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Effect of Ozone and Ascorbic Acid on the Anatomical, Physiological and Biochemical Parameters of Pepper (*Capsicum frutescens* L.)

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In the present study to investigate the anatomical changes, physiological and biochemical parameters, *Capsicum frutescens* L. grown under different levels of ozone (two different polluted locations) with or without combination of ascorbic acid (AA) were investigated. The results indicate that the AA treatment as well as the combination of the second power plant (SPP II) +AA had the great effect on the growth responses of pepper. The highest plant height was affected significantly (P<0.0001) by SPP II+AA (52.66±7.50 cm plant^{-1}) followed by AA (27.33±2.51 cm plant^{-1}) and the lowest highest was found by SPP II (16.66±2.08 cm plant^{-1}). Most of the growth parameters were enhanced with the combination of AA with SPPII. most of the stomatal parameters and wood vessels of the planted grown in the locations with high level of O_3 in comparisons with the control plants. For example the St. U.E. No and St. U.E.L were highly affected in plants grown in SPP II with the values of 6.00±0.50 μ and 20.44±1.71 μ, respectively. The plants planted in SIC II which exposed to approximately 92 ppb of O_3, exhibited significantly (P<0.0001) higher Chlorophyll a (24.85±1.72 mg g^{-1} FW), Chlorophyll b (7.52±2.05 mg g^{-1} FW) and total Chlorophyll (39.77±4.84 mg g^{-1} FW). The carotenoid level was affected significantly by the treatment of SPP II+AA (i.e. 8.12±0.90 mg g^{-1} FW), while the plants didn't show any significant for the determination of proline (P=0.3447). Under O_3 stress, plants showed lowest growth parameters.

Key words: *Capsicum frutescens*, ascorbic acid, Ozone, Anatomical, Physiological, Biochemical parameters.

Surface level (tropospheric) ozone (O_3) is all pervasive and is considered to be the most important phytotoxic air pollutant across many parts of the US and rest of the world (Krupa et al. 2001; US EPA, 2006). Some plants can be used as bio-indicators because they show characteristic responses when exposed to O_3 (De Temmerman *et al.* 2004; Klumpp *et al.* 2001) and initiated when the O_3 enters the leaf through stomata and reacts with the intercellular water forming reactive oxygen species (ROS). Primarily, the ROS act on the plasma membrane and depending on the capacity of the plant to neutralize the ROS, they can cause oxidative stress (Iriti and Faoro 2009; Roschina and Roschina 2003; Bray *et al.* 2000). Ascorbic acid (AA) is generally protecting plants from ROS formed as part of normal metabolism in the chloroplast and mitochondria or during periods of environmental stress associated with ozone exposure (Runeckles and Chevone, 1992; Smirnoff, 1996; Conklin and Barth, 2004; Burkey et al., 2006).

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The report of US EPA (2006) found that the national air quality regulations aimed at controlling ground level O_3 pollution and continues to be of major concern for crop production and forest health. In addition to causing visible foliar injury on sensitive plant species, chronic exposures to O_3 can result in reductions in crop yield and quality and in forest growth and productivity (Krupa *et al*. 2001; Chen and Gallie 2005). The Current estimates are that O_3 alone causes $3-5 billion in crop loss annually (US EPA, 2006; Fiscus *et al.,* 2005). Root production appears particularly susceptible to O_3 exposure (Cooley and Manning, 1987; Grantz *et al.,* 2006). Allocation of carbon and energy resources to detoxification and repair processes in O_3-stressed plants likely detracts from growth as well (Amthor, 1988; Heath and Taylor, 1997). At the present time for forests and native vegetation, the similar estimates are not available, although the adverse effects of O_3 on parks and forests have been documented (Chappelka *et al.*, 1999; Chappelka 2002; Kohut *et al.,* 1997; Percy *et al.,* 2003; EPA, 2006). It have been reported that the effects of O_3 on plants are numerous, varying with the intensity and duration of exposure (Pasqualini *et al.*, 2003). One-year-old leaves of *Quercus ilex* were much less sensitive to O_3 than current-year leaves, suggesting that the low stomatal conductance observed in aging leaves limited O_3 uptake (Velikova *et al.,* 2005). Previously, the rate of blackberry (*Rubus cuneifolus*): broomsedge (*Andropogon virginicus*) litter decomposition was reduced with increasing O_3 concentrations, with implications in altering nutrient cycling and thus, biological diversity (Kim *et al.*, 1998). Moreover, the decomposition of soybean leaf residues was also found to be slower following growth at elevated O_3 (Booker *et al.,* 2005). Similarly, decreased yield and quality of O_3-exposed Bahia grass (*Paspalum notatum*) (Muntifering *et al.,* 2000) and sericea lespedeza (*Lespedeza cuneata*) (Powell *et al.,* 1999) were of sufficient magnitude to have nutritional implications in their utilization by mammalian herbivores (Krupa *et al.,* 2004). On the other hand, with elevated O_3, the initial acceleration in flowering in blackberry Chappelka (2002) was found and more flowers were initiated and peak production occurring sooner than all other treatments. Also, the total numbers of fruits were not significantly different among treatments; however, numbers of large and ripe fruits were greatest in charcoal-filtered air and nonfiltered air and least in the added O_3 treatments (Chappelka, 2002). It has been reported that as a highly reactive oxidizing agent, the superoxide production by ozone induces cell death lesions in plant leaves when it enters the symplast (Overmeyer and Kangasjarvi, 2003). In the study of Abeyratne and Ileperuma (2006) the 10 days old *Capsicum annuum* L. species showed yellowing of leaves after exposure to 5 days while it took 1 day for similar symptoms to appear in 30 days old plants. Similarly 10 day old *C. frutescens L.* showed yellowing of leaves after exposure to 5 days while it took 11 days for similar symptoms to appear in 30 day old plants. No symptoms were observed in young leaves at this time and further exposure of these plants leads to leaf drop followed by plant death. Some studies indicated to its effect on stomatal density, leaf thickness, intercellular spaces, substomatal chambers, and fine-structure for leaves. The relationship between compactly arranged mesophyll and O_3 sensitivity in plants has already been confirmed by some authors. Ferdinand *et al.,* (2000), analyzing two *Prunus serotina* genotypes, found a higher compact arrangement of the parenchyma cells within the tolerant genotype. The same was verified by Pedroso and Alves (2008) in two tobacco cultivars, one sensitive (*Nicotiana tabacum* 'Bel-W3) and the other tolerant (*N. tabacum*'Bel-B), the last with mesophyll comparatively more compact. The sensitive cultivar of *N. tabacum* L. (Bel-W3) showed lower stomatal density on the abaxial leaf surface when compared to the tolerant cultivar (Bel-B) and slightly prominent stomata on both leaf surfaces when exposed to ozone. On the other study, no stomatal conductance differences of the two tobacco cultivars when exposed to ozone (Pasqualini *et al.,* 2002). Additionally, Giacomo *et al.*, (2010) observed that there was no significant difference between the stomatal density of the upper epidermis between the two clones *Populus deltoides x maximowiczii*, sensitive and *Populus xeuramericana*, tolerant. Despite the vast amount of data on the effects of physiological, biochemical and molecular ozone on plants, but the anatomical studies using microscopy methods are few. In the present study, the anatomical studies and physio-biochemical characteristics of *Capsicum frutescens*

L. grown under different levels of ozone with or without of ascorbic acid were investigated.

MATERIALS AND METHODS

Experimental

This experiment was carried out under different condition using pepper (*Capsicum frutescens* L.). The seeds were obtained from a local market in Riyadh, Saudi Arabia. Healthy seeds were surface sterilized with 1% sodium hypochlorite for 10 min, and then vigorously rinsed with sterilized double distilled water (DDW) before sowing. The seeds were sown in plastic pots on December 10, 2012 at a temperature of 9/20ºC, air humidity 66%, and natural light. (30 cm diameter, 30 cm height) filled with sterile sandy alluvial soil, ratio 1:1 were added a fungicide to prevent fungus and supplied with Raukura's nutrient solution (Smith *et al.,* 1983). The pots were arranged in a simple randomized design in the different location with a single factor and four replicates. One week after sowing, seedlings were thinned so that each pot contained healthy plants of uniform size. When the plants were at the stage of two to three true leaves and then start treatments.

Treatments

The plants were divided to six groups; control (Cont.) group was left in King Saud University site without treatment, one group treated with ascorbic acid (AA), two groups were under ozone stress (O_3) one of them transferred to the second industrial city (SIC II), and other one to the second power plant (SPP II), and two groups under ozone stress which treated by ascorbic acid (AA) concentration (300 mg/L) (O_3+AA) one of them transferred to the (SIC II), and other one to the (SPP II). Table 1 presents the measuring of the concentration of ozone gas in the study sites.

Irrigation was done at every 15 days.The plants were sampled at 25 days after sowing to assess their growth characteristics [plant height, stem fresh plant^{-1} (stem FW), dry weight plant^{-1} (stem DW), root fresh plant^{-1} (root FW), dry weight plant^{-1} (root DW), leaves fresh plant^{-1} (leaves FW), dry weight plant^{-1} (leaves DW)], leaf number, root length, and area leaf^{-1}, relative water content (RWC) and physio-biochemical attributes [chlorophyll (Chl) a and b, carotene, total chlorophyll, proline (pro)].

Plant growth characteristics

Plant height (PH) was measured using a meter scale after removal from the pots. After recording fresh weight (FW) with balance, plants were placed in a 60°C oven for 48 h and then were weighed for dry weight (DW). Leaf area (LA) was measured using a LI-3000 Portable Leaf Area Meter (LI-COR, Lincoln, NE, USA). The relative water content (RWC) was expressed as percentage of the water content at a given time and tissue as related to the water content at full turgor (Slatyer, 1967). The relative water content was calculated using the following formula given by González and González-Vilar (2001): RWC (%) = [(FW - DW) / (TFW- DW)] x 100.

Physiological and biochemical parameters

The Chl was extracted from fresh leaves of experimental plants using the DMSO method based on Barnes *et al.,* (1992). Chl absorption in the extract was measured using UV-VIS spectrophotometer. Contents of the Chls were calculated using the following formulas:

Chl a = $14.85\ A_{664.9}\ 6\ 5.14\ A_{648.2}$

Chl b = $25.48\ A_{648.2}\ 6\ 7.36\ A_{664.9}$

Total chlorophyll = $7.49\ A_{664.9} + 20.34\ A_{648.2}$

Proline concentration was determined spectrophotometrically by adopting the ninhydrin method of Bates *et al.,* (1973). Firstly 300 mg of fresh leaf samples were homogenized in sulphosalicylic acid. To the extract, 2 mL each of acid ninhydrin and glacial acetic acid were added. The samples were heated at 100°C and mixture was extracted with toluene and the free toluene was quantified spectrophotometrically at 528 nm using L-proline as a standard.

Anatomical characters

A leaf surface was cleaned with distilled water, and then silicon rubber imprinting was made for studying epidermis characters according to Lloyd (1908). For studying internal structures, leaf portions was divided into 5 mm pieces, then fixed in FAA (formalin-acetic acid-ethanol 10:5:85), dehydrated in a graded ethanol series and embedded in paraffin wax at 58°C. Sections (25µm thick) were stained with safranin and light green and mounted in Canada balsam. These sections were examined and photographed using Zeiss photomicroscope III. The following parameters were recorded; Stomata Upper epiderm Number (St. U. E. No), Stomata Upper epiderm Length (St.

U. E.L), Stomata Lower epiderm No (St. L. E. No), Stomata Lower epiderm Length (St. L. E. L.), Stomata Lower epiderm Width (St. L. E.W.), Palisade cells Length (P. C. L.), Palisade cells Width (P. C. W.), Upper Epidermis Cell Length (U. E. C. L.), Upper Epidermis Cell Width (U. E. C. W.), Lower Epidermis Cell Length (L. E. C. L.), Lower Epidermis Cell Width (L. E. C. W.) Wood Vessels Number (W. V. No.) and Wood Vessels Diameter (W.V. D.).

Measurements and photographs

Measurement of concentrations of ozone gas was a day for three months for each of the study sites using a device (AEROQUAL Series Monitor with multihi). Epidermis cells dimensions, stomatal number, stomatal dimensions, palisade cells dimensions, and wood vessels were taken at Magnification x 40 using leitz light microscope with motic 2000 cam. All measurements and description were taken in the last vegetative growth before flowering.

Statistical analysis

Most ozone studies, however, have been single factor or two-way interaction experiments. However, plant responses to ozone are highly influenced by site conditions, and comprehensive assessment of their relative influences needs attention (Reid and Fiscus, 2008). Each pot was treated as one replicate and all the treatments were repeated five times. All data from morpho-anatomical investigations were expressed as means ± SD and the means were subjected to a one-way analysis of variance (ANOVA). When the ANOVA indicated a significant difference among treatments, a comparison of the means was done employing Duncan's multiple-range test at a 0.05 level of probability. The data were statistically analyzed using SAS version8.2 (SAS, 2001) in a completely randomized design (CRD) to test the differences among treatment levels.

RESULTS AND DISCUSSION

Growth responses

Because the reactivity of O_3, it makes the study of plant responses to elevated O_3 difficult (Karnosky *et al.,* 2003). Measured of growth and yield were significantly affected by the different applications of O_3 (Table 2). The highest plant height was affected significantly (P<0.0001) by SPP II+AA (52.66±7.50 cm/plant) followed by AA (27.33±2.51 cm/plant) and the lowest highest was found with SPP II (16.66±2.08 cm/plant). Furthermore, the treatment of SPPII+AA showed the highest LN, LA, LR, RFW, SFW, SDW, LFW, LDW and RWC values with 20.66±8.32 cm^2, 80.41±5.25 cm2, 25.17±1.05 cm $plant^{-1}$, 4.83±0.19 g $plant^{-1}$, 16.17±4.67 g $plant^{-1}$, 2.16±0.62 g $plant^{-1}$, 1.60±0.25 g $plant^{-1}$, 0.32±0.13 g $plant^{-1}$ and 114.71±7.17 %, respectively. On the other hand, the treatment AA showed a significant effect on RDW with 0.67±0.02 g $plant^{-1}$, RFW with 4.33±0.57g $plant^{-1}$, LFW with 1.66±0.15 g $plant^{-1}$ and LDW with 0.37± 0.13 g $plant^{-1}$. Previously, it was reported that O_3 significantly affected the relative area of leaf epidermis also significantly decreased the relative area of palisade intercellular space in colonies of *Betula pendula* (Pääkkönen *et al.*, 1995) at low O_3 exposure. Most of the growth parameters were enhanced with the combination of AA with SPPII. On the hand, significantly, the plants grown under the O_3 stress were showed the lowest growth parameters.

Table 1. Average monthly reading of ozone gas in the study sites

Study sites	Month	Ozone gas conc. (ppb)	Average Ozone conc. (ppb)
Control	January	42	44.33
	February	44	
	March	47	
the second industrial city (SIC II)	January	132	136.66
	February	137	
	March	141	
The second power plant (SPP II)	January	85	92
	February	98	
	March	93	
Limit air pollution to global gas ozone (ppb)	30-25		

In the study of Takemoto *et al.,* (1988), responses were monitored the depressed green pepper growth and yield responses via the inhibition of photosynthesis but enhanced leaf buffering capacity, and caused no visible injury. The results from Table 2 reveals that the AA treatment as well as the combination of SPPII+AA had the great effect on the growth responses of pepper.

Anatomical features

The Diffuses of O_3 through the mesophyll intercellular space to reach the spongy and palisade mesophyll after passing through the stomata has been discussed (Pääkkönen *et al.,* 1995). The amount of cell surface able to interact with the O_3 is an important leaf characteristic in the injury process (Evans and Ting, 1974; Bennett *et al.,* 1992). In agricultural plants, the percentage of intercellular space, especially in the palisade layer, has been shown to be related to the sensitivity of O_3 (Evans and Ting, 1974). The total leaf thickness and the palisade mesophyll thickness were smaller and the percentage of spongy mesophyll layer was higher for O_3 sensitive trees (*Fraxinus pennsylvanica* Marsh. and *Prunus serofina* Ehrh.) compared to tolerant individuals (Bennett *et al.,* 1992). Similarly, in the present study most of the stomatal parameters and wood vessels of the planted grown in the locations with high level of O_3 in comparisons with the control plants were significantly affected (P<0.0001) (Table 3). The anatomical changes occurred in plants grown under ozone/or ascorbic acid application. (Fig. 1). For example, the stomata U.E. No and Stomata U.E.L were highly affected as plant in the location SIC II with the values of 6.00±0.50 µ and 20.44±1.71 µ, respectively. The high stomatal densities and high percentage of intercellular space among palisade mesophyll cells are associated with ozone sensitivity (Evans *et al.,* 1996). Furthermore, the ozone responses were assessed in simulated summer and autumn conditions of central Finland in order to elucidate whether the time of occurrence

Table 2. Statistical analysis of the effects of different applications of treatments of O_3 on pepper growth responses

Growth responses [a]	Treatments						
	Cont.	ASA	SICII	SICII+ASA	SPPII	SPPII+ASA	P value [b]
PH (cm plant^{-1})	24.66± 4.04bc	27.33± 2.51b	24.66± 0.57bc	17.66± 4.61c	16.66± 2.08c	52.66± 7.50a	<0.0001***
LN (cm^2)	5.33± 1.15b	8.00± 1.00b	9.00± 1.00b	10.66± 3.78b	10.66± 3.05b	20.66± 8.32a	0.0093***
LA (cm^2)	32.53± 1.74b	35.20± 4.01b	30.46± 4.30b	31.90± 1.63b	30.63± 3.18b	80.41± 5.25a	<0.0001***
RL (cm plant^{-1})	16.50± 4.82b	19.46± 4.80b	14.54± 1.53b	17.26± 1.12b	17.06± 1.22b	25.17± 1.05a	0.0131*
RFW (g plant^{-1})	3.00± 0.19b	4.33± 0.57a	2.32± 0.15c	1.36± 0.26d	2.46± 0.16c	4.83± 0.19a	<0.0001***
RDW (g plant^{-1})	0.43± 0.03b	0.67± 0.02a	0.23± 0.03c	0.13± 0.03d	0.24± 0.006c	0.45± 0.04b	<0.0001***
SFW (g plant^{-1})	3.49± 0.24b	5.49± 1.15b	3.64± 0.90b	2.31± 0.33b	3.54± 0.14b	16.17± 4.67a	<0.0001***
SDW (g plant^{-1})	0.56± 0.05b	0.62± 0.18b	0.53± 0.12b	0.28± 0.04b	0.41± 0.01b	2.16± 0.62a	<0.0001***
LFW (g plant^{-1})	1.00± 0.08b	1.66± 0.15a	1.03± 0.09b	1.06± 0.07b	1.06± 0.20b	1.60± 0.25a	0.0004***
LDW (g plant^{-1})	0.10± 0.03b	0.37± 0.13a	0.11± 0.03b	0.12± 0.02b	0.12± 0.06b	0.32± 0.13a	0.0042***
RWC (%)	79.91± 3.22b	82.58± 3.20ab	87.53± 8.83ab	80.51± 1.63b	81.55± 5.70ab	114.71± 7.17a	<0.0001***

a: Mean (± SD) for growth responses. Dissimilar letter designations indicate significant differences at P < 0.05 (Duncan's new multiple range test). b; Results of the ANOVA: NS = not significant at P < 0.05; *, *** = significant at P <0.05, 0.001, respectively.

Table 3. The anatomical characters of leaf and stem of *Capsicum frutescens* as affected by different O_3 exposure treatments

Anatomical feature (μ)[a]	Treatments						
	Cont.	ASA	SICII	SICII+ASA	SPPII	SPPII+ASA	P value [b]
St. U.E. No	4.52± 0.87bc	5.46± 1.01ab	6.00± 0.50a	5.88± 1.22a	2.52± 0.67d	4.00± 0.37c	<0.0001
St. U.E.L	17.63± 1.54b	18.43± 1.64b	20.44± 1.71a	16.87± 0.61b	20.38± 1.40a	13.27± 0.60c	<0.0001
St. U.E.W	11.49± 0.73b	12.89± 0.88a	10.68± 0.93b	10.68± 1.23b	11.69± 0.96b	7.73± 0.59c	<0.0001
St. L.E. No	4.44± 0.84c	6.04± 0.66b	7.96± 0.89a	6.44± 0.89b	2.24± 0.08d	3.52± 0.54c	<0.0001
St. L.E.L	21.02± 1.86a	22.22± 1.83a	18.99± 0.96b	16.68± 0.61c	21.51± 0.54a	14.91± 1.78c	<0.0001
St. L.E.W	13.49± 0.71a	14.29± 0.50a	10.12± 0.35b	10.50± 0.39b	10.87± 0.72b	8.62± 2.39c	<0.0001
P.C.L.	30.73± 2.23d	36.13± 2.96d	105.40± 5.03a	64.90± 1.98b	44.08± 3.97c	44.97± 12.48c	<0.0001
P.C.W.	6.62± 1.01c	10.22± 1.50c	23.26± 2.20a	21.68± 3.70a	15.21± 1.83b	15.68± 4.96b	<0.0001
U.E.C.L.	28.96± 2.94d	31.76± 1.66cd	33.20± 0.98bc	36.39± 2.41b	41.95± 2.18a	28.95± 5.04d	<0.0001
U.E.C.W.	16.22± 1.50c	18.62± 1.75bc	21.40± 3.98b	15.82± 1.98c	27.46± 4.10a	18.42± 2.97bc	<0.0001
L.E.C.L.	22.67± 1.42	24.67± 1.16cd	28.61± 0.90bc	32.95± 2.22b	46.59± 4.57a	30.16± 5.77b	<0.0001
L.E.C.W.	13.58± 0.97d	15.98± 0.97c	19.65± 0.85b	14.03± 0.93d	29.82± 1.15a	15.87± 2.49c	<0.0001
W.V. No.	26.12± 1.31d	26.12± 1.31d	29.44± 2.16c	42.60± 0.87a	34.12± 2.19b	32.40± 2.39b	<0.0001
W.V. D.	16.20± 1.20c	18.40± 0.41b	12.68± 0.43d	18.30± 0.81b	20.55± 0.89a	18.10± 1.74b	<0.0001

a: Mean (± SD) for Anatomical feature (μ). Dissimilar letter designations indicate significant differences at P < 0.05 (Duncan's new multiple range test). b Results of the ANOVA: *** = significant at P <0.001.

Table 4. Statistical analysis of different applications of treatments of O_3 on pepper chl a, chl b, T chl (a+b), carotenoid and proline contents

Biochemical parameters	Treatments						
	Cont.	AA	SIC II	SIC II+AA	SPP II	SPP II+AA	P value
chl a (mg g-1 FW)	16.47± 1.19cd	18.13± 1.72bc	19.57± 2.25b	19.64± 1.26b	24.85± 1.72a	14.18± 0.53d	<0.0001***
chl b (mg g-1 FW)	4.89± 1.62a	5.41± 2.01a	6.82± 1.46a	4.71± 1.60a	7.52± 2.05a	7.40± 2.71a	0.3389[ns]
Caro (mg g-1 FW)	5.89± 0.52bc	6.69± 0.75abc	6.39± 1.10abc	4.86± 1.18c	7.39± 1.10ab	8.12± 0.90a	0.0185*
T chl (mg g-1 FW)	27.26± 3.03b	28.59± 2.80b	32.79± 4.73b	29.22± 3.13b	39.77± 4.84a	29.71± 3.75b	0.0175*
Pro (g/100 g Fw)	2.57± 0.04a	3.04± 0.30a	2.47± 0.91a	2.88± 0.89a	3.06± 0.12a	3.47± 0.41a	0.3447[ns]

a: Mean (± SD) for biochemical parameters. Dissimilar letter designations indicate significant differences at P < 0.05 (Duncan's new multiple range test). b Results of the ANOVA: NS = not significant at P < 0.05; *, *** = significant at P <0.05, 0.001, respectively.

of ozone episodes can affect the ozone responses. Special emphasis was put on chloroplast ultrastructure because alterations in the chloroplast could lead to changes in carbon assimilation and biomass accumulation. Furthermore, it is well established that chloroplast structure is altered by ozone, usually before other cell organelles (Sutinen *et al.*, 1990; Holopainen *et al.*, 1996). On the other hand, the planted treated with AA showed a high Stomata U.E.W (12.89±0.88µ), Stomata L.E.L (22.22±1.83µ) and Stomata L.E.W (14.29±0.50µ). There were significant differences in stomatal density, palisade mesophyll thickness and total mesophyll thickness, and the percentage of spongy mesophyll layer relative to total leaf mesophyll, also, the total leaf thickness was less for sensitive trees (Bennett *et al.*, 1992). Morpho-anatomical characters, such as amphistomatous lamina, higher stomatal density and relaxed mesophyll cell packing (evaluated by the palisadeness coefficient), observed in the sensitive clone leaves, may favor a greater O_3 uptake in the apoplast and increase the cumulative dose of pollutant per mesophyll cell, with respect to tolerant clone leaves (Giacomo *et al.*, 2010).

In another study the three ozone sensitive species, *Sassafras albidum*, *Rudbeckia laciniata*, and *Rubus canadensis* had higher stomatal densities and more intercellular spaces among palisade cells compared with less sensitive species (*Magnolia tripetela*, *Aster divaricatus*, and *Liquidamber styraciflua*) (Evans *et al.*, 1996). There were no statistically significant relationships between ozone sensitivity and cross-sectional area of spongy mesophyll cells, number of palisade parenchyma cell layers or thicknesses of epidermis or mesophyll tissues. These data support the hypothesis that ozone sensitivity is associated with leaf characteristics that relate to the ability of ozone to diffuse into leaves (high stomatal densities) and the ability of ozone to diffuse among the target cells (high percentage of intercellular spaces among palisade parenchyma cells). On the studied species, the increase of stomatal density can be related to a higher efficiency on the stomata opening closure mechanism. Relations between O_3 sensitivity, and different parameters of stomata were discussed by some authors. The increase of stomatal density in *Eugenia uniflora* exposed to the same local of our study, in comparison to control plants kept in a place little affected by air pollutants (Alves *et al*., 2008). The physiological and structural parameters in symptomatic leaves of *Fraxinus ornus*, observed a stomatal conductance reduction due to the changes in stomata or in other epidermis cells (Paoletti *et al*., 2009). Furthermore, the most sensitive cultivar of *Nicotiana tabacum* 'Bel-W3' with the most tolerant (K63) did not observe significant differences in stomatal density (Saitanis and Karandinos 2002).

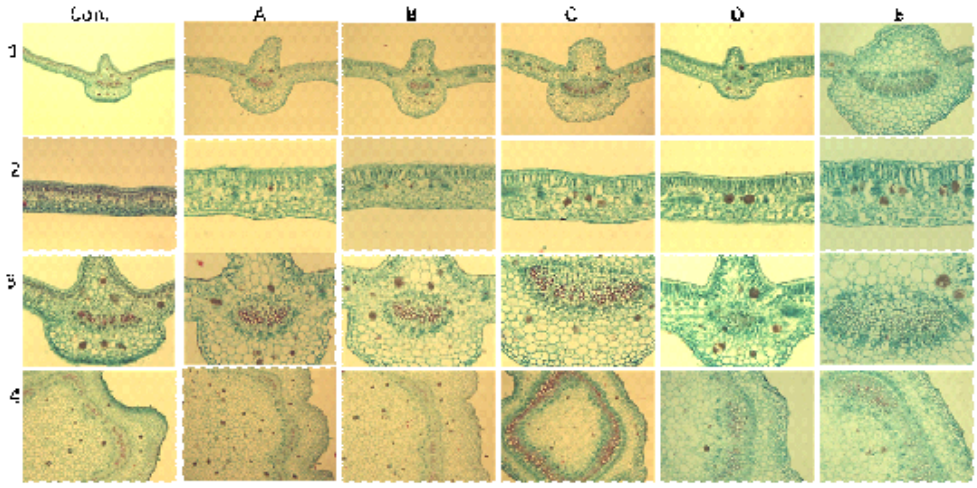

Fig. 1. The anatomical changes of *Capsicum frutescens* as affected by ozone with or without combination of ascorbic acid. Cross-sections in leaf and stem of pepper (Con.): Control. (A): ascorbic acid (AA), (B): Ozone stress (SIC II). (C): Ozone stress and ascorbic acid (SIC II+ AA). (D): Ozone stress (SPP II). (E): Ozone stress and ascorbic acid (SPP II+ AA). Cross-sections in (1) leaf 4X. (2) Intercostal Region 10X. (3) Midrib Region 10X. (4) Stem 4X

While Pedroso and Alves (2008), also comparing tobacco cultivars, 'Bel-W3' (O_3 sensitive) and 'Bel-B' (O_3 tolerant), registered a higher stomatal density in the tolerant cultivar.

Leaf chlorophylls, carotene and proline concentrations

Leaf Chl, carotene and proline concentrations were affected significantly by the different amount of O_3 (Table 4). The plants planted in SPP II which exposed to approximately 92 ppb (Table 1) of O_3, exhibited significantly ($P<0.0001$) higher Chl a (24.85±1.72 mg g^{-1} FW), Chl b (7.52±2.05 mg g^{-1} FW) and T Chl (39.77±4.84 mg g^{-1} FW). The carotenoid level was affected significantly by the treatment of SPP II+AA (i.e. 8.12±0.90 mg g^{-1} FW), while the plants didn't show any significant for the content of proline ($P=0.3447$). Relative to the effects of the treatments application, the Chl b didn't affected significantly ($P=0.3389$). The results were in consistent with Takemoto *et al.*, (1988). In the chlorophyll of leaves, ozone exposure led to a decrease in chloroplast area and in granum stack thickness and various changes in plastoglobuli and cell wall thickness, depending on the species and the experiment (Rinnan and Holopainen, 2004; Gallie, 2013).

CONCLUSIONS

In the present study, the anatomical studies and physic-chemical characteristics of *Capsicum frutescens* L. grown under different levels of ozone (two different polluted locations) with or without combination of ascorbic acid (AA) were investigated. The results indicate that the AA treatment as well as the combination of the second power plant (SPP II +AA) had the great effect on the growth responses of pepper. The carotenoid level was affected significantly by the treatment of (SPP II+AA), while the plants didn't show any significant difference for the content of proline. On the other hand, significantly, the plants grown under the O_3 stresses were showed the lowest growth parameters. Under exposure of O_3, plants showed a significant changes in anatomical as well as physio-biochemical parameters.

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