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Enhanced Tolerance to Cowpea Mosaic Virus in *Vigna unguiculata* L. Plants Pretreated with Salicylic Acid

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COWPEA mosaic virus (CPMV) has been identified as an economically critical pathogen infecting cowpea plants. Recently, eco-friendly strategies to enhance the tolerance level of crops against virus infection have been developed. The present investigation evaluates the effect of salicylic acid (SA) on the response of three cultivars of *Vigna unguiculata* plants [Libyan black eye (LB), Libyan red eye (LR), and Egyptian black eye hybrid (Kafr El Sheikh1cultivar; (EBH)] to CPMV. The leaves of 15-day old cowpea plants were foliar sprayed with 50 or 100 µM SA, 24 h before inoculation with CPMV. Most measured growth indices, total photosynthetic pigment, and photosynthetic efficiency were diminished in CPMV-inoculated plants of all three cultivars. SA utilization could enhance cowpea growth and lower virus severity, most notably in the Libyan cultivars. Furthermore, the CPMV-induced oxidative damages and phenolics accumulation were noted to decrease upon SA application to all infected cowpea cultivars. CPMV infection triggered an increase in catalase (CAT), guaiacol peroxidase (GPX), and polyphenol oxidase (PPO) activity in all cultivars, except for EBH where PPO was insignificantly changed. Interestingly, SA pretreatment was observed to significantly inhibit CAT activity in the Libyan cultivars compared to infected plants. It also induced GPX and PPO in all three tested cultivars, most obviously in the Libyan ones. In general, SA was effective in inducing of systematic resistance to CPMV in the two Libyan cowpea cultivars but not in the Egyptian one.

Keywords: Antioxidant enzymes, Cowpea mosaic virus, Salicylic acid, Oxidative stress, Phenolic.

Introduction

Cowpea, *Vigna unguiculata*, has been identified as a major food legume cultivated commercially in most tropic and sub-tropic environments (Kareem & Taiwo, 2007). It is a valuable leguminous crop because of its ability to fixate atmospheric nitrogen, and is extensively utilized as a protein source with high nutritive value worldwide, including in Egypt (Abd El-Aziz, 2015). Cowpea production and maintenance are essential to meet market needs. Like most food crops, cowpea is subjected to a variety of pests and pathogens among which infection with cowpea mosaic virus (CPMV) is the most widely reported in Egypt (Mazyad et al., 1981; Younes et al., 2018). CPMV is the type member of the genus *Comovirus* in the family Comoviridae which causes a wide array of symptoms ranging from light green mottle to distinct yellow mosaic, distortion of leaf, reduction of leaf area and premature death of the plant (Bliss & Robertson, 1971). Infection allows the virus to invade the cellular machinery and counteract plant defenses. However, plants are known to have complex defense mechanisms triggered by microbial pathogen attack, including biochemical, molecular, and morphological changes, such as expression of pathogenesis-related genes, production of antimicrobial compounds, programmed cell death, and oxidative burst (Thomas et al., 2000).

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Mosaic infection of cowpeas resulted in a great reduction in the growth and yield parameters as well as the nutritive content of the seeds (Aliyu et al., 2012; Khalil et al., 2014). Reduction in chlorophyll contents and disorder of chloroplast structure and function have also been reported in several host plant-virus combinations including CPMV (Sinha & Srivastava, 2010; Pazarlar et al., 2013). Viruses are known to interfere with photochemical reactions by inhibiting the electron transport of photosystem II (PSII) and CO_2 fixation as has been demonstrated in tobacco mosaic virus (Rahoutei et al., 2000) and cassava common mosaic virus (Zanini et al., 2021). In this context, alterations in electron transport might generate reactive oxygen species (ROS), which in turn, elicit oxidative stress and produce cellular damages (Rodríguez et al., 2010; Chávez-Arias et al., 2020). Increased levels of ROS can influence pathogenesis and development of symptoms in compatible interactions such as zucchini yellow mosaic virus and cucumber mosaic virus with plants of *Cucurbita pepo* and *Cucumis sativus* (Riedle-Bauer, 2000; Sofy et al., 2020). Plant cells, however, are defended against ROS oxidative damage through the production of non-enzymatic antioxidants, phenolics, and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), polyphenol oxidase (PPO), and peroxidase (POD The activity of various antioxidant enzymes were significantly increased in several host plant-virus combinations encompassing bean yellow mosaic virus (Radwan et al., 2010), tomato leaf curl virus (Mishra et al., 2014), and cucumber mosaic virus (Sofy et al., 2020).

SA is a phenolic phytohormone formed by phenylpropanoid pathways in plants (Radwan et al., 2010). It affects a number of key physiological processes such as seed germination, growth, photosynthesis, stomatal closure, and fruit yield; most importantly, it can induce plant defense reactions against different viruses without any harmful environmental effects (Radwan et al., 2010; Le Thanh et al., 2017). SA may activate a molecular signal transduction pathway that is often characterized by enhancement of pathogenesis-related (PR) proteins and the subsequent induction of a systemic acquired resistance (SAR) defense response (Carr et al., 2019). Zhang et al. (2016) reported that SA application decreased leaf lesions caused by *Glomerella cingulata* and increased the total antioxidant capacity in *Malus domestica* Borkh plants. SAR-mediated by SA has been reported in many host plants infected with different viruses such as zucchini yellow mosaic in *Cucurbita pepo* (Alawlaqi, 2014) and bean common mosaic virus in *Phaseolus vulgaris* (Mardani-Mehrabad et al., 2020).

Currently, the lack of control strategies has led, in some cases, to significant crop loss and increased attention to the development of alternative methods for the soil pathogen management (Chávez-Arias et al., 2020). In this regard, the present study was designed to assess the efficacy of foliar application of SA in the protection of Egyptian and Libyan *V. unguiculata* cultivars against CPMV. The changes in some growth parameters, photosynthetic pigments, photosynthetic efficiency, and changes in the behavior of antioxidative metabolism were evaluated.

Materials and Methods

Plant material, growth conditions, and treatments

Three *V. unguiculata* cultivars were used in this study: the Egyptian black eye hybrid (Kafr El Sheikh1cultivar; EBH) from the Agricultural Research Center, Giza, Egypt, and two Libyan cultivars, that is, black eye cowpea (LB) and red eye cowpea (LR) from the Agriculture Research Center, Sabha, Libya. The seeds were surface sterilized with 4% sodium hypochlorite for 10min, washed with distilled water several times, and soaked for 24h at 25°C in aerated water. To investigate the effect of salicylic acid (SA) on germination, a preliminary experiment was conducted in which 20 seeds were allocated at random in Petri dishes (15 cm diameter) containing filter paper moistened with 20mL of various SA concentrations (0, 10, 25, 50, 75, 100, and 150µM) covered, and incubated at natural environmental conditions (28°C ± 2°C) for 4 days. The germination percentage was then calculated after 4 days as a standard of radicle emergence, and the effective concentrations of SA were 50 and 100 µM. Seeds of uniform size were sterilized, as previously mentioned and then transferred to weighed plastic pots (10cm in diameter and 20cm length) filled with acid-washed quartz sand and clay (2:1). Ten seeds were germinated in each pot and the pots were then placed in separated controlled growth chambers under a 16-h photoperiod, 28/25°C± 2˚C light/dark temperature and relative humidity of about 60% ± 5%. The plants were kept at 80% water holding capacity and irrigated with distilled water every

2-day interval throughout the whole experimental period. After 15 days of growth, the pots were divided into four groups of 3 replicates. The first group was left as a control without any treatment; the leaves were sprayed with distilled water. In the second group, SA treatment was applied by spraying the leaves with 100 µM SA until runoff. In the third group, the plants were left for more 24h and then inoculated with CPMV without any other treatment. The fourth and fifth groups were sprayed with 50 and 100µM SA, respectively 24h prior inoculation with CPMV. All leaves were mechanically inoculated. For virus inoculation, infected leaves of cowpea plants (Kafr El Sheikh cultivar showing characteristic CPMV symptoms and positive by ELISA) were homogenized in 0.1M phosphate buffer pH 7.0 (1:2 W/V), and the homogenate was filtered through two layers of muslin. The leaves of healthy plants were then dusted with carborundum, and rubbed gently with cotton swab previously dipped in the suspension of virus inoculum. Twenty-one days post inoculation (36 day-old cowpea plants), homologous plants were harvested, washed thoroughly from adhering soil particles, gently plotted, dissected to shoots and roots and quickly saved for estimation of the various growth parameters and chemical analyses. All chemical analyses were performed on roots and shoots.

CPMV detection by DAS-ELISA

The double antibody sandwich (DAS)-ELISA technique was applied as described by Lister & Rochow (1979) for CPMV concentrations in the infected and (SA+V) treated leaves using an ELISA kit (Art.No.0012 –DSMZ, Germany).

Growth parameters

Roots and leaves were utilized for determination of fresh and dry biomass. Shoot height was measured. Leaf relative water content (RWC) was determined as described by Silveira et al. (2003) based on the following equation:

$$[(FM - DM) / (TM- DM)] \times 100$$

where, FM is the leaf fresh mass, DM is leaf dry mass (after drying at 80°C for 48 h), and TM is the turgid mass of leaves (after soaking in water for 4h at room temperature).

Estimation of photosynthetic pigments

Photosynthetic pigments were analysed after grinding of leaf samples with acetone as described by Metzner et al. (1965).

Measurement of chlorophyll fluorescence

Measurements of chlorophyll fluorescence was performed with OS-30P pulse modulated chlorophyll fluorimeter (Opti-Sciences, Hudson, USA) following the procedure described by van Kooten & Snel (1990).

Estimation of H_2O_2, lipid peroxidation, and total phenolics

Hydrogen peroxide content was estimated according to Velikova et al. (2005). Tissue was homogenized in 0.1% (w/v) TCA, 0.5mL of the supernatant was mixed with 0.5mL of 10mM potassium phosphate buffer (pH 7.0) and 1mL of 1M KI, and the absorbance was read at 390nm. Lipid peroxidation was monitored by spectrophotometric measurements of malondialdehyde (MDA) using thiobarbituric acid (TBA) as shown by Valentovič et al. (2006). Tissue samples (100 mg) were homogenized in 5mL of TCA (0.1%, w/v), and aliquots of the supernatant were heated in 0.5% TBA in 20% TCA. MDA content was determined from the absorbance at 532 nm, followed by correction for non-specific absorbance at 600nm. The extinction coefficient used was 155mM^{-1} cm^{-1}. Total phenolic contents were determined using the modified Folin-Ciocalteu reagent method, as described by McDonald et al. (2002) and expressed as mg gallic acid g^{-1} FM.

Enzymes assay

For estimation of some antioxidant enzymes activity, frozen plant tissues were homogenized in ice-cold 0.1M potassium phosphate buffer (pH 6.8) containing 0.1mM EDTA. The homogenate was centrifuged at 15,000g for 20min at 4°C, and the supernatant was used for the determination of catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (GPX, EC 1.11.1.7), and polyphenol oxidase (PPO, EC 1.10.3.1) activity. CAT activity was determined according to Azevedo Neto et al. (2006). The reaction mixture (1.5mL) consisted of 100mM phosphate buffer (pH 7.0), 0.1µM EDTA, 20mM H_2O_2, and 50µL enzyme extract. The decomposition of H_2O_2 was monitored at 240nm and CAT activity was calculated using the extinction coefficient of 36 M^{-1} cm^{-1} and was expressed as µmol H_2O_2 g^{-1} FM min^{-1}. Total GPX activity was determined as described by Urbanek et al. (1991) in a reaction mixture (2.0mL) containing 100mM phosphate buffer (pH 7.0),

0.1µM EDTA, 5.0mM guaiacol, 15.0mM H_2O_2 and 50µL enzyme extract. The guaiacol oxidation to tetraguaiacol was followed for 5min at 470nm GPX activity was calculated using the extinction coefficient of 26.6mM^{-1} cm^{-1} and expressed as µmol guaiacol g^{-1} FM min^{-1}. PPO was assayed following the method described by Kumar & Khan (1982) where the assay mixture contained 2mL of 0.1M potassium phosphate buffer (pH 6.0), 1mL of 0.1 M catechol, and 0.5mL of enzyme extract and the reaction was stopped by adding 1mL of 2.5N H_2SO_4. The formation of the purpurogallin was followed at 495nm and PPO activity was expressed in U min^{-1} g^{-1} FM.

Statistical analysis

Statistical analysis of the results was done using SPSS software package version 20.0 to obtain the mean, and the standard error. ANOVA was used to assess the significant difference among the control and treated groups. LSD was estimated P≤ 0.05.

Results

CPMV symptoms and growth parameters

As per findings of this study, CPMV-infected cowpea leaves (LR, LBH, and EBH cv.) showed severe symptoms compared to healthy leaves (Fig. 1). Generally, characteristic symptoms were observed 7 days post inoculation (dpi) and became stronger on the two Libyan cultivars at 21 dpi. These symptoms included intense mosaic and reduced leaf size in LR (Fig. 1 C), intense interveinal chlorosis, and mosaic in LBH (Fig. 1H), and chlorotic spots and mosaic in EBH (Fig. 1M). The effect of SA treatment on CPMV symptom expression was noted to vary in the three tested cowpea cultivars at 21 dpi. For the LR cultivar at V + 50 µM SA treatment, the virus spread was restricted to localized necrotic small areas on the leaf blade (Fig. 1D). This result is indicative of a hypersensitive response (HR) in the form of infected cell death, which restricts virus translocation in the plants. In the V +100 µM SA treatment, the lower new leaves of LR showed no symptoms while the older ones showed HR resistance type (Fig. 1E). For the LBH cultivar, the effect of SA as inducer of resistance to CPMV was well demonstrated as the leaves of V + 50 µM SA- and V + 100 µM SA-treatments showed typical necrotic local lesion pinpoints indicative of the HR type of resistance (Figs. 1I, J). In contrast to the two Libyan cultivars, both 50 and 100 µM SA treatment increased the CPMV symptoms development from 7 to 21 dpi in EBH, thus there was no signs of HR resistance. It started as chlorotic spots at 7 dpi, increased to intense mosaic at 14 dpi and intense mosaic, chlorosis, leaf distortion, and bleaching in the lower leaves at 21 dpi (Figs. 1N, O).

CPMV accumulation in infected cowpea leaves decreased significantly in plants treated with SA compared to CPMV–infected, non treated plants as measured by ELISA (Table 1), most obviously in Libyan cultivars, whereas SA had almost no effect in EBH.

CPMV had a significant inhibitory effect on most growth attributes of the three cowpea cultivars such as RWC, shoot height, leaf area, FM, and DM whereas the leaf number was insignificantly changed in comparison to control un-inoculated plants (Table 2). The reduction of growth biomarkers was more obvious in EBH than in Libyan cultivars. For example, the FM of the shoots of LR, LBH and EBH at 21 dpi was 45%, 32%, and 59% respectively, compared to controls. The corresponding values for roots were 38%, 43%, and 44% respectively. Spraying cowpea plants with 50 or 100 µM SA 24 h before inoculation has resulted in significant maintenance of measured growth parameters, compared to control, most obviously the 100 µM SA treatment. At 21 dpi, the total FM of both roots and shoots of plants sprayed with SA without inoculation were 8.60, 8.42, and 9.53 gm seedling^{-1} for LR, LBH and EBH, respectively compared to 8.29, 7.83 and 9.18 gm seedling^{-1} for their controls, respectively (Table 2).

Photosynthetic pigments and photosynthetic efficiency

The total pigment content significantly declined in response to CPMV, particularly in EBH, and this decline was mainly attributed to the reduction in chl. a (Table 3). However, SA spraying could enhance the chl. a, chl. b and carotenoid content in CPMV- inoculated LR and LBH cultivars, while EBH was insignificantly affected. The ratio F_v/F_m which reflects the quantum efficiency for photochemistry of PSII was slightly deceased in infected plants of both LR and LBH cowpea cultivars while it was markedly inhibited in EBH. Spraying cowpea plants with 50 and 100µM SA could shift off the inhibitory effect of CPMV on F_V/F_M ratio in LR and LBH cultivar, while it had almost no effect in EBH (Fig. 2).

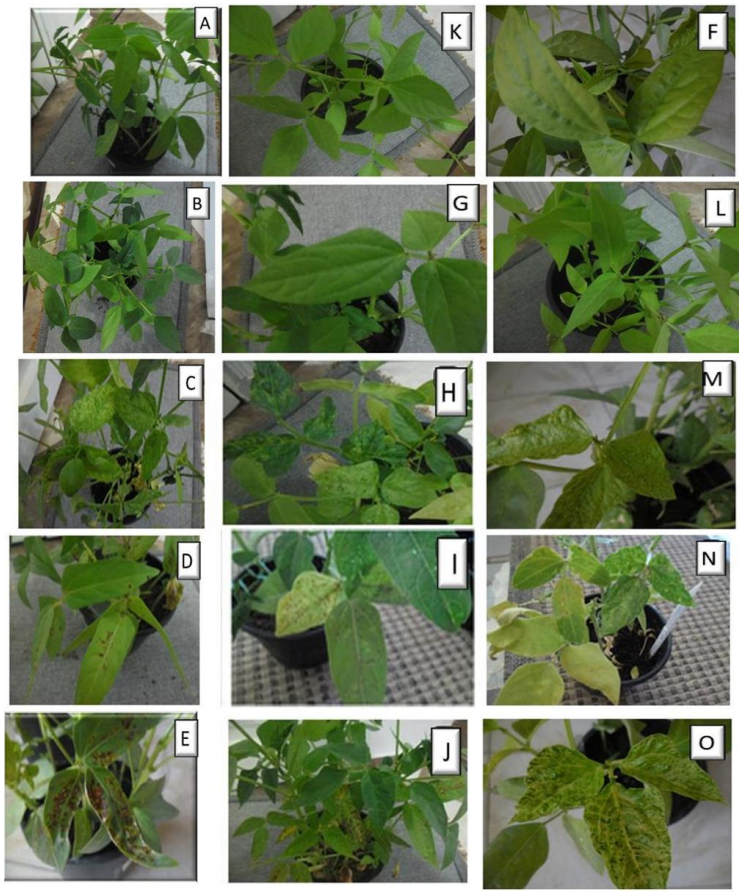

Fig. 1. Effect of CPMV and SA treatment on symptoms development in *V. unguiculata* cultivars at 21 dpi of Libyan red eye (LR), Libyan black eye hybrid (LBH) and Egyptian black eye hybrid (EBH) cultivars [A, K, and F: control buffer inoculated cowpea of LR, LBH and EBH respectively; B, G and L: 100µM SA treated LR, LBH and EBH respectively; C, H and M: CPMV inoculated cowpea of LR, LBH, and EBH respectively; D, I, and N: CPMV inoculated cowpea and treated with 50µM SA in LR, LBH, and EBH, respectively; E, J, and O: CPMV-inoculated cowpea and treated with 100µM SA in LR, LBH, and EBH, respectively]

TABLE 1. Detection of CPMV in different V. unguiculata cultivars treated with 50 and 100µM SA (cultivar Libyan redeye, Libyan blackeye hybrid and Egyptian blackeye hybrid) by ELISA

Cowpea cultivar	Treatment	Abs. at 405nm
Libyan Redeye	Control	0.455[a]
	100µM SA °	0.438[a]
	CPMV infected	3.99[b]
	50µMSA+ V •	2.56[b*]
	100µMSA+ V •	1.080[c*]
Libyan Blackeye hybrid	Control	0.504[a]
	100µM SA *	0.510[a]
	CPMV infected	3.142[b*]
	50µMSA+ V •	2.029[c**]
	100µMSA+ V •	1.566[c*]
Egyptian Blackeye hybrid	Control	0.70[a]
	100µM SA *	0.877[a]
	CPMV infected	2.99[b**]
	50µMSA+ V •	3.02[b**]
	100µMSA+ V •	2.94[b**]

The cut off value for positive samples was twice the negative control

TABLE 2. Effect of CPMV and SA treatment (50 and 100µM) on leaf number (N seedling -1), shoot height (cm), leaf relative water content (RWC), fresh biomass (FM) and dry biomass (DM) of roots and shoots (g seedling^{-1}) of *V.unguiculata* cultivars

Cowpea cultivar	Treatment	Time (21 dpi)						
		Leaf number	Shoot height	RWC	FM		DM	
					Root	Shoot	Root	Shoot
Libyan Redeye	Control	4.600±0.451	28.50^{a}±2.794	95.10^{a}±9.324	0.81^{a}±0.079	7.48^{a}±0.733	0.21^{a}±0.021	1.72^{a}±0.169
	100µM SA *	5.00±0.446	30.0^{a}±2.679	96.90^{a}±^{8}.652	0.72^{a}±0.064	7.88 ^{a}±0.704	0.15^{a}±0.013	1.89^{a}±0.169
	CPMV infected	4.00±0.328	23.90^{b}±1.959	80.70b*±6.615	0.50^{b}*±0.041	4.1^{b}*±0.336	0.08^{b}*±0.007	0.90^{b}±0.074
	50µMSA+ V •	4.00±0.392	30.10^{a}±2.951	86.50b*±8.480	0.52^{b}*±0.051	6.47^{a}±0.634	0.10^{b}*±0.010	1.34^{ab}±0.131
	100µMSA+ V•	4.30±0.352	30.40^{a}±2.492	88.4^{ab}±7.246	0.54^{b}*±0.044	7.52^{a}±0.616	0.11b*±0.009	1.63^{a}±0.134
Libyan Blackeye hybrid	Control	5.00^{a}±0.446	28.0^{a}±2.500	95.30^{a}±8.509	0.86^{a}±0.077	6.97^{a}±0.622	0.19^{a}±0.017	1.27^{a}±0.113
	100µM SA *	6.00^{a}±0.536	30.80^{a}±2.750	95.80^{a}±8.554	1.10^{a}±0.098	7.32^{a}±0.654	0.1 ^{a}±0.009	1.45^{a}±0.129
	CPMV infected	3.30^{b}±0.295	26.50^{b}±2.366	81.00b*±7.232	0.49^{b}*±0.044	4.70^{b}*±0.420	0.08^{b}*±0.007	0.80^{b}*±0.071
	50µMSA+ V •	5.00^{a}±0.379	28.40^{a}±2.152	83.30b*±6.311	0.52^{b}*±0.039	6.23^{a}±0.472	0.09^{b}*±0.007	1.02^{ab}±0.077
	100µMSA+ V•	5.600^{a}±0.459	29.90^{a}±2.451	87.5^{ab}±7.172	0.78^{ab}±0.064	6.52^{a}±0.534	0.15 ^{a}±0.012	1.15^{ab}±0.094
Egyptian Blackeye hybrid	Control	5.00^{a}±0.446	27.50^{a}±2.455	94.0^{a}±8.393	0.62^{a}±0.055	8.56^{a}±0.764	0.17^{a}±0.015	1.36^{a}±0.121
	100µM SA*	5.500^{a}±0.417	28.00^{a}±2.121	95.60^{a}±7.242	0.65^{a}±0.049	8.88^{a}±0.673	0.12^{ab}±0.009	1.55^{a}±0.117
	CPMV infected	3.50^{b}±0.343	22.20^{b}±2.176	77.60^{b}*±7.608	0.35^{b}*±0.034	3.49^{b}*±0.342	0.07^{b}*±0.007	0.60^{b}*±0.059
	50µMSA+ V •	3.600^{b}±0.391	25.40^{b}±2.761	80.20^{b}±8.717	0.50^{a}±0.054	5.17^{c}*±0.562	0.09^{b}*±0.010	0.8^{ab}±0.087
	100µMSA+ V•	3.500^{b}±0.313	25.10^{ab}±2.241	77.50^{b}*±6.920	0.47^{a}±0.042	5.32^{c}*±0.475	0.08^{b}*±0.007	0.87^{ab}±0.078

°: Control healthy plants were sprayed with 100µM SA (15-d old seedling).

•: Plants were sprayed with either 50µM or 100µM SA 24hrs before virus inoculation (15-d old seedling).

Values are means of 3 independent replicates ± SE; means followed by different letters are significantly different at P≥ 0.05 according to the least significant difference.

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

TABLE 3. Effect of CPMV and SA treatment (50 and 100µM) on photosynthetic pigments (mg g^{-1} FM) in the leaves of *V.unguiculata* cultivars

Treatment	Libyan Redeye cultivar				Libyan Blackeye hybrid cultivar				Egyptian Blackeye hybrid cultivar			
	Chl. *a*	Chl. *b*	Carot.	Total	Chl. *a*	Chl. *b*	Carot.	Total	Chl. *a*	Chl. *b*	Carot.	Total
Control	76.42^{ab}± 7.492	26.69^{a}± 2.617	14.50^{a}± 1.422	117.61^{a}± 11.530	82.02a± 8.041	27.45^{b}± 2.691	22.09^{a}± 2.166	131.56^{a}± 12.898	86.2^{a}± 8.451	23.21^{a}± 2.275	12.6^{a}± 1.235	122.01^{a}± 11.962
100µM SA *	80.31^{a}± 7.171	28.87^{a}± 2.578	16.02^{a}± 1.430	125.18^{a}± 11.177	83.98^{a}± 7.498	30.54^{b}± 2.727	22.18^{a}± 1.980	136.70^{a}± 12.205	86.09^{b}± 7.687	24.81^{a}± 2.215	14.31^{a}± 1.278	125.21^{a}± 11.179
CPMV infected	46.03^{b}**± 3.773	17.29^{b}*± 1.417	10.50^{b}*± 0.861	73.82^{b}*± 6.051	52.20^{b}± 4.279	19.70^{b}± 1.615	10.30^{b}± 0.844	82.20^{b}± 6.738	48.25^{b}± 3.955	13.78^{b}± 1.130	8.78^{b}± 0.720	70.81^{b}± 5.804
50µMSA+ V •	69.5^{b}*± 6.814	23.83^{a}± 2.336	14.50^{a}± 1.422	107.83^{a}± 10.572	74.85^{a}± 7.338	20.50^{b}± 2.010	14.23^{b}± 1.395	109.58^{b}± 10.743	49.433^{b}± 4.846	16.4^{b}± 1.608	10.3^{a}± 1.010	76.133^{b}± 7.464
100µMSA+ V •	74.54^{a}± 6.110	26.12^{a}± 2.141	15.88^{a}± 1.302	116.54^{a}± 9.552	80.77^{a}± 6.620	23.56^{b}± 1.931	19.17^{a}± 1.571	123.50^{a}± 10.123	52.97^{b}± 3.522	14.73^{b}± 1.207	11.09^{a}± 0.909	78.79^{b}± 5.639

°: Control healthy plants were sprayed with 100µM SA (15-d old seedling).

•: Plants were sprayed with either 50 µM or 100µM SA 24hrs before virus inoculation (15-d old seedling).

Values are means of 3 independent replicates ± SE; means followed by different letters are significantly different at P≥ 0.05 according to the least significant difference.

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

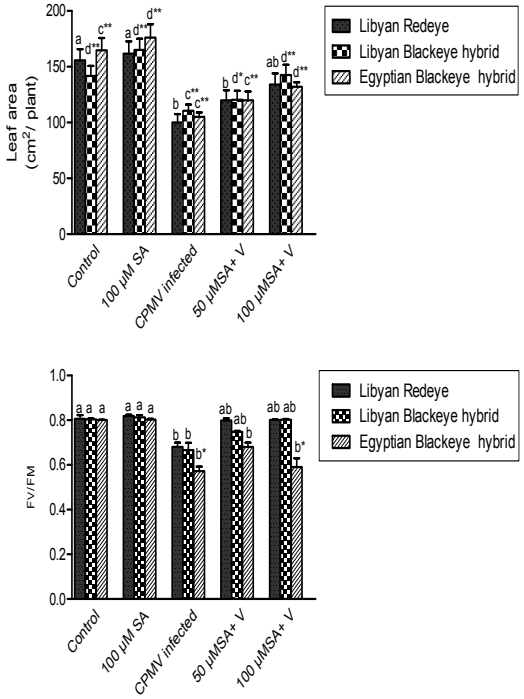

Fig. 2 Effect of CPMV and SA treatment (50 and 100µM) on leaf area and photosynthetic efficiency in the leaves of *V. unguiculata* cultivars [The ratio FV/FM correspond to the potential quantum efficiency of PSII Optimal quantum yield]

Lipid peroxidation, H_2O_2, and total phenolic content

A significant accumulation of H_2O_2 was noted in the leaves of both SA-sprayed and unsprayed cowpea plants in response to CPMV (Fig. 3). At 21 dpi, the H_2O_2 concentration in the leaves of CPMV-infected LR, LBH and EBH was 2.3, 4.2, and 5.3 fold of untreated control plants, respectively. The corresponding values for MDA content were 3.0, 2.4, and 3.2 fold of untreated control plants, respectively. SA spraying (50 and 100µM) before virus inoculation brought about a significant depression in H_2O_2 and MDA accumulation in the three tested cowpea cultivars compared to infected plants but the attained values were still higher than those of their respective controls. For example, the most significant decline of H_2O_2 was noticed for 100µM SA + V treatment in LR (which decreases the H_2O_2 concentration almost to the control value), followed by LBH and EBH (Fig. 3). CPMV resulted in a significant accumulation of total phenolic compounds in the leaves of all tested cowpea cultivars compared to their respective infected plants; the highest accumulation was recorded in EBH Nonetheless,

SA spraying before virus inoculation could alleviate the increase of phenolics in the leaves of the three tested cowpea cultivars (Fig. 3).

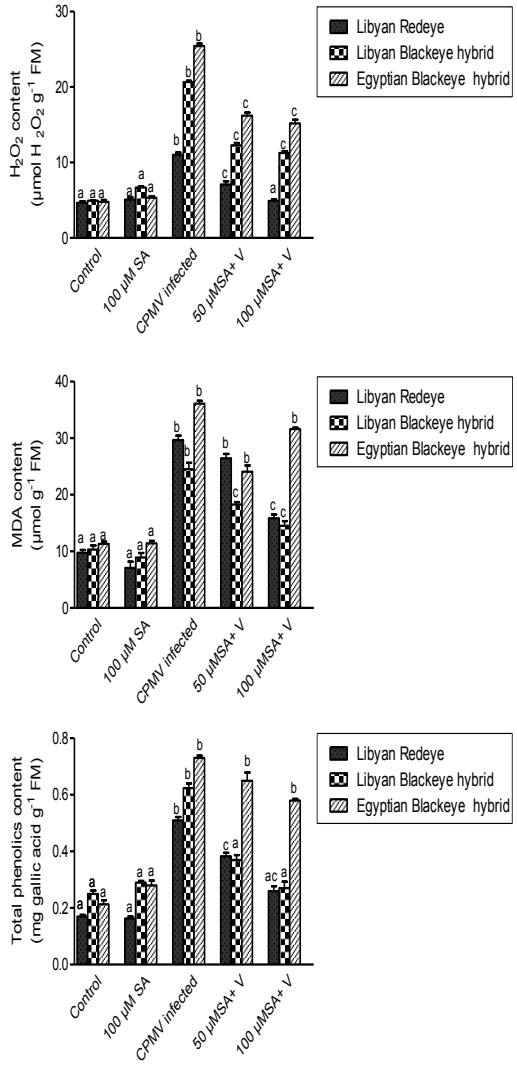

Fig. 3. Effect of CPMV and SA treatment (50 and 100µM) on hydrogen peroxide (H_2O_2) content, malondialdehyde (MDA) content and total phenolics in the leaves of *V. unguiculata* cultivars 21 dpi.

Enzyme activities

Catalase activity increased significantly in the leaves of CPMV-infected cowpea cultivars in comparison to control (Fig. 4); the highest activity was recorded in the leaves of infected LBH in comparison to control (55% higher than control). Except for EBH, spraying healthy plants with SA has resulted in a significant inhibition of CAT activity. In addition, spraying infected plants of both LR and LBH cultivars with SA could inhibit the virus-induced CAT activity; the 100µM SA treatment in particular could lower the CAT activity to the control value or even less. On the contrary, SA treatment could not inhibit the virus induced-CAT activity in the EBH cultivar.

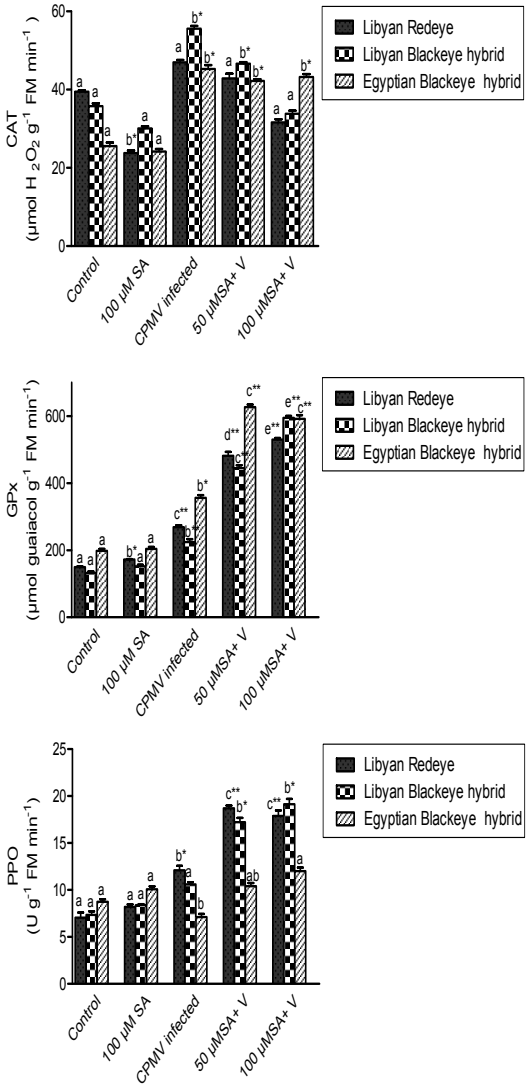

Fig. 4. Effect of CPMV and SA treatment (50 and 100µM) on catalase (CAT), guaiacol peroxidase (GPX) and polyphenol oxidase (PPO) activities in the leaves of *V. unguiculata* cultivars 21 dpi.

As shown in Fig. 4, the GPX activity significantly increased in response to CPMV infection. At 21 dpi, the GPX activity was 1.8, 1.7, and 1.8 fold in LR, BH and EBH respectively, compared to control healthy plants. SA spraying before inoculation resulted in a significant increase in GPX activity in the three tested cowpea cultivars with the greatest increase recorded for LBH cultivar (4.5 fold, compared to control).

PPO activity in the leaves of LR and LBH plants was significantly increased in response to CPMV (Fig. 4). At 21 dpi, the PPO activity was 1.7 fold in LR and BH respectively, compared to control healthy plants. In contrast, CPMV resulted in a marked inhibition of PPO activity in EBH cultivar compared to control plants. When SA was applied, the activities of PPO activity of the three cowpea cultivars were significantly enhanced. With 100µM SA + V treatment, PPO activity was noted to increase by 48%, 80%, and 69% in LR, BH and EBH respectively, compared to their respective infected plants.

Discussion

Several changes were observed in the morphology and metabolic processes of cowpea plants infected with CPMV. Severe symptoms were noted on virus- infected leaves, first on the cotyledonary leaves followed by the sequential trifoliate leaves. These symptoms were accompanied by a significant decrease in growth attributes of all tested cowpea cultivars; the decrease in EBH was observed to be greater than those in the other two Libyan cultivars. Similar results were reported for several plant-virus interactions such as *Capsicum annuum* infected with tobacco mosaic virus (Pazarlar et al., 2013) and *Glycine max*, *Phaseolus vulgaris*, and *Chenopodium amaranticolor* infected with CPMV (Pudake et al., 2015). The results of the present investigation showed that the mosaic symptoms induced in cowpea cultivars by CPMV were associated with a decrease in chlorophyll content and F_v/F_m ratios, which reflect photoinhibition of PSII, more obviously in EBH than the Libyan ones. These observations are in accordance with several studies working with various plant-virus interactions (Alwalaqi 2014; Zanini et al., 2021). The suppression of photosynthetic pigments content has been reported to be related to the inhibitory effect of viral infection on specific enzymes responsible for their synthesis, induction of some degradative enzymes such as chlorophyllase, as well as the destruction of the photosynthetic machinery and pigment-protein complex instability (Sinha & Srivastava, 2010). Moreover, many researchers have shown that viruses can interact with chloroplast proteins and promote the formation of membrane vesicles during viral replication, which then impairs chloroplast functions and disrupts the photosynthetic electron transport chain. This interaction ultimately leads not only to the decline of the carboxylation activity but also to increase in ROS (Zhao et al., 2016; Souza & Carvalho, 2019). Accordingly, the decline of chlorophyll content among CPMV-infected plants could be related to the enhancement of lipid peroxidation of thylakoids and chloroplast membranes as indicated by the increase of MDA content in the leaves. Furthermore, the impaired growth of cowpea plants in response to CPMV infection might be attributed to (i) Disturbance of plasma membrane integrity due to oxidative stress, as evident by increased H_2O_2 and MDA contents, and hence decrease in water content, and (ii) Decrease in the photosynthetic processes, as indicated by the decline in PSII activity and photosynthetic pigments, as well as suppression of photosynthate allocation, and hence reduction in plant growth, (iii) CPMV may have resulted in increased abscisic acid (ABA) that might result in a decrease of photosynthesis due to decrease in leaf area and CO_2 diffusion as well as increasing leaf senescence (Ton et al., 2009).

Recently, eco-friendly approaches have been developed to control viral plant infections and enhance plant defense mechanisms, such as application of synthetic elicitor agents like SA (Chávez-Arias et al., 2020). In the present investigation, spraying cowpea plants with SA 24 h before inoculation has significantly improved the growth and/or delayed CPMV symptoms on the leaves of tested cowpea cultivars, compared to infected plants. The symptoms delay was accompanied by an increase in most growth indices, photosynthetic pigments and photosynthetic rate as well as decreased MDA and H_2O_2 contents. Similar observations were recorded for various plant-virus interactions (Zhao et al., 2016; Mardani-Mehrabad et al., 2020). Alawlaqi (2014) suggested that the inhibition of zucchini yellow mosaic virus disease development in SA-treated *Cucurbita pepo* plants occurred either by obstructing virus replication at the initial point of infection, or by delaying the movement of the virus out of the inoculated tissue. With respect to these views, 100 µM SA+V-treated cowpea plants showed no identifiable symptoms of CPMV disease in the newly formed leaves of the two Libyan cultivars (LR, LBH). Typical necrotic local lesion formation was observed indicating a hypersensitive type of response to restrict virus movement and induce resistance in younger leaves. Conversely, SA pretreatment

could not prevent CPMV spread in EBH, in which numerous necrotic spots were observed in young and old leaves. This observation might reveal the susceptibility of EBH cultivar to CPMV.

It is well known that carotenoids show antioxidant properties besides acting as accessory light harvesting pigments. Thus, in the present investigation, SA may have stimulated the synthesis of carotenoids to conserve chlorophyll and the photosynthetic apparatus from oxidative stress created due to viral infection and finally increased chlorophyll content particularly in the LR and LBH cultivars.

Nogués et al. (2002) reported that inhibition of photosynthesis in several host virus interactions may be related to impairment of electron transport (PSII), inhibition of several enzymes involved in CO_2 assimilation, starch synthesis and photoassimilate transport, reduction in stomatal conductance or a combination of those. Here, the decrease in F_v/F_m ratio in the leaves of CPMV-infected cowpea plants compared to control could be explained on the basis of chlorophyll pigments destruction *via* enhancement of generated ROS and lipoxygenase activity in infected cowpea plants. This destruction could be accompanied by an extensive production of excitation energy under virus stress, leading to photooxidative damage in the reaction centers of PSII (Dubey, 2005). However, SA could reduce to some extent, the inhibitory effect of CPMV on F_v/F_m ratios in the two Libyan cultivars through conservation of chlorophyll molecules and prevention from further degradation during symptom development. In fact, several studies have proposed a strong protection of PSII and chloroplasts by SA treatment (Radwan et al., 2008; Falcioni et al., 2014).

It has been documented that plants respond to stress by boosting ROS levels, which then limits pathogen entry and distribution and induces systemic and local defense responses (Cai et al., 2019). Similarly, CPMV resulted in oxidative stress (measured in terms of MDA and H_2O_2 generation) in leaves of all tested cowpea cultivars, revealing serious damage of plasma membranes due to peroxidation of polyunsaturated fatty acids. Also, the assayed antioxidants enzyme activities were modulated in response to CPMV and/or SA pretreatment.

Radwan et al. (2010) previously reported that one mechanism of SA action is to inhibit catalase, thereby elevating endogenous levels of H_2O_2, which may result from the oxidative burst associated with the HR. They suggested that SA treatment induced the generation of H_2O_2 and/or its products ($OH^{-)}$ that may result from its reaction with transition metals, especially Fe^{3+}, and that might inhibit viral replication and penetration through the cell wall of host plant. More recently, Nadi & Babu (2013) suggested that SA may interact with catalase leading to its inhibition and elevation of H_2O_2, which may act as a signaling molecule and activate plant defense responses. Accordingly, SA + V treatment especially the high concentration of 100 µM SA, significantly inhibited CAT activity in the Libyan cowpea cultivars while it had insignificant effect in EBH, compared to infected plants. Also, the viral spread was greatly inhibited only in the two Libyan cultivars as indicated by the formation of the great necrotic area. These observations might explain the role of SA in the induction of HR in the Libyan cowpea cultivars against CPMV. Moreover, the SA-induced resistance in the Libyan cowpea cultivars might be attributed to enhancement of H_2O_2 content and declined CAT activity compared to control.

It has been reported that acclimation of plants to various pathogens is associated with stimulation of protective secondary plant metabolites such as phenols, flavonoids, alkaloids and terpenes (Huang et al., 2006). Herein, CPMV infection has significantly enhanced the accumulation of the total phenolic content in cowpea plants confirming the results reported for various plant-pathogen interactions (Ashfaq et al., 2014; Sofy et al., 2020). This increase was mainly associated with a highly significant increase in GPX activity indicating that the generated free radicals may be scavenged by peroxidation of synthesized phenolic compounds *via* the donation of their proton for reduction of generated H_2O_2 revealing their adaptive role against the viral infection. On the other hand, foliar application of SA significantly suppressed phenolic accumulation in LR and LBH cultivars rather than the Egyptian one compared to the CPMV-infected plants. This result might indicate the consumption of phenolics in the reduction of H_2O_2, thus decreasing the toxic effect of ROS. However, other studies showed that SA further induces the accumulation of phenolic compounds reducing the damage caused by lipid peroxidation (Ali et al., 2007; Preciado-Rangel et al., 2019).

In the present research, CPMV, particularly those pretreated with SA, has significantly stimulated GPX and PPO activities confirming the results reported for several plant-virus interactions such as tomato leaf curl virus (Mishra et al., 2014) and bean common mosaic virus (Mardani-Mehrabad et al., 2020). Brisson et al. (1994) and Kuvalekar et al. (2011) reported that peroxidases play dual role in disease resistance because they can catalyze the final step of lignin biosynthesis and oxidize phenolic compounds to quinones which are usually more toxic to microorganisms than the authentic phenols. Vidhyasekaran (1988) reported that the PPO enzyme itself is inhibitory to viruses by inactivating the RNA of the virus. Therefore, the significant increase in GPX and PPO in infected and SA + V cowpea treated plants observed in this study might indicate an increase in cell wall lignification and oxidation of phenolics to quinones which resulted in prevention or slowing of viral penetration and induction of HR, particularly in the Libyan cultivars. For the EBH cultivar, only GPX was significantly increased in response to CPMV and SA pretreatment, while PPO was insignificantly changed. This result might explain the sensitivity of this cultivar to CPMV more than the other Libyan cultivars.

Conclusions

The findings of this study suggest that foliar application of SA 24 h before inoculation with CPMV can reduce disease severity and induce hypersensitive response in the two tested Libyan cowpea cultivars but not in the Egyptian one. SA application seems to improve the growth of LR and LBH through modulation of photosynthesis as well as regulation of enzymatic (CAT, GPX, and PPO) and non-enzymatic (carotenoid and phenolics) antioxidants hence protect the plasma membrane integrity from virus-induced oxidative damage. On the other hand, disruption of plasma membranes and a decrease in photosynthetic efficiency of EBH were not greatly enhanced by SA application. The results pointed out that the effective SA treatment of virus-infected plants might be a function of host plant cultivar properties. However, further research is required to determine the specific cascade of changes at the molecular level and the distinct genes that are induced to deliver such an effect

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Authors' contribution: The authors confirm contribution to the paper as follows: study conception and design: Fattouh F.A and Ismail G.S.M., following lab experiments: Omar S.M.S. and Ismail G.S.M. Data analysis and interpretation: Fattouh F.A and Ismail G.S.M., draft manuscript preparation: Ismail G.S.M., and Omar S.M.S., critical revision of the article and final approval of the version to be published: Fattouh F.A, Ismail G.S.M., and Omar S.M.S.

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تعزيز درجة تحمل نبات اللوبيا و المعامل مسبقا بحمض الساليسيليك للأصابة بفيروس تبرقش أوراق اللوبيا

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يعتبر فيروس تبرقش أوراق اللوبيا أحد أهم العوامل الممرضة المؤثرة إقتصاديا على نبات اللوبيا، وفي الآونة الأخيرة تم تطوير استراتيجيات صديقة للبيئة تهدف إلى تعزيز درجة تحمل المحاصيل للعدوى بالفيروسات. يهدف هذا البحث إلي دراسة تأثير المعاملة بحمض الساليسيليك على استجابة ثلاثة أصناف من نبات اللوبيا [الصنف الليبي ذو العين السوداء (LB) ، الصنف الليبي ذو العين الحمراء (LR)، الصنف المصري الهجين ذو العين السوداء (EBH)] للعدوى بفيروس تبرقش أوراق اللوبيا، حيث تم معاملة أوراق النباتات فى عمر 15 يوم بالرش بكميات متفاوتة 50 و100 ميكرومول من حمض الساليسيليك وذلك 24 ساعة قبل العدوى بالفيروس. وقد اوضحت النتائج أن الاصابه بفيروس تبرقش أوراق اللوبيا ادى إلى انخفاض معظم مؤشرات النمو، محتوى أصباغ التمثيل الضوئى وكفاءة التمثيل الضوئى في كل الاصناف المختبره وبالرغم من ذلك فإن المعاملة بحمض الساليسيليك استطاعت أن تعزز نمو اللوبيا وتقلل من شدة الأصابة الفيروسية، فى جميع الأصناف تحت الدراسة، وخاصة الأصناف الليبية. كذلك، فإن أضرار الإجهاد التأكسدي وتراكم الفينولات الناتج عن الأصابة بالفيروس كانت أقل فى حال المعاملة بحمض الساليسيليك في جميع أصناف اللوبيا. أدت الأصابة بالفيروس إلى إنتاج زيادة معنوية فى نشاط أنزيمات الكتاليز، الجواياكل بيروكسيديز والبوليفينول أوكسيديز فى الأصناف الليبية فقط وليس فى الصنف المصري حيث لم يحدث تغيير معنوى فى نشاط أنزيم البوليفينول أوكسيديزفي هذا الصنف. ومما يدعو للإهتمام أن المعاملة المسبقة بحمض الساليسيليك قد ثبطت نشاط أنزيم الكتاليز بدرجة ملحوظة فى الأصناف الليبية مقارنة بالنباتات المصابة الغير معاملة، فى حين أنها حفزت نشاط أنزيمى الجويكال بيروكسيديز والبوليفينول أوكسيديز فى الأصناف الثلاثة المختبرة، وبصورة خاصة فى الأصناف الليبية. وإجمالا أوضحت الدراسه أن المعاملة المسبقه بحمض الساليسيليك كانت فعالة بدرجة أكبر على تحفيز المناعة الجهازية للاصابه بفيروس تبرقش اللوبيا في الأصناف الليبية مقارنة بالصنف المصري.