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A novel method of measuring oxidative stress of pepper (*Capsicum annuum var.* Charlee) infected with tobacco mosaic virus

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ABSTRACT

Objective: To evaluate oxidative, antioxidative and the phenol status of pepper plants (*Capsicum annuum* var. Charlee) at an early stage of the growing period using a novel method.

Methodology and results: Tobacco mosaic virus (TMV) which was isolated from infected pepper plants growing in a greenhouse was inoculated to uninfected healthy pepper plants by mechanical inoculation method at an early stage of the growing period, 4 weeks. The infection of inoculated plants was confirmed by ELISA method. Fruits expressing the virus symptoms were collected and used for biochemical analyses. Total antioxidant status (TAS) and total oxidative status (TOS) of the fruits were determined colorimetrically. Total phenol (TP), free phenol (FP) and conjugated phenol (CP) amounts were measured as part of defense response. Vitamin C contents were also measured using ascorbate oxidase to determine the quality of fruits. The percent ratio of TOS to TAS was evaluated as oxidative stress index (OSI). TAS levels, vitamin C contents, and FP were found significantly lower in the infected peppers than those of healthy ones. In contrary, TOS, OSI, CP and TP levels were significantly higher in the infected peppers than those of the control plants. There were significant correlations among oxidative, antioxidative, and phenol parameters.

Actual or potential application of findings: This study showed that the production of low amounts of TAS and high amount of TOS negatively affected the quality of peppers under TMV stress. Quality of fruits and the condition of plants would be determined in advance of the stress development with the use of instant determination of TAS, TOS and OSI with this novel automated system.

Key Words: Antioxidant, Phenol, Tobacco Mosaic Virus, Total oxidative status, Total antioxidant status, Vitamin C.

Abbreviations: TMV, Tobacco Mosaic Virus; 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

Reactive oxygen species (ROS) are produced during metabolic and physiological processes. As a result of those, harmful oxidative reactions may occur in organisms. Under some conditions, the increase in oxidants and decrease in antioxidants cannot be prevented, and the

oxidative/antioxidative balance shifts towards the oxidative status. Consequently, oxidative stress develops (Halliwell & Gutteridge, 2000; Dikilitas et al., 2009).

Pepper (*Capsicum annuum* L.) is a perennial vegetable species cultivated in greenhouses and fields. *C. annuum* is susceptible to several pathogens including tobacco mosaic virus (TMV). Symptoms of the diseases in pepper plants may usually develop within 1-3 weeks of infection. Infected plants show mosaic vein clearing, distortion of leaves, deformation of fruits, stem necrosis and stunting of plants (Martelli & Quaqcuarelli, 1988; Gottula & Fuchs, 2009). The variety Charlee is highly sensitive to infection of TMV. Infected plants express severe systemic symptoms such as leaf chlorosis, stem and fruit necrosis, and the appearance of leaf drop.

TMV has a rod-shaped particle, 300 nm in length, which consists of 2130 identical protein subunits arranged as a helix around a RNA molecule. The virus has a wide range of hosts and world-wide distribution. The virus is easily transmitted by mechanical inoculation, but not by insects or other common vectors (Zaitlin & Israel, 1975; Gayoso et al., 2004).

When plants are attacked by pathogens they respond by activating a variety of defense mechanisms, including the rapid production and accumulation of ROS, primarily the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Low & Merida, 1996; Zhao et al., 2007). It has been well established that the rate of the production of ROS increases and antioxidant metabolism changes when plants are inoculated with viral pathogens (Zhanlin & Burritt, 2003; Kiraly et al., 2008). It has also been shown that total phenolic contents increase as a response of defense mechanism against infection (Gayoso et al., 2004; Katoh et al., 2009).

There is no report related with TAS, TOS and phenol status of pepper fruit *C. annuum* var. Charlee) infected with TMV. In this study, oxidative, antioxidative status and total and free phenol contents of pepper fruits infected with TMV were investigated and compared with those of non-infected healthy pepper fruits to determine the relationships between oxidative stress and fruit quality.

MATERIALS AND METHODS

Plant material and growth condition: Seedlings of *C. annuum* with six to seven leaves were purchased from a commercial supplier in Southern part of Turkey. The plants were then grown in a greenhouse where they were maintained in pots (25 cm in diameter) containing horticultural sand and peat (1:1). The average day/night temperature during the trial was arranged as 23-25°C/15-18°C at 16 h photoperiod. Plants inoculated with distilled water were used as control. The fruits of control plants remained symptom-free during the experimental trial and gave a negative enzyme-linked immunosorbent assay (ELISA).

Inoculation of plant with TMV: The virus isolate, which was isolated from naturally infected pepper plants raised in a greenhouse, was propagated in *Nicotiana tabacum* var. Samsun plants. Sap was extracted from the tobacco plants showing symptoms of TMV infection by grinding young leaf tissue in 0.3 M potassium phosphate buffer (1:10; w/v, pH 7.2) using a prechilled mortar and pestle. The homogenate was centrifuged at 10000*g* for 5 minutes. Fifteen plants were then inoculated with supernatant containing TMV by gently rubbing the three bottom leaves of the plants with diatomaceous earth with supernatant-wetted gloved fingers. Control plants were treated with the same manner using distilled water. After 15 days of the inoculation, the infection was confirmed by ELISA test.

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

Sampling for oxidative stress: Fruit samples, expressing TMV symptoms such as deformation, necrotic lesions and rolling and healthy ones, were collected after 55 days of inoculation from infected and control groups. Fifteen samples were evaluated for TAS, TOS, OSI, vitamin C and TP and FP contents.

Sample preparation: Two g of the pepper tissues were cut into small pieces and homogenized with 10 ml of 50% aqueous ethanol and sonicated. The sonicated homogenate was filtered through four layers of

cheesecloth and centrifuged at 10000*g* for 10 min. The supernatant was collected and used directly for the TAS, TOS and vitamin C assays.(Velioglu & Mazza, 1998; Zhang & Hamauzu, 2003).

Measurement of total antioxidant status (TAS): TAS levels of infected and control groups of peppers were determined using a novel automated measurement method developed by Erel (2004a). In this method, hydroxyl radical, which is the most potent radical, is produced via Fenton reaction. In the classical Fenton reaction, the hydroxyl radical is produced by mixing ferrous ion solution and hydrogen peroxide solution. In the most recently developed assay by Erel (2004a), ferrous ion solution, which is present in Reagent 1, is mixed with hydrogen peroxide, which is present in Reagent 2. The sequential produced radicals such as brown colored dianisidinyl radical cation, produced by the hydroxyl radical, are also potent radicals. In the assay, the antioxidative effect of the sample against the potent-free radical reactions, which is initiated by the produced hydroxyl radical, is measured. Antioxidants present in the sample, supress the oxidation reactions and color formation. In another way, they accelerate the bleaching rate to a degree proportional to their concentrations. This reaction can be monitored spectrophotometrically and the bleaching rate is inversely related with the TAS of the sample. The assay has excellent precision values lower than 3%. The reaction rate is calibrated with Trolox, which is widely used as a traditional standard for TAS measurement assays, and the assay results are expressed in mmol Trolox equiv/g fresh weight (Erel, 2004b). After manual spectrophotometric optimization processes, the method was applied to an automated analyzer, Aeroset. The assay was carried out as follows: 200 µl of Reagent 1 [(*o*-dianisidine (10 mM), ferrous ion (45 AM) in the Clark and Lubs solution (75 mM, pH 1.8)] was mixed with 10 µl Reagent 2 [H_2O_2 (7.5 mM) in the Clark and Lubs solution] and 5 µl sample extract was added to it. The mixture was then read at 444 nm.

Measurement 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. The ferric ion makes a colored complex with xylenol orange in an acidic medium. 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 (xylenol orange 150 µM, NaCl 140 mM and glycerol 1.35 M in 25 mM H_2SO_4 solution, pH 1.75) was mixed with 11 µl Reagent 2 (ferrous ion 5 mM and *o*-dianisidine 10 mM in 25 mM H_2SO_4 solution) and 35 µl sample extract was added to it. The mixture was then read at 560 nm.

Measurement of vitamin C: Reduced ascorbate concentration was measured by FRASC assay using ascorbate oxidase (Benzie and Strain, 1999).

Measurement of oxidative stress index (OSI): Percent ratio of TOS to TAS level was accepted as oxidative stress index (OSI) (Kosecik et al., 2005; Aycicek et al., 2006). 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].

Measurement of total and free phenolic contents: Total phenols (TP), Conjugated phenols (CP) and free phenols (FP) of the fruits were determined by the Folin-Ciocalteu assay that involves reduction of the reagent by phenolic compounds, with concomitant formation of a blue complex. Its intensity at 760 nm increases linearly with the concentration of phenols in the reaction medium (Singleton et al., 1999). In this study, gallic acid and catechin were used as spectrophotometric standards separately to compare the efficiency of each standard chemical. The phenolic contents of the fruits were determined from calibration equations and were expressed as gallic acid and catechin equivalents (Imeh & Khokhar, 2002).

Chemicals: Vitamin C (L (+) ascorbic acid), ascorbate oxidase, gallic acid, [,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, sadium acetate, hydrochloride acid, orthodianisidine dihydrochloride, glycerol, ethanol, ferrous ammonium sulfate, catechin, hydrogen peroxide and Folin-Ciocalteu's phenol reagent were purchased from Sigma and Merck Co. 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. All chemicals were ultra pure grade, and type I reagent-grade deionized water was used.

Statistical analyses: The means of the groups were compared with student's *t* test since the groups showed Gaussian distribution type. The relationships were investigated by correlation analyses test by using SPSS for Windows computing program (SPPS Inc. Version 12.0).

RESULTS

As seen from the results of the experiment summarized in Table 1, antioxidants TAS, vitamin C and FP contents decreased while oxidants TOS, OSI and TP and conjugated phenol (CP) contents significantly increased in the infected peppers. There were significant positive correlations among TAS, vitamin C and FP and inverse correlation with TOS, OSI and CP (Table 2). In the measurement of phenol contents, catechin gave better results with regard to reflection of phenol contents than that of gallic acid.

Table 1: Comparison of oxidative-antioxidative parameters and phenol contents of the peppers.

Parameters	Infected Peppers n=15	Control Peppers n=15	*p*
TAS, µmol Trolox Eq g^{-1} Fwt	5.84 ± 1.78	10.43 ± 1.58	<0.001
Vitamin C, µmol g^{-1} Fwt	3.10 ± 0.36	4.90 ± 1.22	<0.001
TOS, µmol H_2O_2 Eq g^{-1} Fwt	0.416 ± 0.08	0.384 ± 0.03	<0.001
OSI, Arbitrary Unit	7.12 ± 0.99	4.320 ± 1.17	<0.001
Total phenol, µmol catechin Eq g^{-1} Fwt	0.223 ± 0.01	0.198 ± 0.02	<0.007
Free phenol, µmol catechin Eq g^{-1} Fwt	0.112 ± 0.01	0.124 ± 0.01	<0.002
Conjugated phenol, µmol catechin Eq g^{-1} Fwt	0.111 ± 0.01	0.074 ± 0.01	<0.001
Total phenol, µmol gallic acid Eq g^{-1} Fwt	0.139 ± 0.01	0.124 ± 0.02	<0.007
Free phenol, µmol gallic acid Eq g^{-1} Fwt	0.070 ± 0.01	0.078 ± 0.01	<0.002
Conjugated phenol, µmol gallic acid Eq g^{-1} Fwt	0.069 ± 0.01	0.046 ± 0.01	<0.001

TAS: Total Antioxidant Status; TOS: Total Oxidant Status; OSI: Oxidative Stress Index; *p:* Significance was defined as $p < 0.05$.

Table 2: The correlations between the parameters of peppers infected with TMV.

	TAS	Vitamin C	TOS	OSI	TP	FP	CP
TAS		*r*=0.81 *p*<0.001 *n*=30	*r*=-0.72 *p*<0.001 *n*=30	*r*=-0.77 *p*<0.001 *n*=30	*r*=-0.28 *p*=0.141 *n*=30	*r*=0.45 *p*=0.014 *n*=30	*r*=-0.45 *p*=0.018 *N*=30
Vitamin C			*r*=-0.61 *p*<0.001 *n*=30	*r*=-0.54 *p*<0.003 *n*=30	*r*=-0.14 *p*=0.468 *n*=30	*r*=0.48 *p*=0.007 *n*=30	*r*=-0.35 *p*=0.05 *n*=30
TOS				*r*=0.81 *p*<0.001 *n*=30	*r*=0.49 *p*=0.008 *n*=30	*r*=-0.39 *p*=0.037 *n*=30	*r*=0.58 *p*=0.001 *n*=30
OSI					*r*=0.49 *p*=0.008 *n*=30	*r*=-0.39 *p*=0.037 *n*=30	*r*=0.58 *p*=0.001 *n*=30
TP						*r*=-0.21 *p*=0.275 *n*=30	*r*=0.88 *p*<0.001 *n*=30
FP							*r*=-0.65 *p*<0.001 *n*=30

TAS: Total Antioxidant Status; TOS: Total Oxidant Status; OSI: Oxidative Stress Index; TP: Total Phenol; FP: Free Phenol; CP: Conjugated Phenol; r: Correlation; n: Number of Samples; *p:* Significance was defined as $p < 0.05$.

DISCUSSION

Fruits and vegetables contain significant levels of biologically active components that impart health benefits beyond basic nutrition (Oomah & Mazza, 2000; Perucka et al., 2010). They are a major source of

dietary antioxidants that increase the plasma antioxidant capacity resulting in inhibition of atherosclerosis related diseases in humans (Cao et al., 1998; Krishna et al., 2010). Consumption of fruits and vegetables has thus been associated with lower incidence and lower mortality rates caused by cancer in several human cohort and case-control studies for all common cancer types (Doll, 1990; Willet, 1994). There have been numerous reports on the antioxidant activity of vegetables (Prior & Cao, 2000; Perucka et al., 2010). Garlic, broccoli (Al-Saikhon et al., 1995; Cao et al., 1996; Pengelly et al., 2010), mushroom, white cabbage and cauliflower (Gazzani et al., 1998), kidney and pinto beans (Vinson et al., 1998; Pengelly et al., 2010), beans, beet and corn (Kahkonen et al., 1999) have been reported to have high antioxidant activities. Beside these, other vegetables such as kale, spinach, brussel sprouts, alfalfa sprouts, red bell pepper, onion, corn, eggplant and cucumber are also rich sources of antioxidants (Prior & Cao, 2000; Gorinstein et al., 2010). The reports about antioxidant capacity of peppers are seldom (Zhang & Hamauzu, 2003; Krishna et al., 2010; Perucka et al., 2010) and in particular, infected fruit of peppers is not reported.

In recent years, a lot of efforts have been made about the possible involvement of ROS in symptom development and pathogenesis in plant-virus interactions. A common feature of abiotic and biotic stress factors is the generation of ROS such as O^{-}_{2} and H_2O_2 in plant cell. ROS formation also accompanies normal metabolic processes, in particular, photosynthesis and respiration in all cellular compartments. To ameliorate the danger posed by the presence of cellular oxidants, plant cells have evolved complex defense mechanisms (Baker & Orlandi, 1995; Dikilitas, 2003; Diwan et al., 2010). Plants possess several mechanisms that detoxify O^{-}_{2} and H_2O_2 called antioxidant systems. The primary components of antioxidant systems include non-enzymatic antioxidants (carotenoids, ascorbate, glutathione and tocopherols) and enzymes such as SOD, catalase, glutathione peroxidase, and those involved in the ascorbate-glutathione cycle; ascorbate peroxidase, dehydroascorbate reductase, monodhydroascorbate reductase and glutathione reductase. The components of these antioxidant defense systems can be found in different subcellular compartments (Jimenez et al., 1998; Hernanadez et al., 2000; Panda & Khan, 2009).

Several groups, as a related to antioxidant status, have reported the effects of virus infection on plant antioxidant systems. Catalase activity has been shown to decline following virus infection of a susceptible host and this is believed to lead to increased H_2O_2 levels (Chen et al., 1993; Neuenschwander, 1995; Kiraly et al., 2008). SOD activity declines following the infection of resistant soybean with soybean mosaic virus (Zhuang et al., 1993), while glutathione reductase activity increases in tobacco plants reacting hypersensitively to TMV (Fodor et al., 1997). Virus infection has also been shown to increase peroxides (Moshati et al., 1993; Montalbini et al., 1995).

An increasing body of data supports the hypothesis that a fine regulation of antioxidant systems is part of the signaling pathways activating defense responses. However, the diversity of the systems used to study plant pathogen interplay make it difficult to formulate a clear picture of whether, and to what extent, changes in antioxidant systems are directly involved in the actuation of plant defense responses, or whether they are a mere consequence of the oxidative stress occurring in the attacked cells (De Gara et al., 2003). Hernandez et al. (2004) suggest that long-term effect of plum pox (PPV) virus infection produces an oxidative stress, and that an antioxidative metabolism imbalance may be related to the progress of PPV infection and symptoms in peach plants. In this study, OSI was found increased in infected fruit of plants because of decreasing of TAS and increasing of TOS (Table 1). These explain that oxidative stress is an important element in systemic virus infections where virus particles are distributed within the whole fruit and the whole fruit showed an increased OSI.

Green pepper fruits have vitamin C content between 52.8-115.5 mg in 100 g fresh weight (Howard et al., 2000; Suntomsuk et al., 2002). Plant cells contain both enzymatic and non-enzymatic antioxidants (Mittler et al., 1998; Riedle-Bauer, 2000; Kaya et al., 2010). Ascorbic acid or vitamin C, which is the most abundant water-soluble non-enzymatic antioxidant in plants, has the ability to scavenge a wide range of ROS such as superoxide anion, singlet oxygen and hydrogen peroxide (Foyer et al., 1991; Suza et al., 2010). Ascorbate provides the first line of defense against damaging reactive oxygen species (ROS), and helps protect plant cells from many factors that induce oxidative stress, including wounding, ozone, high salinity, and pathogen attack. Ascorbate works in cooperation not only with glutathione (Halliwell-Asada cycle), but also maintains the regeneration of α-tocopherol, providing synergic protection of the membranes (Thomas et al., 1992). In this study, it was found that vitamin C of fruits significantly decreased in

infected fruits (Table 1). To the best of our knowledge, this result is the first report about the decrease of vitamin C in the TMV-infected pepper fruits.

When phenolic compounds are considered it is important that these compounds are commonly found in both edible and non-edible plant parts. They are important in the plant growth and defense against infection and injury. Also, the presence of phenolic compounds in injured plants may have an important effect on the oxidative stability and microbial safety (Karakaya et al., 2001). Although phenolic compounds do not have any known nutritional function, they may be important to human health because of their antioxidant potency (Hertog *et al.*, 1995; Shadidi & Nazck, 1995; Hollman *et al.*, 1996). Some studies have reported on the relationships between phenolic content change and disease infection (Candela et al., 1995). Plants may not accumulate the phenolic contents after infection, however, in some cases, the accumulation of phenols might be sufficient to avoid the pathogen attack as in the case of *Phytophthora cinnamomi* and avocado plants (Garcia-Pineda et al., 2010). The beneficial health-related effects of phenols or their potential antinutritional properties, especially when these compounds are present in large quantities in foods, are of importance to consumers (Karakaya et al., 2001). In low concentrations, phenolics may protect food from oxidative deterioration however, at high concentration, they are involved in discoloration of foods, interact with proteins, carbohydrates, and minerals (Robarbs et al., 1999). Phenolic compounds are therefore good antioxidants. However, if discoloration exceeds a certain level, then the phenolic compounds could be toxic. Phenols in foods are of importance because of their biological activities in prevention of cancer and cardiovascular diseases (Pearson et al., 1999).

In this study, it was found that while TP and CP increased, FP decreased in infected peppers. This change may be regulated as a defense mechanism of the fruit against the infection. The alterations of the phenolic contents are related with increasing oxidants and decreasing antioxidants (Table 2). For example, in a study of Velioglu et al. (1998) indicated that there was a positive and highly significant relationship between total phenolics and antioxidant activities in fruits, vegetable and grain products.

CONCLUSION

As a conclusion, the fruits infected with TMV have lower TAS and FP levels and lower vitamin C contents than those of control plants. The infected peppers have also higher contents of TOS levels. The OSI levels are also higher in infected plants which help us to determine the quality of fruits. For better determination of phenol contents, catechin standard gave much better results than that of gallic acid although the pattern for TP, CP and FP were similar.

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