nm wavelength for 60 sec.

Peroxidase (POD) Assay

POD (EC 1.11.1.7) activity was measured according to Kato and Shimizu (1987). A 3 ml reaction mixture containing 1.5 ml of 0.1 M potassium phosphate buffer (pH 7), 600 μl of 10 mM guaiacol and 800 μl of 4 mM H_2O_2 was added to 100 μl of enzyme extract and the absorbance was recorded at 470 nm wavelength.

Phenylalanine ammonia lyase (PAL) activity

PAL activity (EC 4.3.1.5) was measured according to the method described by Assis et al. (2001). An extraction solution containing 50 mM sodium borate, 1 g PVP, 5 mM MEP and 2 mM EDTA was added to a 200 mg leaf sample and then centrifuged at 20000 g for 20 min at 4 °C. The solution was finally filtered and a crude extract was achieved. A 250 μl aliquot of the crude extract was added to the reaction mixture containing 500 μl of 30 mM L-phenylalanine, and 750 μl of 30 mM sodium borate buffer (pH 8.7). After 10 min, the substrate was added and the reaction was stopped with 0.1 ml 6 N HCl. PAL activity was then determined

for 90 min at 30 °C by the production of cinnamate at 290 nm wavelength.

Lipid peroxidation (MDA) and H_2O_2 content

Lipid peroxidation was measured as malondialdehyde (MDA) content of tomato leaves as described by Stewart and Bewley (1980) in a colorimetric method. Samples were homogenized in 2 ml of 0.1% trichloroacetic acid (TCA) and centrifuged. Then, 0.5 ml of supernatant was mixed with 2 ml of 20% TCA containing 0.5% thiobarbituric acid. The mixture was incubated at 95°C for 30 minutes and followed by centrifugation at 10,000 × g for 10 minutes. The absorbance of the supernatant was read at 532 and 600 nm. The amount of MDA was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1}.

H_2O_2 content was determined according to Creissen et al. (1999). Briefly, leaf discs (0.2 g) were cut and immediately frozen in liquid nitrogen. The tissue was extracted in 500 mL of 25 mM HCl in a mortar and centrifuged at 5000 g for 5 min at 48°C. Pigments were removed by vortexing and addition of activated charcoal. Then, 50 mL of the extract was mixed in a 3 mL fluorescence cuvette with 2.89 mL of 50 mM HEPES, pH 7.5, and 30 mL of 50 mM homovanillic acid in 50 mM HEPES, pH 7.5. The reaction was started by the addition of 30 mL of 4 mM horseradish peroxidase. The amount of H_2O_2 was calculated by measuring relative fluorescence (excitation of 315 nm; emission of 425 nm) and reported as nM g^{-1} FW.

Chlorophyll content

Chlorophyll content was measured according to Arnon (1949). About 1 g of the fresh leaf sample was added in 80% acetone solution and kept overnight at room temperature. The extract was then centrifuged for 5 min at 14000 g and the supernatant was used for chlorophyll measurement at 645, 663 and 480 nm wavelengths. Chlorophyll a and b contents were calculated by the following formula:

$$Chl\ a = [12.7\ (OD\ 663) - 2.69\ (OD\ 645)] \times V/1000 \times W$$

$$Chl\ b = [22.9\ (OD\ 645) - 4.68\ (OD\ 663)] \times V/1000 \times W$$

Where V= Volume of extract, OD= optical density and W= Weight of sample.

Measuring rate of photosynthesis

Net photosynthesis (NP) rate was measured using a photosynthesis measurement apparatus (LI-6400XT Portable Photosynthesis System, Li-Cor, USA). Three measurements were taken per leaf and 1 leaf per plant in five similar plants in each replicate (on the average, 15 records were used for statistical analysis).

Proline content

Proline content was determined according to Bates et al. (1973). Leaf samples (0.1 g) were extracted in 10 ml of 3% sulfosalicylic acid overnight and centrifuged at 1500 rpm for 10 min. Supernatant (2 ml) was mixed with 2 ml of ninhydrin solution and 2 ml glacial acetic acid for 1 h at 100 °C in a water bath. Then, the reaction was stopped in an ice bath and the mixture was extracted with 4 ml toluene. The light absorbance was read at 520 nm and the proline content was expressed as mM g^{-1} on a fresh weight basis.

Total soluble carbohydrates (TSC)

TSC was determined using anthrone sulfuric acid method described by Scott and Melvin (1956). Briefly, 1 g of dried sample was homogenized with 80% ethanol for 15 min in a water bath. The extract was filtered and oven-dried at 60°C and then added to 10 ml of 1.5 N sulfuric acid and heated at 100 °C for 6 h. TSC content was calculated as mg g^{-1} dry weight.

Evaluation of virus replication

Total RNA was extracted from tomato leaves by using RNX-plus kit (Cinnagen Co. Iran). For RT-PCR, the first strand cDNA was prepared using the RevertAid First Strand cDNA kit (Thermo Scientific USA). To further assay the replication levels of the virus, quantitative real–time PCR analysis was performed on a Light Cycler 96 (Roche, Germany). CMV coat protein gene (CMV-CP) was amplified with the primers CMV-F (5'- AGGGTTGC GTGCTTTGACTC 3') and CMV-R (5'- ATTTCAGGCGGTTTCAGGGT -3'). Relative quantitation of the target gene expression level was performed using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001). Three technical replicates were performed for each experiment. Amplification of actin gene (Vitti et al. 2015) was used as an internal control.

Statistical analyses

Data were subjected to normality test and analysis of variance (ANOVA) considering factorial experiment arranged in CRD using SAS 9.1 software. Mean values were compared using least significant differences (LSD) test at 1% probability level with Slice order in SAS 9.1. MDA content records were subjected to data conversion (1/x) in order to satisfy a normal distribution. Student's t test was used to compare the different lengths of time in each treatment.

Results

Antioxidant enzymes activity and H_2O_2 and MDA content

CAT activity decreased over the time in the control and CMV-infected plants, but in hormone and the combination of hormone and virus treatments, CAT activity increased (Fig. 1a). The highest CAT activity was observed in SA+JA+CMV treatment at 15 dpi (439 U min^{-1} g^{-1} FW).

POD activity also decreased in the control over the time, but in the treatments, POD activity increased over the time. The highest POD activity (440 U min^{-1} g^{-1} FW) was related to SA+JA treatment at 15 dpi (Fig. 1b).

SOD activity decreased in the control up to 6 dpi, but increased again at 8 dpi. In the other treatments, SOD activity had the same trend which increased over the time from 0 to 15 dpi. The highest SOD activity (192 U mg^{-1} protein) was related to SA-treated plants at 15 dpi (Fig. 1c).

H_2O_2 concentration increased in both the control and CMV-infected plants over the time, but in hormone treatments, it decreased from 0 to 15 dpi (Table 2). In SA+CMV and SA+JA+CMV treatments, the levels of H_2O_2 increased up to 2 dpi and then decreased until 15 dpi. In JA+CMV treatment, the H_2O_2 content increased over the time and the highest content of H_2O_2 was observed in CMV-infected plants at 15 dpi (303.3 nM g^{-1} FW).
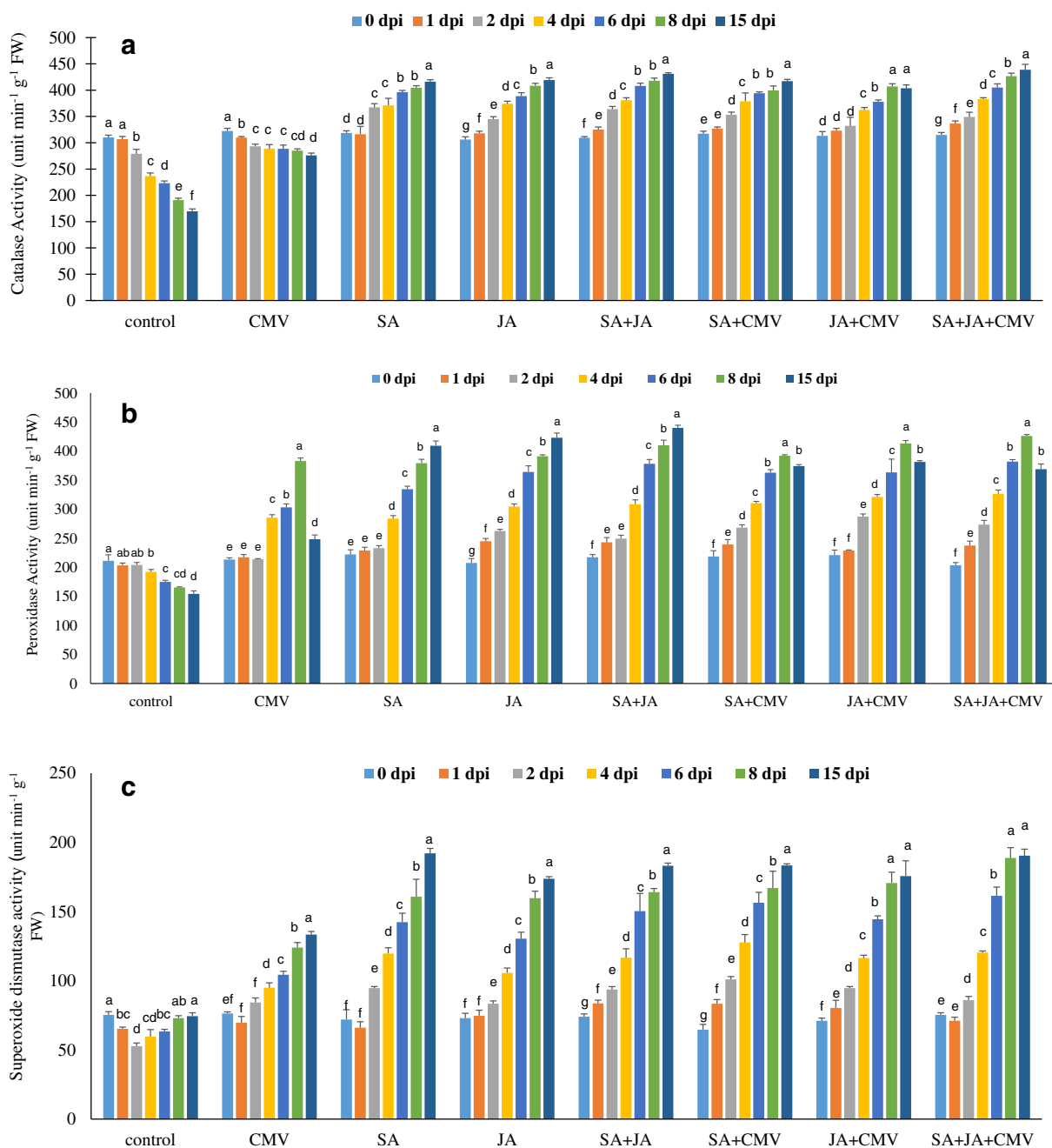
Fig. 1 Effect of *Cucumber mosaic virus* (CMV) and hormone on activity of the antioxidant enzymes (**a**, catalase; **b**, Peroxidase and **c**, Superoxide dismutase) in tomato seedlings at different days post inoculation (dpi). Each treatment consisted of three plants per replicate (9 samples per treatment) which are compared by LSD test at p<0.01 (LSD values for charts **a**, **b** and **c** are 10.47, 16.1 and 8.07, respectively). Error bars indicate standard errors from an average of 9 measurements. Values with the same letter are not different significantly at 1% probability level according to student *t*-test for period of time using 0 dpi as the control. SA, salicylic acid; JA, jasmonic acid

MDA content increased over the time in all the treatments except for SA+CMV and SA+JA+CMV. In these treatments, MDA content increased until 4 dpi and then decreased to 15 dpi. The highest MDA content was related to CMV-infected plants at 15 dpi (7.42 nM g^{-1} FW) which was about 2
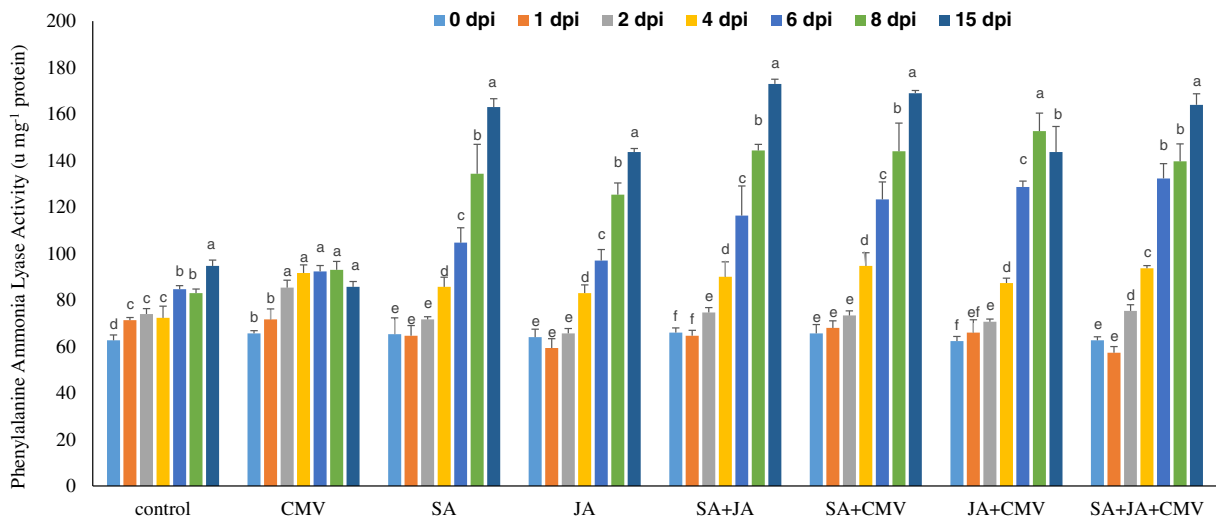
Fig. 2 Phenylalanine Ammonia Lyase changes in response to CMV and hormone application in tomato leaves at different sampling days (0-15 dpi). Values are the average of 9 records which compared using LSD (8.1) at p<0.01. Error bars indicate standard errors from an average of 9 measurements. Values with the same letter are not different significantly at 1% probability level according to student *t*-test for period of time using 0 dpi as a control

folds greater than the control at the same time (Table 2).

Changes in the levels of MDA and H_2O_2 are in agreement with the activity of CAT and POD.

PAL activity

PAL activity increased in all the treatments over the time (Fig. 2) and the highest activity (173 U mg^{-1} protein) was observed in SA+JA treatment at 15 dpi (Fig. 2).

Net Photosynthesis (NP) and chlorophyll content

A significant interaction was observed between the treatments and length of time for NP and chlorophyll content (Table 1). In the control treatment, NP increased over the time period and reached its highest amount (11.43 μM CO_2 m^{-2}s^{-1}) at the final sampling (15 dpi). NP was decreased in other treatments. Virus infection and hormone application led to the NP reduction over the time (Table 2). The lowest NP was observed in the combination of virus and JA application at 15 dpi (6.53 μM CO_2 m^{-2}s^{-1}).

Virus infection decreased Chl a content over the time but had no significant effect on Chl b content (Table 2). Hormones increased both Chl a and b contents in comparison to the control over the time even in the virus-infected plants, but the levels of Chl a was decreased only

in the CMV-treated plants. In the control treatment, chlorophyll content also increased over the time (Table 2).

Proline and total soluble carbohydrate (TSC) content

Proline content did not change over the time in the control, but in the rest treatments it was increased 2.5-3.5 folds at 15 dpi in comparison to 0 dpi. The highest proline content (4.35 mM g^{-1} FW) was observed in JA+CMV-treated plants at 15 dpi which was 3 fold greater than at 0 dpi (Table 2).

TSC content decreased over the time in all the treatments except for the control, but the reduction was not significant with the exception of the virus-infected plants. CMV reduced the TSC content by about 50% in the infected plants at 15 dpi in comparison to 0 dpi. In the control plants, 29% increase in TSC was observed from 0 to 15 dpi. The highest TSC content was related to the control treatment at 15 dpi (4.47 mg g^{-1} DW). TSC reduction speed was slower in the hormone-treated plants than in the CMV-infected plants (Table 2) and it seemed that the hormone treatments inhibited the reduction of TSC either alone or in combination with CMV.

Virus replication

SA and JA treatments reduced the virus replication significantly in comparison to the CMV- infected plants. The highest reducing effect was observed in SA+JA+

Table 1 Analysis of variance for the effects of CMV and hormone treatments on the physiological traits of tomato leaves

Mean Squares

SOV	df	NP	CAT	POD	SOD	H_2O_2	MDA	Chl a	Chl b	Proline	PAL	TSC
Treatment	7	17.75**	49584.5**	44044.8**	9145.1**	23396.1**	0.075**	0.219**	0.066**	5.07**	2303.2**	400.349**
Time period	6	10.3**	12377**	91180.3**	32686.1**	841.36**	0.197**	0.0804**	0.0044ns	13.8**	23163.1**	290.526**
Treatment*time	42	1.79**	4268.9**	4878.2**	870.6**	3417.2**	0.14**	0.064**	0.0081**	0.44**	729.9**	29.243**
Error	112	0.128	41.904	99.095	24.494	6.59	0.0034	0.0216	0.0034	0.0132	25.066	0.9418
CV (%)		4.5	1.9	3.4	4.4	1.82	12.6	10.1	9.99	5.29	5.23	3.28

**represents the significant effect at 1% probability level, ns, non-significant. SOV: source of variation; df: degree of freedom; CV: coefficient of variance; NP: net photosynthesis; CAT: catalase; POD: peroxidase; SOD: superoxide dismutase; MDA, malondialdehyde; Chl a and b: chlorophyll a and b; PAL: Phenylalanine ammonia lyase; TSC: total soluble carbohydrates

CMV treatment which was about 34% at 15 dpi in comparison to the CMV-infected plants. The control and hormone-treated plants without CMV had no detectable CMV-*CP* gene as determined by qRT-PCR (Fig. 3).

Since the effects of hormones on CMV levels and antioxidant enzymes activity were best observed at 2 and 4 dpi, we compared the means for these two days and the results are shown in Table 3. Application of SA+ JA had the highest reducing effect on CMV-*CP* gene expression at 2 and 4 dpi (47 and 29% reduction of virus replication, respectively). POD and SOD activity increased in all treatments except the control, but CAT activity decreased in CMV and the control plants (Table 3). Therefore, increase in virus replication from 2 to 4 dpi resulting in oxidative burst was accompanied with the increase in the activity of antioxidant enzymes.

Discussion

Reactive oxygen species (ROS) are produced in all organisms even under normal conditions and also in response to biotic and abiotic stresses. Superoxide radical is the initially-produced ROS which is scavenged by SOD resulting in the production of secondary ROS molecule H_2O_2. CAT and POD can catalyze H_2O_2 to H_2O and O_2 with different mechanisms (Sedghi et al. 2012). Virus infection accompanied with an increase in cell metabolism enhances the production of ROS which subsequently damages the cell compartments. But, it is proven that in the early days of infection an increase in H_2O_2 content is accompanied by reduction in antioxidants activity thus restricting growth and spread of pathogen, and then the cells undergoing programmed cell death (PCD) produce signals that activate defense responses in adjacent cells. SA, ethylene and JA, and its derivatives, are signal molecules involved in the amplification of plant defense responses (Hayat and Ahmad 2007). In this study, CMV infection decreased CAT activity until the second week of the inoculation, but POD activity increased slowly in the first week (Figs 1a and b). It shows that scavenging H_2O_2 in tomato is probably related to increase in POD activity and reduction in the CAT activity leads to increase of H_2O_2 content as the secondary messenger in LAR (Table 2). Since POD is widely distributed in plants and has more affinity for H_2O_2 than CAT, it is an efficient scavenger of H_2O_2 under stress (Das and Roychoudhury 2014). There are contradictory reports regarding the CAT

Table 2 Comparison of the means for the combined effects of hormone treatment and CMV infection with time in the studied traits of tomato leaves

Treatment	Time (day)	NP (μM CO$_2$ m^{-2} s^{-1})	Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)	Proline (mmol g^{-1} FW)	TSC (mg glucose g^{-1} DW)	H$_2$O$_2$ (nM g^{-1} FW)	MDA content (nM g^{-1} FW)
Control	0	8.53	1.32	0.58	1.34	3.466	134.67	1.61
	1	9.3	1.46	0.68	1.18	3.623	140.3	1.92
	2	9.4	1.54	0.65	1.22	3.74	149.6	3.18
	4	10.5	1.75	0.65	1.21	4.043	165.7	2.32
	6	10.9	1.51	0.73	1.23	4.123	185.3	2.65
	8	11.13	1.69	0.73	1.30	4.26	190.7	3.44
	15	11.43	1.57	0.74	1.23	4.473	204.4	3.46
CMV	0	7.96	1.70	0.55	1.27	3.436	139	1.58
	1	8.30	1.30	0.58	1.25	3.076	152	2.05
	2	8.33	1.32	0.54	1.48	2.846	175.3	2.22
	4	7.63	1.24	0.52	1.81	2.636	191.6	3.23
	6	7.60	1.21	0.53	2.10	2.163	215	4.46
	8	7.36	1.20	0.52	2.23	1.83	254.7	5.42
	15	6.33	1.17	0.48	2.56	1.703	303.3	7.42
SA	0	8.60	1.31	0.51	1.13	3.48	133.4	1.53
	1	8.16	1.16	0.54	1.41	3.26	138.3	1.7
	2	7.63	1.29	0.57	1.63	3.15	136.7	1.87
	4	8.10	1.54	0.54	1.89	2.91	124.7	1.84
	6	7.70	1.55	0.61	2.33	2.67	105.3	2.19
	8	6.66	1.50	0.66	2.75	2.59	87	2.42
	15	6.03	1.34	0.56	3.03	2.48	49	2.22
JA	0	8.40	1.29	0.49	1.30	3.466	141	1.83
	1	8.66	1.50	0.51	1.32	3.313	146	1.89
	2	8.50	1.54	0.54	1.62	3.04	139.7	2.29
	4	8.13	1.39	0.45	1.89	2.683	133.6	2.16
	6	7.60	1.43	0.57	2.28	2.586	115	2.42
	8	7.50	1.34	0.46	2.7	2.42	102.7	2.64
	15	6.20	1.83	0.49	3.1	2.19	96.3	2.31
SA+JA	0	9.43	1.38	0.62	1.30	3.493	140	1.43
	1	8.13	1.36	0.58	1.58	3.416	144.7	1.72
	2	8.33	1.50	0.64	1.80	3.266	130.4	1.79
	4	7.70	1.82	0.64	2.13	3.09	115.4	2.23
	6	7.56	1.77	0.62	2.58	2.866	99.3	2.37
	8	7.93	1.57	0.67	3.13	2.65	64.7	2.29
	15	6.26	1.70	0.70	3.51	2.51	46.3	2.45
SA+CMV	0	8.33	1.66	0.54	1.23	3.44	135.6	1.65
	1	8.73	1.49	0.55	1.65	3.086	147.7	1.76
	2	8.16	1.64	0.54	2.05	2.856	154	2.23
	4	8.16	1.44	0.51	2.37	2.513	145.7	2.49
	6	7.63	1.46	0.62	2.80	2.613	132.6	2.33
	8	6.66	1.73	0.64	3.49	2.503	130	2.12
	15	6.53	1.54	0.53	4.26	2.48	125	1.67
JA+CMV	0	8.70	1.15	0.55	1.26	3.373	135.3	1.6

Table 2 (continued)

Treatment	Time (day)	NP (μM CO$_2$ m^{-2} s^{-1})	Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)	Proline (mmol g^{-1} FW)	TSC (mg glucose g^{-1} DW)	H$_2$O$_2$ (nM g^{-1} FW)	MDA content (nM g^{-1} FW)
	1	8.13	1.39	0.61	1.58	3.156	145	1.66
	2	7.80	1.35	0.64	1.92	2.763	151.4	2.03
	4	7.36	1.55	0.64	2.60	2.526	156	2.35
	6	6.30	1.49	0.54	2.83	2.383	162	2.72
	8	6.33	1.36	0.52	3.41	2.316	162.4	2.32
	15	5.40	1.44	0.59	4.35	2.16	169	2.39
SA+JA+CMV	0	9.40	1.34	0.63	1.21	3.616	134.4	1.51
	1	8.43	1.28	0.65	1.74	3.44	147.3	2.06
	2	8.60	1.32	0.66	2.30	3.143	158.4	2.46
	4	7.93	1.38	0.61	2.63	2.85	124.4	2.69
	6	7.10	1.43	0.53	3.29	2.796	104.7	2.19
	8	6.63	1.44	0.47	3.91	2.486	90	2.09
	15	6.06	1.54	0.53	4.34	2.226	74.3	1.67
LSD		0.581	0.24	0.095	0.19	1.57	4.15	0.095

Means in each column that are not greater than LSD have no significant difference at p<1%. NP: net photosynthesis; Chl a and b: chlorophyll a and b; TSC: total soluble carbohydrates; MDA: malondialdehyde content. Each treatment is the mean of three replicates and each replicate consisted of three plants except of NP which is the mean of 15 records in each replicate. Means were compared as interaction between time period and treatments used in the experiment (simple effects were not compared because of significant effect of interactions based on ANOVA table)

activity under virus infection in the literature. For example, Kobeasy et al. (2011) about *Peanut mottle virus* infection in *Arachis hypogaea*, Siddique et al. (2014) about *Cotton leaf curl burewala virus* in cotton, and

Sarkar et al. (2010) about *Yellow vein mosaic virus* in *Hibiscus cannabinus,* demonstrated increase in the activity of CAT during HR. In contrast, other reports have dealt with decreased CAT activity in *Phaseolus vulgaris*
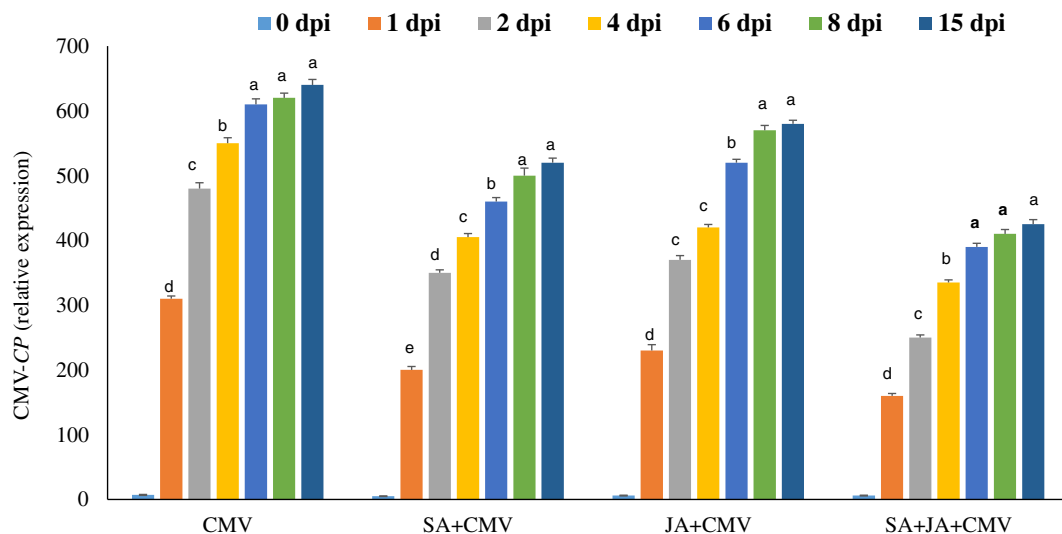

Fig. 3 CMV-*CP* gene expression levels in the tomato leaves infected by CMV and treated with SA and JA. The CMV replication levels were monitored by quantitative real-time PCR at 0, 1, 2, 4, 6, 8 and 15 days post inoculation (dpi). Error bars indicate standard error for 3 replicates and letters are the grouping of each treatment in the time intervals according to Student *t* test (p<0.01). Uninfected control plants and SA, JA and SA+JA treated plants had no virus infection and are not shown in the figure

Table 3 Comparison of means for CMV-*CP* gene expression and antioxidant enzymes activity at 2 and 4 dpi

Treatment	CAT (U min^{-1} g^{-1} FW)		POD (U min^{-1} g^{-1} FW)		SOD (U min^{-1} g^{-1} FW)		CMV-*CP* (relative expression)	
	2 dpi	4 dpi	2	4	2	4	2	4
Control	279	236.7	204	192.3	52.6	59.6		
CMV	293.3	288.7	214	285.6	84.3	95	480	549.67
SA	367.3	371	233	284	94	119.6		
JA	345	374	262.3	304.6	83.3	105.6		
SA+JA	364	381	249.3	308.6	93.6	116.6		
SA+CMV	353.3	379	268.3	310.3	101	127.6	354.67	400
JA+CMV	332	362.3	287.3	321.3	94.6	116.3	330.7	424
SA+JA+CMV	349	383	273.6	326.6	86	120.3	254.3	339
LSD 5%	10.47		16.1		8.07		8.03	

Means in each column that are not greater than LSD have no significant difference at p<1%. CAT: catalase activity, POD: peroxidase activity, SOD: superoxide dismutase activity, CMV-*CP*: CMV coat protein gene expression. Each treatment is the mean of three replicates and each replicate consisted of three plants. Means were compared as interaction between time period (2 and 4 dpi)

infected with *White clover mosaic potexvirus,* WCIMV (Clarke et al. 2002). JA protective effect is related to the regulation of pathogen-induced proteins (Burkhanova et al. 2007). Liao et al. (2012) demonstrated that in tomato plants infected with TMV, both CAT and POD activities were increased and the highest activities was observed when SA was used. Yang et al. (2016) also reported an increase in SOD and CAT activities in *Momordica charantia* plants infected with CMV, but increase in POD activity was insignificant. Also, JA improved plant resistance to CMV and therefore it is concluded that ROS are involved in JA-mediated CMV resistance (Yang et al. 2016). ROS are involved in incompatible plant-virus interactions (Kiraly et al. 2008), but their involvement in compatible plant-virus interactions has not been fully demonstrated in literature (Yang et al. 2016). The accumulation of ROS in incompatible plant-pathogen interactions is regarded as an early and important mechanism for killing pathogens and/or suppressing symptoms development (Hernandez et al. 2015). It has been reported that in the JA-treated plants the content of ROS was the highest, but the damage was the least. This is an evidence that confirmed the role of ROS as the secondary messengers which are mediated by JA during virus resistance (Yang et al. 2016).

PAL activity in plant tissues is responsible for the formation of phenolic compounds which uses phenylalanine as a precursor in the phenyl propanoid pathway and is therefore involved in the biosynthesis of the polyphenol compounds such as SA, flavonoids, phenyl propanoids, and lignin in plants (Ali et al. 2007). The activity of PAL is induced dramatically in response to various stimuli such as tissue wounding, pathogenic attack, light, low temperatures, and hormones (Aldesuquy et al. 2015). Falcioni et al. (2014) demonstrated that PAL activity in tomato leaves increases by SA application under PVX infection. Don et al. (2010) also reported that triggering accumulation of phenolic compounds in response to SA is dose-dependent. It seems that SA and JA activate PAL and its activity leads to the production of phenolics which together with other antioxidants establishes the local and systemic resistance network (Asghari and Hasanlooe 2015).

There are some reports about the effect of SA on photosynthesis. Many of them concluded that SA concentration can influence photosynthesis (Stevens et al. 2006; Falcioni et al. 2014). It is proposed that accumulation of SA can induce PCD in tomato and bean (Senaratna et al. 2002). SA can promote alternative oxidase pathway (AOX) to protect plants against oxidative stress (Lee et al. 2011). This respiratory burst may be the reason for a decrease in net photosynthesis rate. Falcioni et al. (2014) concluded that the reason for contradictory results of SA application is due to the concentration of SA used in the treatment. In other words, SA only positively influences tomato photosynthetic activity when it is used under 1 mM concentration. Yang et al. (2016) observed that the use of ibuprofen (a JA inhibitor) significantly decreased photosynthesis in *Momordica charantia* and its resistance against CMV infection, but not influenced by 1-aminobenzotriazole (ABT, a SA inhibitor). They concluded that JA protects photosynthetic apparatus in CMV-infected plants.

CMV infection did not decrease chlorophyll b content in comparison to the control from 1 to 15 dpi, but chlorophyll a content is significantly decreased by CMV infection (Table 2). It is reported that chlorophyll a is more sensitive to viral infection than chlorophyll b (Sinha and Srivastava 2010). In hormone treatments, either single applications or combination with virus infection, a significant recovery was observed in subsequent days except for the combination of SA, JA and virus for chlorophyll a. Montasser et al. (2012) reported a reduction in the chlorophyll content in tomato plants infected by *Tomato yellow leaf curl bigeminivirus* (TYLCV-K) 2 weeks after appearance of visual symptoms. However, the positive effect of SA on photosynthetic activity is not always due to an increased chlorophyll level. In cowpea (*Vigna unguiculata*) plants, SA treatment increased or decreased chlorophyll content depending on the genotype (Chandra and Bhatt 1998) or the applied concentration, as 0.001-10 M SA increased while 1 mM SA decreased both chlorophyll and carotenoid contents in the cotyledons of sunflower plants (Çag et al. 2009). Infection of *Vicia faba* plants with *Bean yellow mosaic virus* (BYMV) decreased Chl a and Chl b content significantly and the application of 10-100 μM SA compensated this reduction in the presence of the virus, but SA application on uninfected plants at 100 μM caused significant increase in chlorophyll content (Radwan et al. 2007). In potato plants infected by PVY qualitative composition of pigments in healthy and infected leaves was similar, but quantitative amounts were genotype-dependent such that cultivar Desiree had no significant difference in chlorophyll a and b content whether infected and uninfected (Anzlovar et al. 1996).

Proline accumulation is a typical plant response to environmental stresses and also activates a hypersensitive response to biotic infections (Radwan et al. 2007). SA can also induce proline accumulation in healthy and infected plants. Proline contributes to scavenging ROS (Fabro et al. 2004) and may be involved in the prevention of the PCD mediated by ROS (Chen and Dickman 2005). Pazarlar et al. (2013) reported a significant increase of proline content in response to TMV in pepper. Virus infection improves plant tolerance to abiotic stress, which correlates with increased osmoprotectants such as proline and antioxidant levels in infected plants with BMV, CMV, TMV and *Tobacco rattle virus* (TRV) (Xue et al. 2008). It may be possible that SA activates the enzymes of proline metabolism and consequently enhances the accumulation of proline (Hayat et al. 2008).

There was a drastic decrease in TSC under the virus infection, but TSC content reduction was not significant in the hormone treatments (Table 2) and it appeared that the hormone treatments inhibit the reduction of TSC either alone or in combination with CMV. Carbohydrates are the main organic constituents of dry matter which are derived from photosynthesis and they are affected by biotic and abiotic stresses. The reduction of TSC content is in agreement with NP and chlorophyll content (Table 2) which shows that virus infection reduces the plant production capacity while defense hormone application alleviates the reduction of TSC and inhibits the significant reduction. Many researchers have reported the reduction in chlorophyll content, photosynthesis rate and soluble and insoluble sugars in infected plants. For example such results were demonstrated by Radwan et al. (2007) in *Cucurbita pepo* infected by ZYMV (*Zucchini yellow mosaic virus*) and Hemida (2005) in bean plants infected by BYMV. Khalil et al. (2014) also reported that TSC content decreased in the leaves of tomato plants, but there was an increase in TSC content in the roots when infected by TYLCV (*Tomato yellow leaf curl virus*). It has been demonstrated that in PVX-infected tomato plants, SA application increased the amount of photosynthesis and carbohydrate content, but this increase could not reach the amounts in healthy plants, suggesting activation of metabolic pathways related to energy availability and molecule biosynthesis by SA (Cueto-Ginzo et al. 2016).

The highest reducing effect was observed in SA+JA+CMV treatment, then SA+CMV and finally in JA+CMV treatments (Fig. 3). The expression levels of CMV-*CP* gene in *Arabidopsis* leaves infected by CMV was shown to be the lowest when JA application was followed by SA. Combination of SA with JA and application of SA and JA alone had the next order in the reduction of virus replication (Luo et al. 2011). Another study demonstrated that *SlMAPK3* transgenic tomato plants compared with the wild type had greater expression of SA- and JA-defense-related genes and less *Tomato yellow leaf curl virus* (TYLCV) content (Li et al. 2017). Exogenous application of both SA and JA induced stronger resistance in tobacco against virus attack compared with application of SA or JA alone (Shang et al. 2011). SA treatment can delay or prevent the appearance of disease symptoms in BYMV-infected *Vicia faba* plants, suggesting that SA acts as inducer for at least one mechanism of resistance against the virus infection (Radwan et al. 2008). Previous reports indicate

that SA not only has significant roles in the RNA silencing mechanism, but also delays accumulation of RNA pathogens (Lewsey et al. 2009). *MAPK3* may also play a key role in the development of induced resistance against viruses by coordinating the expression of defense genes in SA- and JA-mediated pathways (Li et al. 2017). CMV as a biotic stress enhances the production of ROS and oxidative stress. In this condition, plants activate the antioxidant defense mechanisms such as POD, SOD and CAT enzymes which are responsible for scavenging the ROS. Production of PR and antioxidant enzymes under the application of SA and JA maybe the reason for decreasing virus levels against CMV infected plants in this study. TMV replication was strongly inhibited in mesophyll protoplasts isolated from SA-treated non-transgenic tobacco plants and it is concluded that SA can have fundamentally different effects on the same pathogen in different cell types (Murphy and Carr 2002). The RNA from the CMV particles taken up by or adsorbed to the Cowpea protoplasts during the inoculation procedure could be detected at 0 hours post inoculation (hpi) (Gonda and Symons 1979) and RNA and coat protein synthesis for CMV were detected from 5 hpi in zucchini squash protoplasts (Gal-On et al. 1994). Further detailed studies are required to test SA- and JA-induced resistance against CMV and clarify the mechanisms contributing to viral accumulation, replication or cell-to-cell movement.

From the results of this study, it can be concluded that CMV infection increased ROS production in tomato leaves which is the part of resistance pathway in early days of infection. Hormone treatments decreased the replication of CMV efficiently at least in the first week of infection and established LAR in adjacent cells. Therefore, application of SA and JA may be helpful for tomato plants under CMV infection to improve its resistance mechanisms.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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