ORIGINAL ARTICLE

Physiological and biochemical responses of cowpea (*Vigna unguiculata* (L.) Walp) to ozone

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Abstract

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The aim of this research was to investigate physiological and biochemical responses of cowpea (*Vigna unguiculata* (L.) Walp) to ozone. There were two main factors of the experiment; level of ozone concentration at 40 and 70 ppb and plant ages at 7 and 21 days. Plants were grown in fumigation chambers in which inlet air was filtered by a charcoal filter. Additional ozone was given 8 hours/day for 7 days in ozone fumigating chambers. The ozone concentration in the control chambers was less than 10 ppb. The results showed the biomass of ozone-fumigated plants was significantly lower and leaf injury of ozone fumigated plants was significantly greater compared to the control group. The major visible-injury symptom appeared as chlorosis on the upper surface of the leaves. Antioxidant levels in the charcoal filtered (CF) plants and ozoned plants had significant differences because of their detoxification role in removing ozone and its derivatives. The ozone treatment of 7-day-old plants showed superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) levels significantly higher than in 21-day-old plants and total ascorbate concentrations

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significantly lower than 21-day-old plants. These results showed that different ozone concentrations exhibit different effects on antioxidant production. Analysis of antioxidants daily for 7 days found that antioxidant levels rapidly changed. Notably, SOD and total ascorbate could be selected as indicators for ozone-effect monitoring in plants. This indicates that cowpea is sensitive to ozone and may be usable as an ozone bio-indicator. In conclusion, plant age, ozone concentration and the duration to exposure to ozone were the main physiological or biochemical responses of cowpea. An efficient defense system was generated from a combination of antioxidants.

Key words : cowpea, ozone, antioxidant

บทคัดย่อ

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การตอบสนองทางสรีรวิทยาและชีวเคมีของถั่วพุ่ม (*Vigna unguiculata* (L.) Walp) ต่อก๊าซโอโซน

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ได้ศึกษาความแตกต่างในการตอบสนองทางสรีรวิทยาและชีวเคมีต่อก๊าซโอโซนของถั่วพุ่ม (*Vigna unguiculata* (L.) Walp) ต่อปัจจัย 2 ชนิด ได้แก่ ความเข้มข้นของก๊าซโอโซนและอายุของพืช โดยทดลองให้ถั่วพุ่มอายุ 7 และ 21 วัน ได้รับก๊าซโอโซนความเข้มข้น 40 และ 70 ส่วน/พันล้านส่วน (ppb) เป็นเวลา 7 วัน วันละ 8 ชั่วโมง โดยเปรียบเทียบกับกลุ่มควบคุมที่ได้รับก๊าซโอโซนความเข้มข้นไม่เกิน 10 ส่วน/พันล้านส่วน (ppb) พบว่ากลุ่มที่ได้รับก๊าซโอโซนมีมวลชีวภาพลดลงและมีเปอร์เซ็นต์ความเสียหายของใบเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติจากกลุ่มควบคุม ส่วนความเสียหายของใบพืชที่พบเป็นอาการภาวะพร่องคลอโรฟิลล์ (chlorosis) ปริมาณสารแอนติออกซิแดนท์ของกลุ่มที่ได้รับก๊าซโอโซนมีค่าแตกต่างกันอย่างมีนัยสำคัญทางสถิติจากกลุ่มควบคุมโดยพืชต้องใช้สารแอนติออกซิแดนท์ในการกำจัดก๊าซโอโซน กลุ่มที่ได้รับก๊าซโอโซนในถั่วพุ่มอายุ 7 วัน มีปริมาณ superoxide dismutase (SOD) catalase (CAT) ascorbate peroxidase (APX) และ hydrogenperoxide (H_2O_2) มากกว่าและมีความเข้มข้น total ascorbate น้อยกว่าอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับถั่วพุ่มอายุ 21 วัน โดยอายุพืชที่แตกต่างกันมีผลต่อการผลิตสารแอนติออกซิแดนท์ จากผลการวิเคราะห์ปริมาณสารแอนติออกซิแดนท์รายวันเป็นระยะเวลา 7 วัน พบว่าปริมาณสารแอนติออกซิแดนท์ของกลุ่มที่ได้รับก๊าซโอโซนมีการเปลี่ยนแปลงอย่างรวดเร็ว ซึ่งเป็นการบ่งชี้ว่า ถั่วพุ่มมีการตอบสนองเร็วต่อก๊าซโอโซนซึ่งเป็นคุณสมบัติของดัชนีชีวภาพเฉพาะสำหรับก๊าซโอโซน โดยเลือก SOD และ total ascorbate เป็นดัชนีการตอบสนองทางชีวเคมีต่อก๊าซโอโซน ซึ่งสามารถสรุปผลการวิจัยได้ว่าปัจจัยหลักที่มีอิทธิพลต่อการตอบสนองทางสรีรวิทยาและชีวเคมีต่อก๊าซโอโซนของถั่วพุ่ม ได้แก่ ช่วงอายุของถั่วพุ่ม ความเข้มข้นและระยะเวลาที่ได้รับก๊าซโอโซน กลไกที่มีประสิทธิภาพในการปกป้องพืชจากก๊าซโอโซน เกิดจากการทำงานร่วมกันของสารแอนติออกซิแดนท์หลายชนิด

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Ozone at low atmospheric levels has been found to be gradually increasing due to the constructive substance of ozone (NOX and VOC) from transportation and industries. This could harm vegetation, animals, humans and the environment.

When ozone gas is uptaken into plants it becomes to reactive with oxygen species (ROS) i.e. O_2^-, HO^- and H_2O_2. These damage cell structures and enhance programmed cell death (PCD), and decrease photosynthesis, biomass and gas ex-

change. Moreover, there are reports of increased sensitivity to biotic and abiotic stress changes such as water stress, infection, heavy metal uptake and other air pollutants (Calatayud and Barreno, 2001). Plants first react to ROS by producing antioxidants. The initial reaction to ROS is superoxide dismutase (SOD) which removes O_2^- to H_2O_2. This is to prevent generating high toxicity ROS; HO^- from the reaction of O_2^- and H_2O_2. Then catalase (CAT) and ascorbate peroxidase (APX) remove H_2O_2. Ascorbate acts as an electron donor for APX which reduces H_2O_2 to H_2O and monodehydroascorbate (MDHA) (Rao *et al.,* 1996; Sharma and Davis, 1994).

The increase of antioxidants which has been found in ozone-exposed plants was induced to remove ROS. For instance, *Arabidopsis thaliana* was exposed to ozone at 220 ppb 6 hours per day for 8 days SOD and APX increased by 83% and 87% respectively (Rao *et al*., 1996). I ncreased antioxidants were affected by ozone concentration and sensitivity of plants, including leaf age. This was reported in young leaf of *Nicotiana tobacum* cv PBD6, in which was found a maximum of chloroplast Cu/Zn-SOD which is not indicated in old leaves (Willekenes *et al*., 1994). Generally, antioxidant production in vegetation depends on its ozone resistant capability. Willekenes *et al*. (1994) found increases of 4-5 fold of the gene cyt Cu/Zn-SOD related to visible injuries of ozone sensitive Nicotiana tobacum cv PBD6. There was another report of antioxidant correlation with visible injuries with no increase of mRNA of APX and Cu/Zn-SOD of *Nicotiana tobacum* and *Nicotiana plumbagini* during ozone exposure until visible injury was observed. After that mRNA of APX and Cu/Zn-SOD levels increased (Conklin and Last, 1995).

This research aimed to investigate 1) the differences in physiological and biochemical responses to ozone with plant age, 2) effects of ozone dose on plants, and 3) the relation of physiological and biochemical responses during ozone exposure. Cowpea was selected following preliminary experiments for screening of plants sensitive to ozone. The results will be useful for bio-indicator development in the further experiment.

Material and Method

Plant materials

Cowpea seeds were germinated in charcoal-filtered air chambers. Temperature was controlled at 30-35ºC day and night. 2x400 W light from Metal Halide lamp provided PPFD of 300 µmol $m^{2-}s^{-1}$ with a 10-h photoperiod. After germination in compost beading soil, seedlings were planted in plastic cups (one plant / cup) and transferred into six chambers (50 cups / chamber) which were maintained under the same environmental conditions. Two groups of plant ages were established, 7- and 21-day-old.

Ozone fumigation

Six fumigating chambers were allocated for three ozone levels, (a) charcoal-filtered air as a controlled ozone concentration <10 ppb, (b) charcoal-filtered air with 40 ppb ozone added, and (c) charcoal-filtered air with 70 ppb ozone added. Two replicates of each level were used. Ambient air passed through a charcoal-filter was supplied to the chambers. Ozone was generated by an ozone generator (Model OZ-3020). The ozone concentration was continuously monitored by an ozone monitor (2B Technologies model 202, USA). Plants were placed in fumigation chambers 72 h before ozone exposure. They were fumigated by ozone 8 hr/d (9.00-17.00) for 7 days.

Dry weight

The final harvesting was performed at 7 days after exposure. Roots and shoots of 15 plants per chamber were collected and dried at 70ºC for 48 hours in a hot air oven before weighing.

Leaf injuries

Visible leaf injuries of each plant were noted daily. The percentage of leaf damage was calculated as

$$\% \text{ damage} = \frac{\text{Number of leaves damages}}{\text{Total leaves}} \times 100$$

Extraction

The youngest expanded leaf was cut and 0.1 g fresh weight placed in a 2 ml Eppendorf tube. Measurements of SOD, CAT, APX and H_2O_2 were made daily through out the experiment. Three replicate samples were collected and ground in 1 ml 67 mM phosphate buffer (pH 7.0) containing 1 mM EDTA and 100 % w/w PVPP, 5 mM. Ascorbate was added to the extraction medium used for the determination of APX. The extraction of total ascorbate, fresh leaf 0.1 g was ground in 1 ml of 6% metaphosphoric acid containing 0.25 mM EDTA and 100% PVPP. The samples were centrifuged at 10,000 g for 10 min at 4ºC and the supernatant obtained was continuously determined in each assay.

Antioxidant enzyme assay

Superoxide dismutase (SOD, EC 1.15.1.1) was determined using the nitroblue tetrazolium (NBT) reduction of Winterbourn *et al*. (1975). One unit of SOD was defined as the amount of leaf extract resulting in 50% inhibition of NBT reaction.

Catalase (CAT, EC 1.11.1.6) was determined from the decrease in absorbance at 240 nm, following the consumption of H_2O_2 (Aebi, 1984). Catalase content was calculated using an extinction coefficient of 39.4 mM^{-1} cm^{-1} for H_2O_2 at 240 nm.

Ascorbate peroxidase (APX, EC 1.11.1.11) was determined from the decrease in absorbance at 290 nm (Nakano and Kozi, 1981). APX content was calculated using an extinction coefficient of 2.8 mM^{-1} cm^{-1} for ascorbate at 290 nm.

Total ascorbate determination

Total ascorbate content was determined using the spectrophotometric method described by Takahama and Oniki (1992). An extinction coefficient of 14.3 mM^{-1} cm^{-1} for ascorbate at 265 nm was used to calculate concentration.

Hydrogen peroxide determination

H_2O_2 content was determined by adding 1 volume of leaf extract to 10 volumes of working reagent, mixing 1 volume of R 1 (solution containing 25 mM Ammonium Iron (II) Sulfate and 2.5 M H_2SO_4) with 100 volumes of R 2 (solution containing 100 mM Sorbital and 125 µM Cyrenolorange), mixing well and incubating at room temperature for 30 min, then measuring the absorbance at 560 nm, determining H_2O_2 concentration from the standard curve.

Statistical analysis

Multivariate Analysis of Variance (MANOVA) was performed on the experimental data, statistical significance ($P \leq 0.05$) and was judged by the Duncan's Multiple Range Test (DMRT) method.

Results and Discussion

1. Physiological responses

1.1 Leaf-visible injuries and percent damage

Ozone-fumigated plants showed visible injuries as chlorosis symptoms (Figure 1). This is commonly found in ozone-exposed plants in which chlorophyll and carotenoid were damaged (Rao *et al*., 1996). Leaf-visible injuries of 7-and 21-day-old cowpea plants were seen on day 2 and 4 respectively during 7-day fumigation. The percentage of leaf damage of ozone-fumigated plants was significantly different when compared with the control group. Leaf-visible injuries were caused by the accumulation of ROS from the ozone reaction. Ozone-induced oxidative bursts in tobacco and tomato leaves resulted from H_2O_2 accumulation. A continuously increasing amount of O_2^- was correlated to H_2O_2 accumulation. The leaf-visible injury symptoms of 21-day-old plants were seen faster than 7-day-old plants because of higher accumulations of ROS and the ROS detoxification capability. Thus, the percentage of damage of 21-day-old cowpea plants was significantly higher than in 7-day-old plants.

1.2 Biomass

Roots, shoots and total dry weight of ozone fumigated plants, and 7- and 21-day-old plants were significantly lower than the charcoal group (Figure 2). There are several reports of the

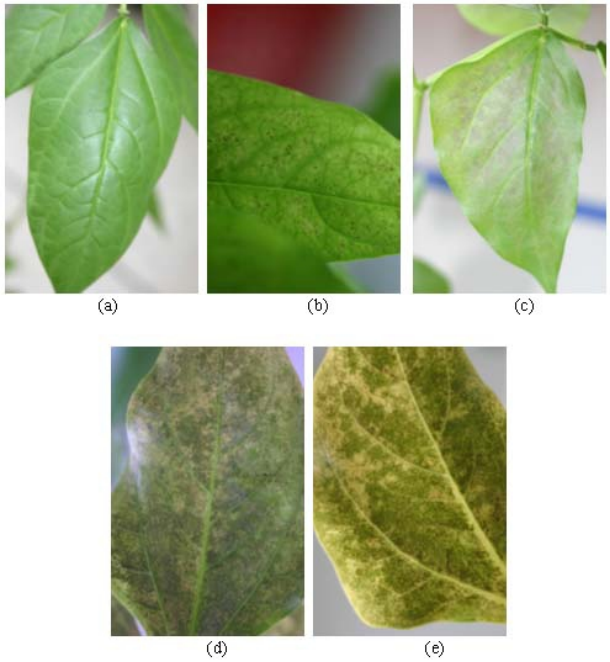

Figure 1. Cowpea leaves before appearance of symptoms (a), chlorosis symptom on upper surface of leaves (b-e)

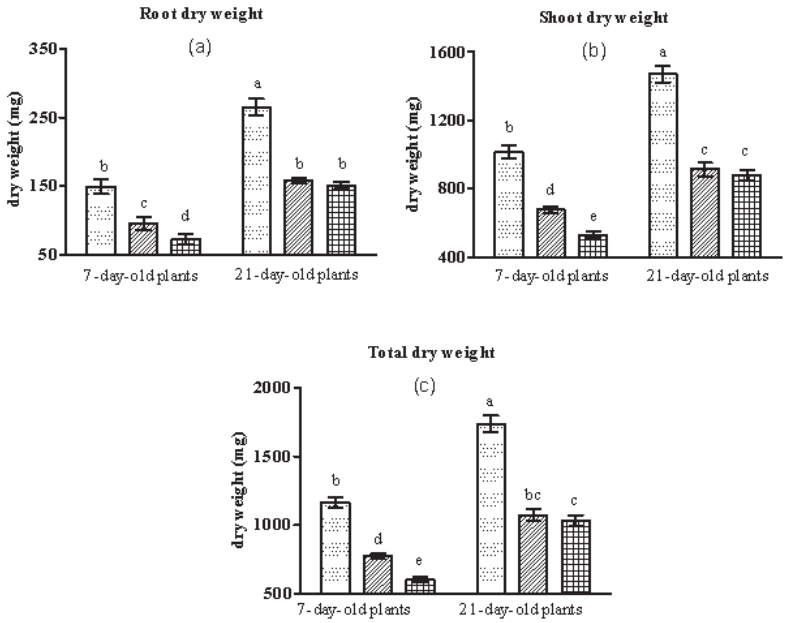

Figure 2. Roots (a), shoots (b) and total dry weight (c) of 7-and 21-day-old plants after exposure to ozone at concentrations of 40 ppb and 70 ppb for 8 hr/d for 7 days, and control group (CF) with ozone concentration ≤ 10 ppb. Data represent the means ± SE, (n=15) and bars with different letters (a-e) indicate significant differences at P≤0.05 (□ CF ▨ 40 ppb ⊞ 70 ppb).

effect of ozone on biomass. For instance in *Arabidopsis thaliana* Columbia, after exposure to ozone at 150 ppb 6 hours per day for 14 days the total dry weight decreased 48% (Sharma and Davis, 1994). Rao *et al*. (1995) found that wheat showed a reduction of shoot dry weight when exposed to ozone at 120 ppb 8 hours per day for 5 weeks. A reduction of biomass in ozone-fumigated plants was typically found because the respiration and transpiration had decreased due to stomata closure, with a possible reduction in photosynthesis and gas exchange which are important to plant growth. However, stomata closure is considered as a defense mechanism of plants to reduce ozone uptake and cell repairing (Sharma and Davis, 1997).

2. Biochemical response

The SOD, CAT, APX and H_2O_2 of ozone-fumigated plants and 7-day-old plants were significantly higher than in 21-day-old plants. Nevertheless, the total ascorbate of ozone-fumigated plants and 7-day-old plants was significantly less than that of 21-day-old plants (Figure 3). This can be explained by the age which causes the

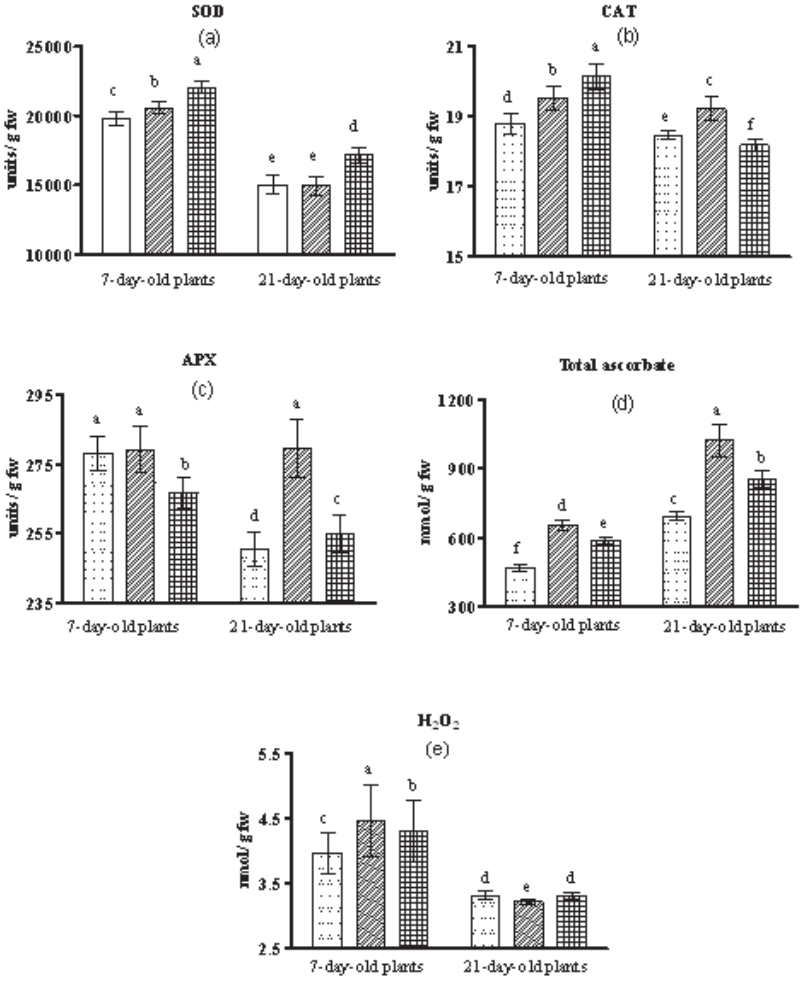

Figure 3. SOD (a), CAT (b), APX (c), total ascorbate (d) and H_2O_2 (e) contents of 7- and 21-day-old plants after exposure to ozone concentrations of 40 and 70 ppb for 8 hr/d, for 7 days, and control group (CF) with ozone concentration ≤ 10 ppb. Data represent the means ± SE, (n=21) and bars with different letters (a-f) indicate significant difference at P≤0.05 (□ CF ▨ 40 ppb ⊞ 70 ppb).

differences in antioxidant production. The concentration of H_2O_2 was from 2 reactions, ozone decomposition and O_2- removed by SOD. Thus, SOD was an important antioxidant which could control H_2O_2 in the plant cells.

The SOD, CAT, APX, total ascorbate and H_2O_2 content of ozone-fumigated plant of both ages and control groups were significantly different, as indicated by the daily results of SOD, CAT, APX, total ascorbate and H_2O_2 content between control and ozoned plants. However, in some durations of exposure, CAT, APX and H_2O_2 did not show statistical differences in either 7- or 21-day-old plants (Figure 4). From these results, the indicators of biochemical response of cowpea to ozone were confirmed as SOD and total ascorbate because daily changes were obviously found through 7 days in both control and ozone-fumigated plants.

Antioxidants might be found either slightly increased or decreased due to insufficient ozone concentration and/or exposure time. Consequently, ROS production would not be sufficient to affect induction of antioxidants in the plant cells. For instance, Rao *et al*. (1996) found, after exposure of *Arabidopsis thaliana* LER to ozone 200 ppb 6 hours for 8 days, there was no effect on CAT. Another report of Tang *et al*. (1999) found slightly increased (15-20%) of CAT in white clover

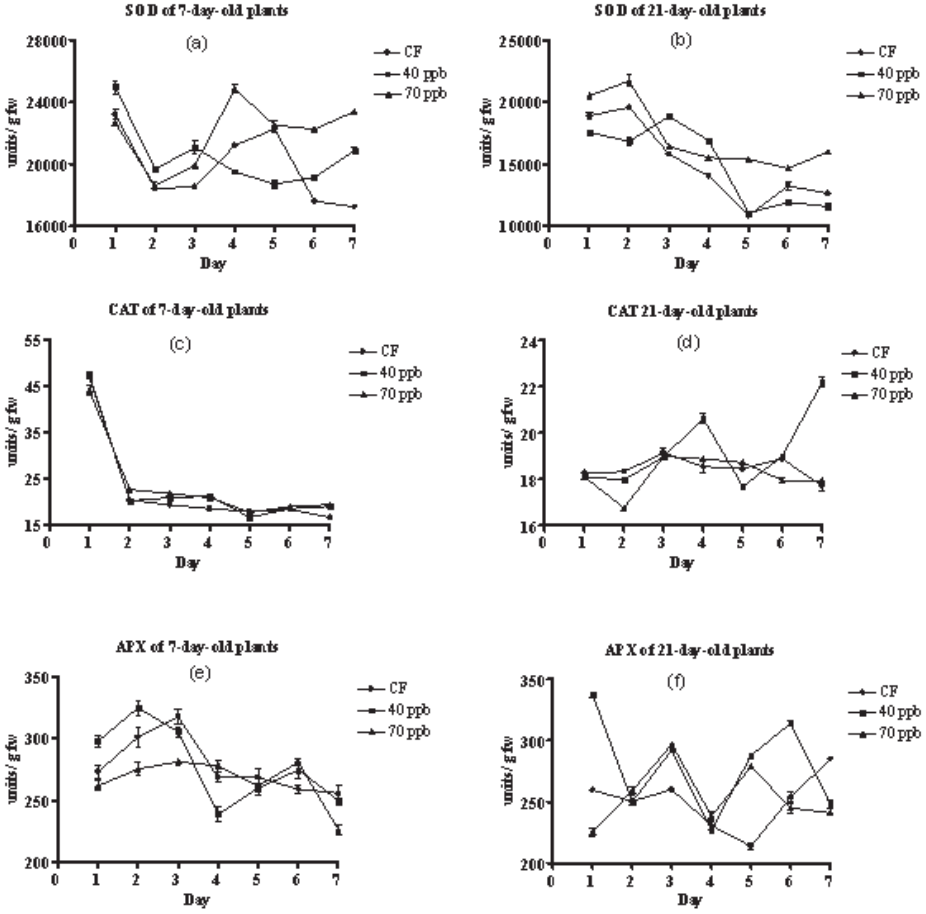

Figure 4. Daily SOD (a-b), CAT (c-d), APX (e-f), total ascorbate (g-h) and H_2O_2 (i-j) contents of 7-and 21-day-old plants after exposure to ozone at concentrations of 40 ppb and 70 ppb for 8 hr/d, for 7 days, and control group (CF) with ozone concentration ≤ 10 ppb. Data represent the means ± SE, (n=3).

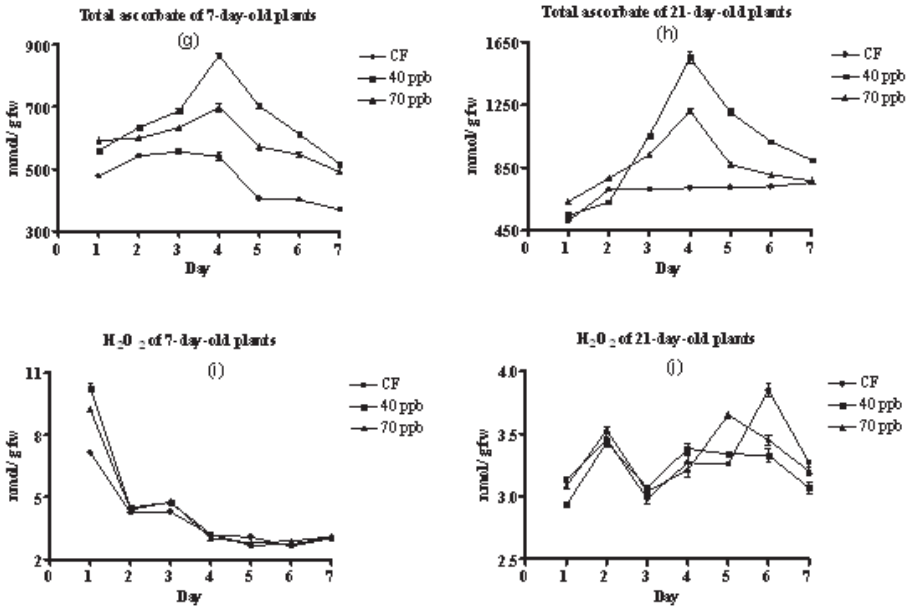

Figure 4. (Continued)

(*Trifolium repens*) after exposure to ozone of 50-150 ppb for 3 days. The slight increase of CAT was due to less H_2O_2 from the ozone reaction.

Moreover, the slight increase and decrease of antioxidants can indicate that cowpea should be considered as an ozone-sensitive species. Generally, high ozone-resistant plants can produce more antioxidants during ozone exposure. Turcsanyi *et al*. (2000) found an increase in ascorbate in *Vicia faba* L. which promoted resistance to ozone. Örvar and Ellis (1997) found lower level of APX in ozone-sensitive (Bel W3) tobacco when compared with ozone resistant Havana. In wheat, increased levels of SOD and GR indicated the resistance to ozone (Rao *et al*., 1995).

3. The relations of physiological and biochemical responses to ozone

When considering leaf damage and antioxidants of ozone-fumigated plants, 7-day-old plants had less than 21-day-old plants because of significantly higher amounts of antioxidants in the younger plants. The visible injuries were caused by the accumulation of ROS. If plants had a better mechanism to remove ROS, the visible injuries would tend to be less. Several researchers have found the same result. For instance, *Trifolium repens* exposed to ozone leading to SOD increase, did not show visible injuries on any levels. In contrast, *Trifolium pretense*, in which SOD decreased, showed visible injuries on the leaves (Scebba et al. 2003). Örvar and Ellis (1997) also found a correlation of APX mRNA and increased of leaf injuries in ozone-exposed plants.

Conclusions

From the experiment the results could be concluded that

1. Plant age, ozone concentration and the duration to ozone exposure were the main factors associated with physiological and biochemical responses of cowpea to ozone.

2. Physiological and biochemical responses of cowpea to ozone were strongly related to decreasing biomass and increasing percentage of leaf damage resulting from accumulation of ROS, which were removed by antioxidants.

3. Better efficiency of defense systems was generated from a combination of antioxidants.

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References

Aebi, H. 1984. Catalase **In** S.P. Colowick and N.O. Kaplan, N.O. (Eds.). Methods of enzymology, Vol 3. Academic Press, New York, p. 273-277.

Calatayud, A. and Barreno, E. 2001. Chlorophyll a fluorescence, antioxidant enzymes and lipid peroxidation in tomato in response to ozone and benomyl. Environ. Pollut., 115: 283-289.

Conklin, L.P. and Last, L.R. 1995. Differential accumulation of antioxidant mRNAs in *Arabodopsis thaliana* exposed to ozone. Plant Physiol., 109: 203-212.

Nakano, Y and Kozi, A. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol., 22(5): 867-880.

Örvar, L.B. and Ellis, E.B. 1997. Transgenic tobacco plants expressing antisense RNA for cytosolic ascorbate peroxidase show increased susceptibility to ozone injury. Plant J., 11(6): 1297-1305.

Rao, V.M., Hale, A.B. and Ormrod, P.D. 1995. Amelioration of ozone-induced oxidative damage in wheat plants grown under high carbon dioxide. Environ. Pollut., 109: 421-432.

Rao, V.M., Paliyath, G. and Ormrod, P.D. 1996. Ultraviolet-B-and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*, Plant Physiol., 110: 125-136.

Scebba, F., Pucciarelli, I., Soldatini, F.G. and Ranieri, A. 2003. O_3 - induced changes in the antioxidant systems and their relationship to different degrees of susceptibility of two clover species. Plant Sci., 165: 583-593.

Sharma, K.Y. and Davis, R.K. 1994. Ozone-induced expression of stress-related genes in *Arabidopsis*. Plant Physiol., 105: 1089-1096.

Sharma, K.Y. and Davis, R.K. 1997. The effects of ozone on antioxidant responses in plants. Free Rad. Biol. Med., 23(3): 480-488.

Takahama, U. and Oniki, T. 1992. Regulation of peroxidase-dependent of phenolics in the apoplast of spinach leaves by ascorbate. Plant Cell Physiol., 33(4): 379-387.

Tang, Y., Chevone, I.B. and Hess, L.J. 1999. Ozone-responsive proteins in a tolerant and sensitive clone of white clover (*Trifolium repens*). Environ. Pollut., 104: 89-98.

Turcsanyi, E., Lyons, T., Plochl, M. and Barnes, J. 2000. Does ascorbate in the mesophyll cell walls form the first line of defence against ozone? Testing the concept using broad bean (*Vicia faba* L.). J. Exp. Bot., 51(346): 901-910.

Willekenes, H., Camp, V.W., Montagu, V.M., Inze, D., Langebartels, C. and Sandermann, H. 1994. Ozone, sulfur dioxide and ultraviolet B have similar effects on mRNA accumulation of antioxidant genes in *Nicotiana plumbaginifolia* L. Plant Physiol. 106: 1007-1014.

Winterbourn, C.C., Haekins, E.R., Brian, M. and Carrell, W.R. 1975. The estimation of red cell superoxide dismutase activity. J. Lab. Clin. Med. 85: 337-341.