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Antioxidant and Oxidant Levels of Pepper (*Capsicum annuum* cv. 'Charlee') Infected with Pepper Mild Mottle Virus

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Abstract

Total antioxidant status (TAS), total oxidant status (TOS), as well as total phenol (TP), free phenol (FP), conjugated phenol (CP), and the vitamin C content of pepper plants (*Capsicum annuum* cv. 'Charlee') infected with *Pepper mild mottle virus* (PMMoV) were determined via a novel automated antioxidant system using test kits. PMMoV was first identified in Turkey in 1994 in commercial pepper (*Capsicum annuum* cv. 'Charlee') fields and caused an outbreak in southern Turkey by 2006, especially in greenhouses. Infected plants were confirmed by ELISA using commercial kits for the virus. The infected plants had stunted growth; and leaves and fruits became mottled, puckered, malformed, deformed, and marked by off-colored sunken areas. Fruits expressing virus symptoms were utilized for biochemical analyses. The percent ratio of TOS to TAS was calculated as the oxidative stress index (OSI). The TAS level and the vitamin C content were found to be significantly lower in infected peppers as compared to healthy peppers. However, TOS, OSI, TP, FP, and CP levels were significantly higher in infected pepper fruits than in healthy ones. In this study, it has been demonstrated that low levels of TAS and high levels of TOS, as well as high OSI status, reflects the quality of peppers. Using this method, the quality of fruits and the condition of plants can be determined in advance of stress development.

Keywords: antioxidant, phenol, pepper mild mottle virus, PMMoV, total oxidative status, total antioxidant status, vitamin C

Abbreviations: TAS: Total Antioxidative Status; TOS: Total Oxidative Stress; OSI: Oxidative Stress Index; TP: Total Phenol; FP: Free Phenol; CP: Conjugated Phenol; ROS: Reactive Oxygen Species

Introduction

Sweet and hot peppers are one of the most important vegetable species in Turkey, as well as in other parts of the world. Turkey ranks fourth in the world in regards to vegetable production and second in rank, by 1.84 million tons, in terms of pepper production (Ozalp, 2009). Pepper crops can be grown in fields and in greenhouses, however, their average yields are severely impacted by the presence of pests and diseases. The main restricting factor in the yield of pepper quantity, as well as quality, is infection of virus diseases (Anandakumar *et al*., 2008). Although several viruses are known to infect peppers, PMMoV has been severely damaging the fruits of peppers, in fields and in greenhouses, in recent years (Aycicek *et al*., 2006; Güldür and Çağlar, 2006). The geographic distribution of PMMoV has been reported to include North America, Australia, Japan, Korea, and Europe (Brunt *et al*., 1996). Infected plants exhibit stunted growth, mottling, puckering, malformed leaves, and the fruits are generally small and deformed and marked by off-colored sunken areas (Güldür and Çağlar, 2006). If plants are infected when they are young symptoms may become more severe. However, symptoms may vary depending on the cultivar. In severe cases, the virus can lead to a 75-95% loss in yield (Güldür and Çağlar, 2006). Symptoms of virus infection in pepper plants usually develops within 1-3 weeks of infection (Anandakumar *et al*., 2008). When plants are attacked by pathogens they respond by activating a variety of defense mechanisms, including the rapid production and accumulation of ROS (reacting oxygen species) (Kiraly *et al*., 2008). On the other hand, the phenolic content of the plants increases as a response of the defense mechanism (Katoh *et al*., 2009). The status of TAS, TOS, and OSI, as well as the phenolic content of virus-infected peppers, reflects the condition and resistance of pepper plants. In this study, wit has been used a novel automated method developed by Erel (2004a; 2005) for measuring TAS and TOS, and determined the level of OSI based on the ratio between TOS and TAS in order to ascertain the health and the antioxidative status of pepper fruits. Infected and healthy (virus-free) plants were compared in order to evaluate the relationships between oxidative stress and fruit quality.

Materials and methods

Plant material and growth condition

Seedlings of *C. annuum* cv. 'Charlee' with six to seven leaves were purchased from a commercial supplier in the southern part of Turkey. Plants were then grown in a greenhouse where they were maintained in pots (25 cm in diameter) that contained horticultural sand and peat (1:1). During the experimental trial the average day/night temperature was determined to be 23-25°C/15-18°C for a photoperiod of 16 h.

Inoculation of plants with PMMoV

Virus isolates were made from the naturally infected pepper plants grown in greenhouses and propagated in pepper plants. Sap was extracted from the pepper plants by grinding young leaf tissue into a 0.3 M potassium phosphate buffer (1:10; w/v, pH 7.2) using a prechilled mortar and pestle. The homogenate was centrifuged at 10,000 g for 5 minutes. Thirty plants were then inoculated with the supernatant containing PMMoV by gently rubbing three of the bottom leaves of the plants with diatomaceous earth while wearing supernatant-wetted gloved fingers. Control plants were treated in the same manner using distilled water. After two weeks of inoculation, the infection was confirmed using ELISA.

The ELISA test

An ELISA test was carried out in order to confirm the existence of PMMoV in inoculated plants. Leaf tissue was ground in phosphate buffered saline with Tween 20 (1/10; w/v) using a pre-chilled mortar and pestle, and the extract was tested using commercial ELISA kits. Absorbance values (A_{405nm}) that were nearly two-fold the maximum negative control value (ELISA index > 2) were considered as positive (Clark and Adams, 1997).

Sampling for oxidative stress

Fruit samples expressing PMMoV symptoms were collected after 60 days of inoculation from infected and control groups. Thirty samples were evaluated for TAS, TOS, OSI, vitamin C, and TP and FP content.

Sample preparation

A 1 g sample of the pepper tissues from fruit samples were cut into small pieces and homogenized with 10 ml of a 50% aqueous ethanol solution and sonicated. The sonicated homogenate was filtered through four layers of cheesecloth and centrifuged at 10,000 *g* for 15 min. The supernatant was re-centrifuged at 10,000 *g* for 10 min and used for the TAS, TOS, and vitamin C assays (Dikilitas *et al*., 2011; Velioglu *et al*., 1998).

Measurements of total antioxidant status (TAS)

TAS levels for the infected and control groups of peppers were determined using a novel automated method developed by Erel (2004a). In this method, the antioxidative effect of the sample as a result of potent-free radicals, which are initiated by the production of hydroxyl radical, is measured spectrophotometrically. Antioxidants present within the sample supress oxidation reactions and color formation. In other words, they accelerate the bleaching rate proportional to their concentrations. The reaction can be monitored spectrophotometrically and the bleaching rate is inversely related to the TAS of the sample. The assay has excellent precision values that are lower than 3%. Suppression of color formation can be calibrated using Trolox, which is widely used as a traditional standard for TAS measurement assays, with assay results expressed in mmol Trolox equiv/g fresh weight (Erel, 2004b). Following a manual spectrophotometric optimization process, the method was applied while using an automated analyzer, Aeroset. The assay was carried out as follows: 200 µl of Reagent 1 [(*o*-dianisidine (10 mM), ferrous ion (45 µM) in Clark and Lubs solution (75 mM, pH 1.8)], was mixed with 10 µl of Reagent 2 [H_2O_2 (7.5 mM) in Clark and Lubs solution], and 5 µl of the sample extract was added to the mixture. The mixture was then read at 444 nm for 3 min.

Measurements of total oxidant status (TOS)

TOS levels were determined using a novel automated measurement method developed by Erel (2005). In this method, oxidants present in the sample oxidize the ferrous ion-*o*-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. In an acidic medium, the ferric ion makes a colored complex with xylenol orange. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide, and the results are expressed as µmol H_2O_2 equiv/l. The assay was carried out as follows: 225 µl solution of Reagent 1 (150 µM xylenol orange, 140 mM NaCl, and 1.35 M glycerol in a 25 mM H_2SO_4 solution, pH 1.75) was mixed with 11 µl Reagent 2 (5 mM ferrous ion and 10 mM *o*-dianisidine in a 25 mM H_2SO_4 solution) and 35 µl sample extract was added to the mixture. The mixture was then read at 560 and 800 nm for 3 min.

Measurements of vitamin C

The reduced ascorbate concentration was measured with the FRASC assay using ascorbate oxidase (Benzie and Strain, 1999).

Measurements of the oxidative stress index (OSI)

The percent ratio of TOS to TAS level has been accepted as the oxidative stress index (OSI) (Benzie and Strain, 1999; Dikilitas *et al*., 2011; Erel, 2004a; Kosecik *et al*., 2005). In order to perform the calculation, the unit of TAS, mmol Trolox equivalent/g, was changed to µmol

Trolox equivalent/g, and the OSI value was calculated according to the following formula:

OSI = [(TOS, μmol H_2O_2 equivalent/g)/(TAS, μmol Trolox equivalent/g) × 100].

Measurements of the total and free phenolic content

Total phenols (TP), conjugated phenols (CP), and free phenols (FP) of fruits were determined by the Folin-Ciocalteu assay which involves a reduction of the reagent by phenolic compounds, with the concomitant formation of a blue complex. Intensity at 760 nm increases linearly with the concentration of phenols in the reaction medium (Singleton *et al*., 1999). In this study, catechin was preferred to gallic acid since the activity of the catechin substrate gave better results (Dikilitas *et al*., 2011). The phenolic content of fruits were determined from the calibration equations and were expressed as catechin equivalents g^{-1} Fwt (Imeh and Khokhar, 2002).

Chemicals

Vitamin C (L (+) ascorbic acid); ascorbate oxidase; gallic acid; 2,2-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS); 2,4,6-tripyridyl-striazine (TPTZ); xylenol orange (o-cresosulfonphthalein-3,3-bis (sodium methyliminodiacetate)); sulfuric acid; acetic acid; sodium acetate; hydrochloric acid; orthodianisidine dihydrochloride; glycerol; ethanol; ferrous ammonium sulfate; catechin; hydrogen peroxide; and Folin-Ciocalteu's phenol reagent were purchased from Sigma (Missouri, USA) and Merck Co (New Jersey, USA). The water-soluble analogue of vitamin E (Trolox; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from the Sigma-Aldrich Chemical Co. (Missouri, USA). All chemicals were the ultra pure grade, and type I reagent-grade deionized water was utilized.

Statistical analyses

Values were expressed as the mean ± SE. The means of the groups were compared using a one way analysis of variance (ANOVA). A p value less than 0.05 was accepted as significant. Data were analyzed using SPSS® (Illinois, USA) for the Windows (Microsoft, USA) computing program (Version 11.0).

Results and discussion

The oxidative, antioxidative, and phenolic content of the peppers infected with PMMoV, as well as the control plants, are presented in Tab. 1. The TAS and vitamin C contents of the control plants were higher than those of the PMMoV infected pepper plants while the TOS, OSI, and phenolic contents, including FP and CP, were found to be lower than the infected pepper plants. For example, the TAS and Vitamin C levels of the control plants were 2.75 and 1.97 times higher, respectively, than those of infected plants. On the other hand, biochemical responses such as TOS, OSI, and phenol levels generated under biotic stress conditions were 1.35, 3.72, and 1.20 times higher, respectively, than that generated under stress-free conditions. Significant negative correlations also existed between vitamin C and OSI in both the control ($r = -0.41$, $p < 0.05$) and infected plants ($r = -0.77$, $p < 0.05$). Infected plants displayed a lower content of vitamin C when the OSI level of infected plants increased (Fig. 1).

Fruits and vegetables contain significant levels of biologically active components that provide a major source of dietary antioxidants that increase plasma antioxidant capacity (Krishna *et al*., 2010). Thus, the consumption of fruits and vegetables has been associated with a lower incidence of and lower mortality rates caused by cancer in several human cohorts, as well as case-control studies for all common types of cancer. For example, Anandakumar *et al*. (2008) stated that the effect of a pungent ingredient of red pepper, capsaicin, restored cellular metabolism by mitigating the oxidative stress that induced changes in the antioxidant defense system by benzo(a)pyrene in the lungs of mice. Pepper and its products are rich in health related food components and are a good source of vitamin C. Numerous reports have been published on the antioxidant activity of vegetables and crop plants (Perucka, *et al*., 2010). For example, potatoes, broccoli (Al-Saikhon *et al*., 1995; Cao *et al*., 1996), mushrooms, white cabbage, and cauliflower (Gazzani *et al*., 1998), and kidney and pinto beans (Pengelly *et al*., 2010) have been reported to have high antioxidant activities.

Reports regarding the antioxidant capacity of peppers have recently been updated and have gained significant attention (Anandakumar *et al*., 2008; Dikilitas *et al*., 2011; Keçeli, 2008; Perucka *et al*., 2010). Peppers are an important crop due to the economic and nutritional value of fruits, and since they are an excellent source of antioxidant compounds such as vitamin C and carotenoids (Navarro *et al*., 2006). However, abiotic and biotic stress factors reduce fruit yields and crop production, as well as quality. The deterioration of fruit quality has commonly been reported to result from abiotic stress factors in pepper plants as a result of biochemical responses. Very few reports describing PMMoV virus infections of pepper plants have been reported. In general, abiotic and biotic stresses generate ROS such as O^-_2 and H_2O_2 in plant cells, and their overproduction is a common consequence of stress factors. However, plants respond to stress by ameliorating the danger that results from the presence of cellular oxidants. Under these conditions, there should be a balance between the generation and the degradation of ROS, otherwise oxidative injuries are inevitable. Therefore, plants, in general, have evolved complex defense mechanisms for detoxifying O^-_2 and H_2O_2 (Dikilitas *et al*., 2009; Diwan *et al*., 2010). The primary components of antioxidant systems consist of antioxidant enzymes (superoxide dismutase, catalase, peroxidase, and glutathione reductase) and non-enzymatic low molecular weight antioxidants (glutathione, proline, caro-

Tab. 1. Comparison between the oxidative-antioxidative parameters and the phenolic content of peppers

Parameters	Control Peppers, n=30	Infected Peppers, n=30
TAS, µmol Trolox Eq g^{-1} Fwt	$8.8^a \pm 1.2$	$3.2^b \pm 0.28$
TOS, µmol H_2O_2 Eq g^{-1} Fwt	$0.174^a \pm 0.015$	$0.235^b \pm 0.012$
OSI, Arbitrary Unit	$1.97^a \pm 0.03$	$7.34^b \pm 0.02$
Vitamin C, µmol g^{-1} Fwt	$4.12^a \pm 0.06$	$2.09^b \pm 0.05$
Total phenol, µmol catechin Eq g^{-1} Fwt	$0.213^a \pm 0.02$	$0.256^b \pm 0.03$
Free phenol, µmol catechin Eq g^{-1} Fwt	$0.152^a \pm 0.01$	$0.175^b \pm 0.03$
Conjugated phenol, µmol catechin Eq g^{-1} Fwt	$0.087^a \pm 0.01$	$0.121^b \pm 0.02$

TAS: Total Antioxidant Status; TOS: Total Oxidant Status; OSI: Oxidative Stress Index; p: Significance was defined as $p < 0.05$. Letters different from each other above the numbers in the same lines are statistically significant. Values were expressed as mean ±SE ($p < 0.05$)

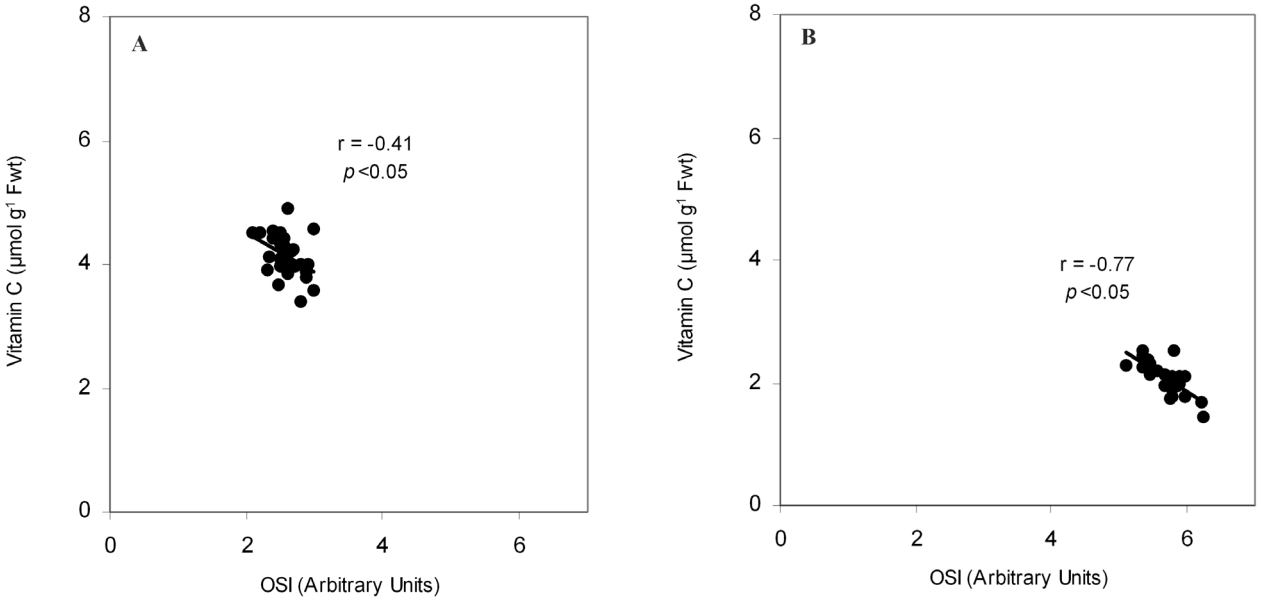

Fig. 1. The correlation between the content of OSI and vitamin C in *C. annuum* cv. 'Charlee' pepper plants-(A) the control group, and (B) the pepper mild mottled virus-infected group

tenoids, ascorbate, and tocopherols) (Panda and Khan, 2009). Many studies regarding the relationship of virus infection on plant antioxidant status have revealed that the antioxidant capacity was significantly reduced. For example, catalase activity has been shown to decline following TMV infection (Brunt *et al*., 1996). Similarly, changes in ascorbate and glutathione levels and changes in the activities of ascorbate peroxidase, catalase, dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione S-transferase (CST), and superoxide dismutase (SOD) declined following a TMV inoculation of tobacco plants (Fodor *et al*., 1997). On the other hand, stress metabolites such as H_2O_2 and MDA (malondialdhyde) tend to increase following virus infections. For example, higher H_2O_2 and MDA concentrations were recorded in bean yellow mosaic virus (BYMV) virus-infected faba bean leaves as compared to corresponding controls (Radwan *et al*., 2010). Additionally, the content of phenolics and flavonoids, as well as either soluble or insoluble proteins, also tend to increase following the occurrence of stress. Radwan *et al*. (2010) reported that BYMV-infected bean leaves had a protein content higher than the control, indicating the accumulation of pathogenesis-related proteins and concluded that changing antioxidant status and the accumulation of some antioxidant metabolites, as well as pronounced alterations in the protein composition, indicated a kind of plant response against pathogen invasion. Similarly, Hernandez *et al*. (2004) reported that a long-term effect of plum pox (PPV) virus infection produced an oxidative stress that was monitored as an increase of lipid peroxidation and protein oxidation and suggested an imbalance in antioxidative metabolism, which eventually resulted in the progress of PPV infection and related symptoms in peach plants.

In this study, an increase in TOS levels in infected pepper plants was evident when they were compared to corresponding control groups. In a similar manner, the total phenolic content, as well as free and conjugated phenols, were found to be higher under stress conditions. Therefore, OSI levels in infected pepper plants was found to be remarkably higher than those of the control group as a result of a decrease of TAS and an increase of TOS levels

in infected pepper plants. The results clearly explain that oxidative stress is an important element in systemic virus infections where virus particles are distributed within the entire fruit (Dikilitas *et al*., 2011).

Vitamin C (ascorbate), which is one of the most powerful non-enzymatic antioxidants in plants, functions as a ubiquitous antioxidant in plants by scavenging a wide range of ROS via enzymatic and non-enzymatic reactions (Suza *et al*., 2010). Vitamin C is also a cofactor in at least eight enzymatic reactions, including several collagen synthesis reactions, which cause the most severe symptoms of scurvy when they are dysfunctional (www.food.gov.uk). Vitamin C provides the first line of defense against damaging ROS, and helps protect plant cells from many factors that induce oxidative stress including wounding, high salinity, and pathogen attack in living organisms. However, not certain is whether or not vitamin C and antioxidant supplements, in general, prevent oxidative stress-related diseases and promote health. In plants, ascorbic acid is associated with chloroplasts and apparently plays a role in ameliorating the oxidative stress of photosynthesis, and has a number of other roles in cell division and protein modification.

In this study, the vitamin C content of infected fruits was found to be significantly lower than those of control plants. The results outlined here regarding a decrease in vitamin C in pepper fruits with PMMoV, and represents the first report. The correlation between the vitamin C content and the OSI of fruits was found to be negatively correlated. However, the negative correlation was more evident in infected fruits than within the corresponding control fruits. Clear is that an increase in OSI reduces the quality and quantity of fruits and crop yields. In general terms, the quality of fruits are measured in regards to their vitamins, as well as to their nutritional value as related to carbohydrates and the content of other organics. In the present case, a reduction in vitamin C resulted in a higher accumulation of oxidants in pepper fruits. As determined via calculations (OSI = TOS/TAS), stress indices were remarkably higher in infected fruits.

When phenolic compounds were examined, infected fruits displayed a higher content of phenolics as compared to controls. Phenolics are important metabolites in plant growth and in the defense against infection and injury. The presence of phenolic compounds in injured plants may have an important impact on oxidative stability and microbial safety (Karakaya *et al*., 2001). Although phenolic compounds do not have nutritious value, they are as important as other antioxidants (Holiman *et al*., 1996). In some cases, their accumulation has been considered as having health-promoting effects for consumers. In fact, phenolic accumulation may be regarded as a sign of the avoidance of abiotic and biotic stress factors. Although the accumulation of phenolic content may prevent the spread of disease agents in fruits or leaves, a higher accumulation of phenolics is not desired as a result of their interactions with proteins and carbohydrates (Robarbs *et al*., 1999). Since phenolic compounds result in discoloration in fruit or leaf tissues, an accumulation that exceeds a certain level could be toxic to consumers, as well as to infecting agents.

Conclusions

In this study, it has been found that both CP and FP increased after virus infection in pepper fruits. The change may result from a defense mechanism against infection by fruit. Although the phenolic content increased, an expected increase in other metabolites, such as antioxidant activity (TAS) in infected pepper plants, was not observed, which may be the result of the insufficient accumulation of antioxidant metabolites due to heavy infection.

In conclusion, fruits infected with PMMoV had lower TAS and TP levels and a lower vitamin C content than those of corresponding control plants. Infected peppers accumulated a greater amount of TOS and, as a result, higher OSI. OSI levels are regarded as a good quality index for fruits as well as the status of plants.

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