

Research Article

A Comparative Study of the Influence of Cucumber Mosaic Virus on Free Radical Scavengers of Tomato and Squash Plants

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Abstract

Cucumber Mosaic Virus (CMV) is a lethal plant virus that results in the loss of crop yield with stark economic consequences. The viral strain with the benign satellite RNA, KU-1 CMV, can be used as a vaccine to protect plants against diseases caused by the severe CMV-16 strain. In order to understand physiological changes in response to CMV-16 and KU-1 CMV, we looked at the antioxidant defences of tomato (*Solanumlycopersicon* L.) and squash (*Cucurbitapego* L.) plants. The strain CMV-16 showed severe stunting symptoms in tomato plants, whereas in squash plants, it caused mild stunting accompanied by severe chlorosis, yellowing and mosaic. However, the KU-1 CMV strain showed milder mosaic symptoms on squash leaves, while the tomato leaves were symptomless. This study appraises the free radical scavengers as markers for stress in tomato and squash plants after infection with KU-1 CMV. In this endeavour, antiradical composites such as carotenoids, total phenolic compounds and membrane fatty acids were investigated in infected plants. The lutein/ β -carotene ratio in squash plant leaves infected with CMV-16 increased by 88.8%, while those infected with KU-1 CMV had only a 22.2% increase in the lutein/ β -carotene ratio. When plants were infected with CMV-16 or KU-1 CMV, the total phenols, markers of oxidative stress, decreased by 31.3% and 22.6%, respectively with a decline in unsaturation index of fatty acids.

Keywords: *Cucurbitapego* L; *Solanumlycopersicon* L; Cucumber mosaic virus CMV-16; KU-1 CMV; photosynthetic pigments; Phenols

Introduction

Cucumber Mosaic Virus belongs to the Cucumovirus genus and Bromoviridae family [1]. Worldwide, it is an economically important virus, causing significant crop losses in as many as 190 host plants from 40 different plant families [2-4]. The virus can be transmitted from plant to plant, both mechanically by sap and by aphids in a stylet-borne fashion [4].

The CMV causes a broad spectrum of symptoms from mild to severe leaf mosaic or yellowing to stunting, chlorosis, necrosis, filiformism and fruit distortion depending on the virus strain and the host [4]. One of the CMV strains is KU-1 CMV, which is a mild strain associated with a benign viral satellite RNA (345bp) that induces mild mosaic symptoms on young leaves, but later the plants start to be symptomless [5]. Thus, the viral strain with the benign satellite RNA, KU-1CMV could be used as a vaccine to protect plants against diseases caused by severe CMV strains such as CMV-16 [5]. Therefore, the KU-1 CMV strain has been used as a biological control agent to protect tomato plants against the diseases induced by severe CMV strains, against Potato Spindle Tuber Viroids (PSTV), and against fungal diseases caused by *Fusariumoxysporum* f. sp. *Lycopersicae*, *Lycopersiconesculentum* and *Allernaria* alternate in tomatoes [4].

In the natural environment, plants are continuously exposed to both biotic and abiotic stresses; in response, plants generate their molecular resistance mechanisms depending upon the nature

of the pathogen or the stress signal [6,7]. Under stress, the plant physiology is entirely changed, with alterations in energy metabolism, biomolecules such as photosynthetic pigments, carbohydrates, lipids, proteins, and signal transduction, [8]. Virus-infected plants show physiological colour changes such as yellowing and chlorosis, indicating that the virus influences the photosynthetic apparatus of the plant [9]. In pathogen-infected plants, an increased production of Reactive Oxygen Species (ROS), causing oxidative stress, has also been reported [10].

Plants scavenge the ROS through two defence antioxidant mechanisms, enzymatic (peroxidase, glutathione, catalase, superoxide dismutase (SOD)) and non-enzymatic. Carotenoids, ascorbate, phenols and vitamin E and carotenoids are regarded as the most effective non-enzymatic scavenger of ROS in plant cells [11,12]. Carotenes play an important role in the complex photosynthetic process by dissipating the light energy that they absorb from chlorophyll. In addition, they also protect plant tissues by helping to absorb energy from the damaging singlet oxygen that is formed during the photosynthetic processes [11-14].

The CMV outbreak has caused huge losses of tomato and pepper crops worldwide, including in several Asian and Mediterranean countries and several parts of the United States [15,16]. Yellowing and chlorosis of the leaves indicate severe damage to the photosynthetic apparatus, severing the energy metabolism by impairing chlorophyll synthesis and the electron transport rate and by altering the thylakoid

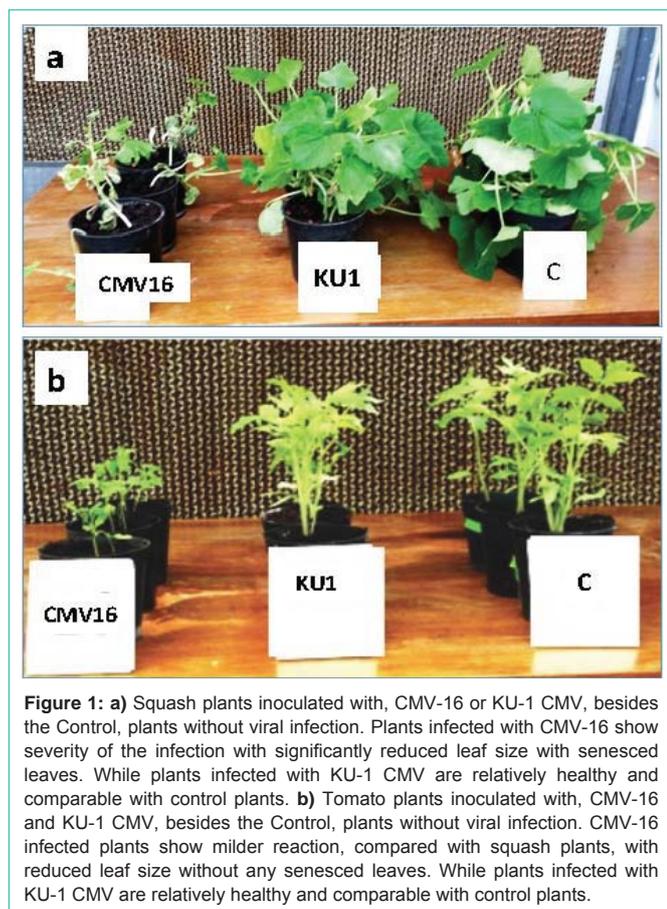


Figure 1: a) Squash plants inoculated with, CMV-16 or KU-1 CMV, besides the Control, plants without viral infection. Plants infected with CMV-16 show severity of the infection with significantly reduced leaf size with senesced leaves. While plants infected with KU-1 CMV are relatively healthy and comparable with control plants. b) Tomato plants inoculated with, CMV-16 and KU-1 CMV, besides the Control, plants without viral infection. CMV-16 infected plants show milder reaction, compared with squash plants, with reduced leaf size without any senesced leaves. While plants infected with KU-1 CMV are relatively healthy and comparable with control plants.

membranes of chloroplasts [17,18]. The KU-1 CMV strain is used as a biological control for the severe CMV-16 strain. However, to our knowledge, there is no published report elucidating the effect of KU1 CMV on the physiology of economic plants. We evaluated the effect of KU-1 CMV on the photosynthetic apparatus of tomato and squash plants by measuring photosynthetic pigments, including carotenoids, total phenolics and membrane fatty acid components.

Materials and Methods

Virus: source, maintenance and inoculum preparation

Cucumber mosaic viral strain, associated with a benign viral satellite RNA (KU-1 CMV), was isolated in Kuwait [19]. This viral strain is symptomless on tomato plants. CMV-16, subgroup II, is a Japanese isolate from tomato plants (kindly provided by H. Sayama, Kikkō Foods Corporation, Japan) that contains no detectable viral satellite when maintained on tomato plants. The virus strains were extracted from the leaves of old desiccated samples that were made available to our molecular virology lab, at University of Kuwait. The viral isolates were invigorated by mechanical passage into fresh squash and tomato plants. The infected leaves were macerated in 10 mM potassium phosphate buffer pH 7.2 with a mortar and pestle. The crude sap was used to inoculate tomato and squash test plants.

Plant growth and groups

Seeds of the tomato cultivars ‘Super Marmande’ and ‘UC82B’ and of squash were sterilized with 2% sodium hypochlorite (4 mL

L⁻¹) followed by Tween 20 (10%) and rinsed three times with distilled and autoclaved water. The sterilized tomato seeds were planted in 15 cm plastic pots filled with sterilized sandy soil mixed with peat moss (2:1 v/v). Following seed germination, the seedlings were transferred individually to the 15 cm pots containing above noted mixture of soil and peat moss to ensure a satisfactory growth under greenhouse conditions, adjusted to 25 °C and 40% humidity with alternating 16 hr light and 8 hr dark periods. The 40w Sun lite bulbs were used in the greenhouse and growth chambers to ensure uniform PPFD.

One hundred and fifty pots of tomato plants were randomly divided into three groups (a-c), with 50 pots in each group. Similarly, 216 pots of squash plants were randomly divided into three groups (d-f), with 72 pots in each group. For replicates, each group of plants (a-f) was further divided into three groups. Three independent experiments, in triplicate, were conducted on each group of squash and tomato plants as follows: a) Plants treated with KU-1 CMV strain; b) Plants treated with CMV-16 and c) Plants left untreated as healthy control.

Mechanical inoculation, maintenance and plant material sampling

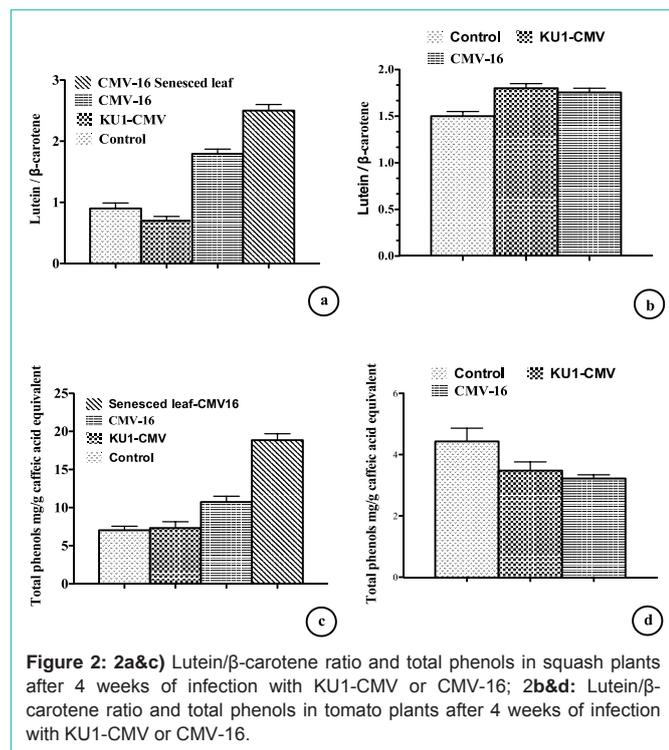
The virus (KU-1 CMV) was mechanically inoculated onto the leaves of healthy tomato and squash plants. Test plants maintained in green house, were dusted with carborundum powder and rubbed with sap extracted from infected leaves.

Plant leaf samples from groups (a-f) were collected from middle of the main trunk (25 g, pooled sample of all plants) at various growth stages and were immediately freeze-dried (Virtis, Vertis Inc. Gardiner, NY, USA). The plant material was sampled on a weekly basis for a period of four weeks. It was noticed that during the 4th week of inoculation, some lower leaves were senesced in squash plants (group d), while the top leaves were relatively fresh and healthy. Both Senesced Squash Leaves (SSL) and relatively Fresh Top Leaves (SFL) were separately sampled for analyses. The tomato plants, group b, showed no senesced leaves at the same age. All individual freeze-dried leaf samples were powdered in a disperser, and a known weight was processed for the extraction of carotenoids, total phenolics, and lipids.

Extraction of carotenoids

Carotenoids were extracted by shaking freeze-dried powdered leaf material (1g) with 80% freshly distilled acetone (4 mL) at room temperature. The extract was centrifuged at 2000 rpm/min. The clear supernatant was collected, and the residue was re-extracted twice with the same extraction solvent. The organic extracts were pooled, filtered through a membrane filter (5µm, GE healthcare Bio-Sciences, Pittsburgh, PA, USA) and concentrated under a gentle stream of N₂ gas. The dark green residue was saponified with 20% methanolic potassium hydroxide in the dark at room temperature. The saponification procedure removed chlorophyll pigments and yielded free xanthophylls.

The saponified material was mixed with normal saline (10 mL) to remove all the soaps and sodium salts of the free fatty acids formed from the lipid hydrolysis. Under a dim light, the free carotenoids were thrice extracted with distilled hexane (5 mL each extraction). The pooled organic extract was dehydrated over dry sodium sulphate,



carefully membrane filtered and concentrated under vacuum using a rotary evaporator and transferred into screw cap brown glass vials. The solvent was then completely evaporated under a gentle stream of nitrogen, under low light intensity. The samples were stored under nitrogen gas at -20 °C until use.

HPLC separation of carotenoids

Sample preparation: A normal phase silica sep-pak (1 mL, sep-pak classic, silica cartridges, Waters, part# WAT051900, Lot#071134091A) was used for sample preparation. The cartridge was equilibrated with distilled hexane, and the crude carotenoid solution in hexane-chloroform (4:1. v/v) (200 μL) was loaded on the Sep-pak cartridge and allowed to adsorb by gravity. The Sep-pak was eluted with distilled hexane (1 mL) followed by 10% methanol in hexane (2 mL). This fraction was injected into HPLC for analyses of the carotenoids. HPLC analyses of the carotenoids were carried out according to Thayer and Bjorkman [20], on a Shimadzu LC-10 system with a PDA detector (model SPD-M20A 230V CAT #NL20154300739VCD, JAPAN) using a reversed phase ZORBAX SB-C-18 column (150 x 4.6 mm, PN 863953-902 SNUSEG015875 LN B11187, US), 3.5 μm particle size. The carotenoid components were eluted with a gradient mixture of two solvents: 80% methanol (solvent A) and 100% ethyl acetate (solvent B).

The total run time of the gradient program was 31 min. Elution began with 20% B, reaching 22.5% after 2.5 min. A linear increase in B to 50% occurred at 17.5 min. At 19 min, eluent B linearly increased to 80%, and this composition was maintained for 2 min. At 26 min, solvent B increased to 100% and held for 3 min before returning to 20% solvent B. The flow rate throughout the run was maintained at 1 mL/min.

All chromatograms were monitored at λ450 nm. The eluted

components were identified from their retention times compared with standard carotenoids run under identical conditions. The identification of carotenoids was also carried out through their specific absorption patterns at λ450 nm. For quantification of the individual carotenoids, standard curves of lutein and β-carotene were drawn at five different concentrations with a correlation coefficient ≥0.99, and the generated linear equation was used to determine the unknown carotenoid concentrations. As the peak area numbers were very large, the peak areas for standards and unknowns were expressed as log values, which resulted in a curvilinear graph.

The total amount of carotenoids was determined by using the specific absorption coefficient 2500 as reported by Britton [21]. The results were expressed as mg% of carotenoids based on the following equation:

$$\text{mg\% carotenoids} = A \times V \times 1000 / 2500 \times l \times 100$$

Where: A - absorbance of the sample at λ450 nm; V - sample volume (mL); 2500 - specific absorbance for carotenoids (A1% 1 cm); and l - optical path length (1 cm) [22,23].

Total phenolic compounds: Phenolic compounds were extracted and quantified by the method reported by Rodriguez-Delgado et al. [24] and modified by Neacsu et al. [25]. Two grams of the freeze-dried, powdered plant material was extracted with 80% ethanol. The extraction was left on a shaker overnight, at room temperature followed by centrifugation at 2000 rpm for 10 min. The clear supernatant was taken, and the remaining pellet was washed 3 times with the same extraction solvent. The organic extract was pooled and concentrated on a rotary evaporator at 35 °C. The total extract was subjected to acid hydrolysis (2N HCl) for 16 hr at 35 °C. Freshly distilled, peroxide-free diethyl ether was used for exhaustive extraction of the organic materials. The total extract was dried over anhydrous sodium sulphate and filtered, and the solvent was removed under reduced pressure at 20 °C. The material thus obtained was stored at -20 °C under nitrogen gas and protected from light. The total phenolic compounds are commonly determined by using HPLC-DAD-MS/MS [26]. In this study we used spectrophotometry for the estimation of total phenolics as described by Singleton et al. [27], using Folin-Ciocalteu reagent. A methanolic solution of caffeic acid was used to produce a standard curve, and its linear equation was used to calculate the amount of total phenols in the plant extracts. The amount of total phenols in the plant extracts was expressed as milligrams of Caffeic Acid Equivalents (CAE) per gram of freeze-dried plant material. The assay as suggested by Golfakhrabadi et al. [28] was followed for quantification of total phenols in the extract.

Gas Chromatographic Analysis of Fatty Acid Methyl Esters (FAMES)

Freeze-dried plant tissue (0.5 g) was powdered and extracted by a modified Folch method as described by [29]. The extract was centrifuged at 2000 rpm for 10 min. The clear supernatant was collected, and the residue was re-extracted twice with the same extraction solvent. The total extract was concentrated under a gentle stream of N₂ gas. Boron trifluoride (BF₃)/methanolic solution (500 μL) and dry benzene (1 mL) were added, and the mixture was heated in an oven at 50 °C for 30 min. After the methylation process, the mixture was cooled on ice, 1 mL of ice cold water was added and extraction

was performed 3 times with distilled hexane (1mL) and Vortexing. The pooled organic extract was dried over dry sodium sulphate, the mixture was filtered using a syringe filter (25 mm, 0.22 μ m, PTFE loop purple, Luer Lock Inlet, Restek, Cat#26144, Lot#T0304112627) and the filtrate was passed through a Sep-pack (Sep-pak classic, silica cartridges, Waters, part#WAT051900, Lot#071134091A). The Sep-pak was eluted with distilled hexane. This fraction was injected into GC for the analyses of FAMES.

Analysis of FAMES was performed on a GC-MS system: Agilent-6890A: ser#US00041476. The FAME separation was achieved using a fused silica SGX BXP capillary 70 m column (25 x 0.22 mm film thickness). Helium was used as the carrier gas at a flow rate of 0.7 mL/min. The oven temperature was programmed at 70 °C and increased to 250 °C at the rate of 4 °C/min for a total run time of 45min. Well resolved FAME peaks were identified by comparing their retention time and equivalent chain length compared with standard FAMES. The standard FAME came from Supelco Inc., Bellefonte, PA (Supelco 37 Component FAME Mix, Catalogue No. 47885-U). 5973N MSD, Agilent, was used for identification of the unknown FAMES. The relative peak areas were quantified using the respective peak area. Both standard mixture and FAMES from the plant samples were chromatographically separated under identical conditions. The identified FAMES in squash and tomato plants were expressed as a molar percentage of saturated and unsaturated fatty acids. The ratio of the molar percentage of the unsaturated/saturated (saturation index) fatty acids was calculated for healthy control C and infected plant samples.

Statistical analysis

Statistical analyses were carried out using Prism software Version 5.0. All values were calculated for 95% confidence intervals. Comparisons between groups were performed using analysis of variance (one way) followed by Turkey's multiple comparison tests used to statistically compare the control and treated groups and the data were expressed as a mean \pm standard error. The results with $P < 0.05$, were considered statistically significant.

Results and Discussion

Squash and tomato plants inoculated with the strains KU-1 CMV and CMV-16 showed the predictable symptoms. The strain CMV-16 produced severe stunting in tomato plants, whereas in squash plants, it caused only a mild stunting accompanied by severe chlorosis, yellowing and mosaic symptoms. However, the KU-1 CMV strain produced milder mosaic signs on squash leaves, while the tomato leaves were symptomless (Figure 1a and 1b). This showed that the KU-1 CMV strain was lesser virulent on both tomato and squash plants compared with the CMV-16 strain.

The production of photosystem damaging ROS is a natural defence mechanism in plants, when challenged by pathogens [30] such as CMV that is known to work through the production of ROS [31]. Carotenoids are not only natural non-enzymatic powerful antioxidants offering defence but they also take part in the photosynthetic process. These are also precursors to hormones and vitamin A, controlling growth and development of the plants [32]. Therefore, for a comparative study, the level of carotenoids, as an antioxidant index, was measured in both tomato and squash

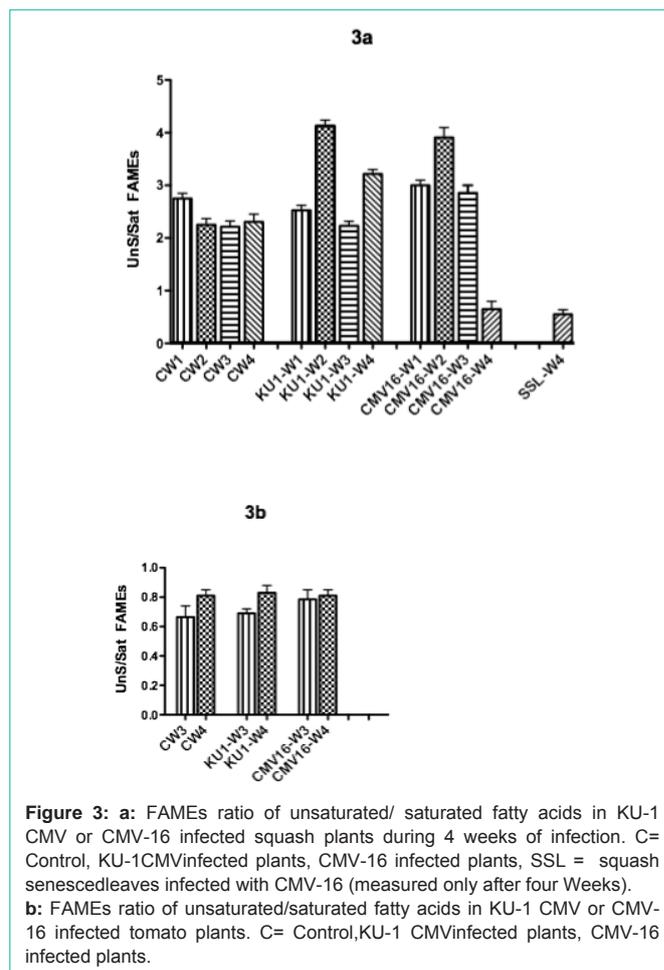


Figure 3: a: FAMES ratio of unsaturated/ saturated fatty acids in KU-1 CMV or CMV-16 infected squash plants during 4 weeks of infection. C= Control, KU-1CMVinfected plants, CMV-16 infected plants, SSL = squash senescedleaves infected with CMV-16 (measured only after four Weeks). b: FAMES ratio of unsaturated/saturated fatty acids in KU-1 CMV or CMV-16 infected tomato plants. C= Control, KU-1 CMVinfected plants, CMV-16 infected plants.

plants, after the infection with CMV-16 and KU-1 CMV. Because of the mosaic symptoms and yellowing of the leaf, chlorophyll content was not measured in plants. After four weeks of inoculation, the carotenoids were analysed on a weekly basis by HPLC and the ratio of lutein and β -carotene was measured as an index of the viral injury in plant tissues. It took two weeks for the symptoms to appear in both squash and tomato plants infected with CMV-16 or KU-1 CMV. Therefore, the carotenoid profiling in both plants was done after the fourth week of infection, as is shown in Figure 2a and 2b.

Zeaxanthin and lutein are both derived from β -carotene [33]. The only difference between lutein and zeaxanthin is the ionone ring (β - or ϵ -ionone), which differs in the position of one of the double bonds in the ionone ring. As lutein was the major product and it was easily separable from β -carotene, we used the lutein / β -carotene ratio as an index of viral damage in tomato and squash plants. Changes, in the ratio of photosynthetic pigments have been suggested as an early indicator of oxidative stress [34], and the ratio involving β -carotene is often used to show the significance of metabolic disorders. Under many environmental stresses, the accumulation of zeaxanthin has been used as an indicator of oxidative stress [35].

The lutein/ β -carotene ratio in squash plant leaves infected with CMV-16 increased by 88.8% ($P < 0.05$) compared with the control plants after the fourth week of infection. In senesced squash leaves,

which only appeared in the fourth week after exposure to CMV-16, the increase in lutein/ β -carotene was 162.6%, indicating severe oxidative stress in senesced leaves. This result also indicated a gross decrease in β -carotene synthesis/degradation and/or its rapid conversion to better antioxidant lutein to fight back against the oxidative stress of the virus. Lutein is known to have a twofold higher antioxidant activity than β -carotene [36]. The increase in lutein or zeaxanthin has been correlated with a simultaneous decrease in β -carotene, as it is the biosynthetic precursor of other carotenoids [33,37]. Therefore, a decrease in β -carotene would suggest plants under higher oxidative stress.

However, the lutein/ β -carotene ratio in the squash plants after four weeks of infection with KU-1 CMV, reduced by $\sim 22.2\%$ ($p < 0.05$) compared with the control plants, indicating the relative low severity of the strain and thus a reduced oxidative stress (increased β -carotene) generated by KU-1 CMV. However, total carotenoids in squash, after four weeks of infection with KU-1 CMV or CMV-16, only increased nonsignificantly by 2.3% and 1.2% respectively, compared with control plants, indicating that total carotenoids may not be taken as markers for oxidative stress. The minor severity and a protective role of KU-1 CMV, the photosynthetic apparatus of the plant is not severely damaged, thereby showing a higher percentage of the total carotenoids. In squash senesced leaves, the total carotenoids increased by 3.8% after four weeks of infection with CMV16. An increase in concentrations of xanthophyll cycle pigments in senesced leaves of *Daphniphyllum humile* Maxim has been reported [38]. In tomato plants, after four weeks of infection with KU-1 CMV or CMV-16, the total carotenoids increased nonsignificantly 3.4% and 1.1%, compared with the control, respectively. These results again suggested that KU-1 CMV strain was relatively less damaging compared with CMV-16 strain.

The changes in tomato leaves infected with CMV-16 were inverse and less pronounced compared with squash plants. The lutein/ β -carotene ratio in these plants, after four weeks of infection, significantly increased by 16.12% compared with the control plants. On the other hand, the lutein/ β -carotene ratio significantly increased by 19.35% in response to KU-1 CMV infection in tomato leaves. This result may be explained by pathogen/host specificity. Tomato plants may not be a specific host for CMV-16, and it is known that KU-1 CMV is less virulent in tomato plants. This result also showed that tomato plants were under less oxidative stress than were squash plants when challenged by either strain of the virus.

Many studies have reported the effect of biotic/abiotic stress on the variation of carotenoids in plants. Thus, Raithak and Gachande [2012] observed a reduction in β -carotene in four tomato cultivars namely Laxmi (NP-5005), Kranti, Priya and Sartaj-plus, after 45 days of plant exposure to the virus. Crosbie and Matthews [39] reported that a white severe strain of Turnip Yellow Mosaic Virus (TYMV) reduces the concentration of all six pigments, including (chlorophylls a and b and the four carotenoids) to a similar extent in all tested plant tissues. It was shown that the reduction of the major chloroplast pigments (chlorophylls, β -carotene and xanthophyll) was mainly due to the virus infection, and the decrease varies with the type of the viral strain, the host species variety and the growth environment. Thus, in our studies, a decrease in β -carotene with a concomitant increase in lutein could be the consequence of CMV-16 severe viral infection.

Plant viruses that cause systemic infections may be particularly important as inhibitors of chlorophyll synthesis as they continuously spread during plant growth and development [40]. Since carotenoids are auxiliary pigments that absorb energy for use in photosynthesis and also protect chlorophyll from photodamage, our results support the view that viral infection moderates the photosynthetic process, as suggested by Rahouteiet al. [2000]. This fact normally leads to a decreased chlorophyll concentration and consequently a lower β -carotene content and related carotenoids in plant leaves. The results obtained in this study are parallel to earlier reports [41].

These results are in agreement with Wise and Naylor [42], who have reported the influence of photo oxidative conditions on the modification of pigments. In detached leaves from cucumber and pea plants, an apparent increase in lutein was found during the first 3 h of treatment with chilling temperatures (5 °C) and high irradiance with 1000 microeinsteins per square metre per second ($\mu\text{E m}^{-2} \text{s}^{-1}$). Wise and Naylor [1987] have proposed that the increase in lutein is probably due to the violaxanthin/zeaxanthin epoxide cycle that is commonly induced under stress conditions, and an increase in lutein could actually be an up-regulation of the synthesis of zeaxanthin, an isomer of lutein. Therefore, the apparent lutein increase (which equalled the amount of violaxanthin lost) could well be a conversion to zeaxanthin. Huang and co-workers [43] confirmed that lutein performs a key role in plants under severe stress conditions and in the xanthophyll cycle in the protection of photosynthetic organisms. Zeaxanthin is more directly involved in the protection of the photosystems, but only in situations where the photo inhibition of photosynthesis occurs [44-46]. The violaxanthin cycle is present in all higher plants and some green algae. Its basic biochemical characteristic is that violaxanthin is reversibly converted to zeaxanthin *via* the intermediate antheraxanthin [47].

A coordinated role of soluble and cell bound phenols has been implicated in the cellular physiology of plants and these mighty antioxidants suffer a decline in response to biotic/abiotic stress [48]. In tomato plants, the total phenols decreased by 22.6% and 31.3% (Significance level $P = 0.090$) compared with control plants after four weeks of exposure to KU-1 CMV or CMV-16, respectively. This outcome again supports the results indicating that CMV-16 is more virulent than KU-1 CMV, and therefore, in defence, the plants produce additional antioxidant phenolic compounds in response to CMV-16. In general plant phenolics, due to their antimicrobial activity, increase in response to pathogen infection [49-50]. However if the ultrastructural changes take place in response to pathogens, the level of plant phenolics is adversely affected [51]. The decrease in phenolic compounds in response to virulent CMV-16 infection may be due to an ultrastructural events Bashan et al. [52] reported that mature tomato leaves had higher Phenol Oxidase (PO) activity and phenol content compared with younger leaves. The increase was justified due to the resistance of the tomato plants to *P. Syringaepv.* tomato (bacteria) involving phenol oxidative processes. Thus, a direct correlation between the disease severity and Phenol Peroxidase (PPO) activity and phenol content in tomato plant tissue was suggested [52]. Similarly, oxidative stress defences of *Carica papaya* are known to elevate phenol peroxidase and superoxide dismutase in response to nitric oxide, *Papaya meleria* and *Saccharomyces cervisiae* [53]. These workers associated the plant defence with a burst

in nitric oxide, production of ROS, and an elevation in antioxidant phenolics, accompanied by changes in osmotic balance, increase in water content with a reduction in sugar and protein content of the plant during the viral infection. An increase in phenolic secondary metabolites such as flavones, and hydroxycinnamic acids, in response to virus-infection in plants, was reported [54,55]. Parallel to these reports, the accumulation of phenols in tomato and squash plants during the third and fourth weeks of infection with CMV-16, in the current study, could be explained by the plant's protective response against the virus producing ROS.

In squash plants, the total phenols increased by 7.8% when challenged by KU-1 CMV, and 52.1% when challenged by CMV-16 virus. In senesced squash leaves, after the fourth week of infection, phenols showed a notable 160.6% increase in the total phenols confirming virulence of CMV-16 in squash plants (Figure 2c and Figure 2d). Many workers have reported [56-58] an increase in the powerful radical scavenger phenolics in *Vicia faba* after inoculation with Bean yellow mosaic virus.

Significant oxidative changes in unsaturated fatty acids and their alterations provide an indirect measure of the extent of lipid peroxidation [59]. Stress-adapted plants respond to abiotic/biotic stress by remodelling membrane fluidity and by releasing Alpha-Linolenic Acid (ALA) from membrane lipids. The modification of the membrane fluidity is mediated by the alterations in unsaturated fatty acids that are the major components of biomembranes and are regulated by fatty acid desaturases [60]. It is believed that fatty acid desaturases are regulated at both the transcriptional and post-translational levels in response to pathogen stress [61]. Therefore, the desaturation index may be used as a disease marker for environmental stress in biological systems. The oxidative stress induced by cucumber mosaic virus and zucchini yellow mosaic virus-infected plants (*Cucumis sativus* and *Cucurbitapepo*) undergo an enhanced peroxidation of polyunsaturated fatty acids, leading to an advanced disintegration of biomembranes [62].

In the current study, we calculated the ratio of total unsaturated fatty acids/to saturated fatty acids (UFA/SFA) as a desaturation index for variations in response to viral stress. The major identified fatty acids in both tomato and squash were 16:0; 16:1; 18:0; 18:1; 18:2 and 18:3. Both viruses had an impact on the desaturation index, and the influence was found to be parallel to carotenoids and phenol variations. The squash plants, after 4 weeks of infection with KU-1 CMV, showed an increase of 45.45% in the desaturation index, compared with the control. Contrastingly, infection to the squash plants with CMV-16 virus showed a sharp decline of 68.18% in the desaturation index value, and in squash senesced leaf, the desaturation index value declined by 77.2% when compared with control healthy squash leaf (Figure 3b). The percentage increase in desaturation index after two weeks of infection with either KU-1 CMV or CMV-16 was observed maximum at 91.30% and 86.95% respectively. It is known that chlorosis, as a consequence of the viral infection, may contribute to an increase in lipids in etiolated leaves and decrease during senescence, suggesting a decrease in the desaturation index [61].

In tomato plants, the desaturation index increased by 5.88% in response to KU-1 CMV, while in response to CMV-16 infection, the desaturation index increased by 9.44% compared with the healthy

tomato plant within the same period of time (Figure 3a). This increase may be due to a stimulation/upregulation of desaturases in response to the virulent CMV-16 infection. It was noticed that the KU-1 CMV strain had a lesser impact on the desaturation index in both tomato and squash plants compared with the CMV-16 strain. Many reports have indicated that there is an increase in oleic, linoleic and/or linolenic acid in plants challenged by biotic stress [61,62].

Conclusion

In conclusion, CMV-16 affected the lutein/ β -carotene ratio by reducing the β -carotene content and increasing lutein accumulation in squash. The change in the lutein/ β -carotene ratio was less pronounced in tomato plants infected with the same virus. The desaturation index (UFA/SFA) increased in response to CMV-16 in both squash and tomato plants, indicating an enhanced defence by an upregulation of the unsaturated fatty acids. This process is accompanied by an increased synthesis of phenolics in response to CMV-16. In CMV-16-infected squash plants, phenolic accumulation sharply increased, while it had a lower impact in tomato plants, possibly indicating host pathogen specificity.

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