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Changes in Antioxidant Defense Capacity in Crop Plants Depend on Leaf Developmental Periods

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Abstract

In this study, chlorophyll, antioxidant compounds and antioxidant enzyme activities of five crop plants (Lycopersicum esculentum Miller, Solanum melanogena L., Capsicum annuum L., Lactuca sativa L. and Cucumis sativus L.) were analyzed in leaves at different development phases (sink, source and senescent). Leaf dry weight decreased, while relative water content increased in all species except C. sativus, depending on leaf development level. Chlorophyll amount significantly decreased with senescence, while carotenoids were found to be well preserved during senescence. It was found that ascorbic acid and tocopherol contents and antioxidant enzyme activities decreased in senescent leaves when compared to young sink and mature source leaves. High GR activities of sink leaves decreased at source and senescent phases. GR activity was preserved in L. esculentum and C. sativus during the senescent phase. It was found that high SOD activity of sink leaves was significantly preserved in source and senescent laves. The findings indicate that young sink leaves need antioxidant protection and after the elimination of this need in the senescent phase, the antioxidant capacity of leaves decreased. We suggest that the relationship between antioxidant defense system and developmental phases show significant variations according to plant species.

Key words: Antioxidant compounds, Antioxidant enzymes, Senescence, Sink leaf, Source leaf.

INTRODUCTION

While young leaves grow out from the apical meristem of the plant, older leaves are shadowed and lose photosynthetic activity. Young leaves consume the carbohydrates and other nutrients they produce by fast photosynthesis for their own growth and development. As the young leaves also use the sources they extract from the phloem of the plant, they have a sink character. On the other hand, leaves which completed their growth transfer the product of photosynthesis to the phloem of the plant and contribute to the development of generative structures of the plant. The leaves in this phase show a source character [1]. Senescence, which starts in old leaves and is thought to be managed by a genetic program, involves the regular destruction of cell structure and re-utilization of a considerable proportion of the sources in other parts of the plant [2].

During metabolic activities in plant cells, as a result of irregularities especially in reduction-oxidation reactions, reactive oxygen species (ROS) can be formed [3]. The leaves are damaged by these reactive oxygen species, which can be formed during photosynthetic activities. As the leaves are aged, the level of damage increases so increasing the necessity for the preservation by antioxidant compounds and antioxidant enzymes. It was suggested that perennial plants have a higher ratio of antioxidant defense elements when compared to annual plants [4].

Leaf senescence can be initiated by external or internal stimulants. Since the receipt of the signals initiating senescence will remove the necessity for the antioxidants, a decrease can be expected in concentrations of antioxidant defense compounds with the symptoms of senescence. Leaf senescence is generally monitored by experimentally measurement of decrease in chlorophyll, protein and nucleic acids [5]. The most significant destruction in the senescence process takes place in chloroplasts. Vacuoles and mitochondria are well-preserved during the senescence period [6].

When the leaves of annual plants are fully expanded, their photosynthesis rates rapidly decrease. It was suggested that the reduction of photosynthesis rates below a certain threshold can stimulate senescence. One of the reasons for initiation and acceleration of leaf senescence is the formation of reactive oxygen species [7]. Loss of antioxidant capacity during the development of senescence was reported in different plants. The relationship between life span of the plants and oxidative stress tolerance is not fully understood [8].

The main sources of ROS in plants are the chloroplasts which induce photosynthesis. In addition, peroxisomes and mitochondria are also significant ROS sources [9]. The leaves also have a developed antioxidant defense system which can prevent the formation of ROS during metabolic activities. The balance between the formation and elimination of ROS determines the life span of the leaf [10]. It was found that long-life mutants of Arabidopsis were more tolerant to various oxidative stress sources. This tolerance may have biochemical and genetic basis [11].

The purpose of this study was to determine whether the antioxidant defense system can be used in delaying or controlling senescence. For this purpose, the study analyzed antioxidant compounds and antioxidant enzyme activities of leaves during different developmental phases. The findings of this study can form the basis for

future research in the fields of physiology, biochemistry and molecular genetics.

MATERIALS AND METHODS

In this study, plants species Lycopersicum esculentum Miller, Capsicum annuum L. and Solanum melongena L. from Solanaceae, Lactuca sativa L. from Asteraceae and Cucumis sativus L. from Cucurbitaceae were used. Registered seeds of these species, which are widely cultivated, were supplied from various seed production and sale centers. The seeds were germinated in plastic pots in a greenhouse environment. After germination, the seeds were transferred to cultivation pots containing equal amounts of soil, sand and organic manure (v/v/v). When seedlings reached flowering period after 8 weeks, leaf samples were collected. During April-May when the plants were cultivated, the temperature of the cultivation greenhouse ranged between 15-35°C and relative humidity ranged between 25-50%.

Three groups of leaf-samples were collected simultaneously and were categorized according to their different developmental stages, as follows: Group 1: sink leaves (did not complete their growth, younger than 1 week, have sink character as they take assimilation products from the phloem). Group 2: source leaves (completed their growth, 1-6 weeks old, have source character as they transfer the assimilation products they produce to sink tissues). Group 3: Senescent leaves (chlorophyll loss depending on programmed destruction process started, leaves older than 6 weeks; destruction products are transferred to young tissues). Leaf dry matter content were determined as the difference between fresh weight and dry weight after drying at 110°C for 24 hours in an oven. Measurements of relative water content (RWC) were taken from middle segments of leaves. After floating on distilled water that allowed the leaf segments to rehydrate for 2 h at 20°C, they were blotted dry and weight. The same segments were dried overnight at 110°C and weighed again. RWC of leaves was calculated according to formula: 100X [(fw-dw / turgid w-dw)].

Biochemical Analysis

Chlorophyll

Chlorophyll extraction from fresh leaf material was carried out with 80% acetone (buffered to pH 7.8 with phosphate buffer). The chlorophyll a, chlorophyll b and total chlorophyll measurements were carried out using a Cecil 5000 spectrophotometer. Chlorophyll contents were calculated according to Porra et al. [12] and chlorophyll a/b ratios were determined.

β-Carotene and xanthophyll

Fresh leaf material (0.5 g) was ground in pre-chilled mortar, in 5 ml acetone containing 200 mg Na2SO4 and then filtered through glass fiber disks (Whatman GF/A) The volume of the acetone extracts was reduced in a rotary evaporator and the material resuspended in 1 ml chloroform. 20 µL of the extracts and standards were applied to silica gel (Sigma Type GF, 10-40 µm) TLC plates (20x20 cm, 0.5 mm thickness). The chromatograms were developed with hexane / diethyl ether / acetone (60/30/20, v/v/v) [13]. B-carotene and xanthophyll spots were scraped from the TLC plates and centrifuged in 5 ml acetone for five min at 5000 X g. The absorbance of supernatants was determined at a wavelength of 450 nm by spectrophotometer.

Total ascorbic acid (TAA)

Frozen leaf material (0.5 g) was homogenized with Ultra Turrax for 30 s in 6 mL of 0.1 M cooled sodium acetate buffer, pH 3. The homogenate was centrifuged for 5 min at 4°C and 7000 g. The supernatant was filtered through a cellulose nitrate filter (Sartorius pore size 0.45 µm) and stored at -70°C. Total ascorbic acid was determined through a reduction of dehydroascorbate to ascorbate by dithiothreitol (DTT). Reaction mixture was incubated with 50 mM DTT at 25°C for 20 min and on ice for 40 min. For the chromatographic separation a Cecil 1200 HPLC system controlled by a personal computer and Data Control software was used. TAA was separated on a RP C18 column (250 X 4.6 mm) using 0.1 M sodium acetate buffer, pH 5 with an isocratic flow of 1.2 mL min-1. Elutes were monitored by an UV detector at 264 nm [14].

α-Tocopherol

Frozen leaf material (0.5 g) was homogenized at 4°C for 30 s in 8 mL ethanol containing 0.1 g insoluble polyvinyl polypyrolidone (PVP) and 0.2 g Na2SO4. The homogenate was centrifuged for 5 min at 4°C and 5000 g. The supernatant was filtered through a cellulose acetate filter, and stored at -70°C [14]. α-Tocopherol was separated at room temperature on a RP-C18 column (250 X 4.6 mm) using solvents A [95% methanol + 5% water (v/v)] and B [95% methanol + 5% ethyl acetate (v/v)] with a flow rate of l.2 mL min-1. The gradient elution started at 100% A and 0% B, changed to 90% A and 10% B within l min and finished with 100% A and 0% B in 15 min. Elutes were monitored by an UV detector at 292 nm. For this chromatographic separation a Cecil 1200-HPLC system controlled by a personal computer including Cecil Data Control software was used. Amounts of α-tocopherol were calculated from a standard curve prepared with tocopherol acetate.

Enzyme extraction

Frozen leaf material (0.5 g) was homogenized in 6 mL 0.1 M potassium phosphate extraction buffer (pH 7, containing 100 mg insoluble PVP and 0.1 mM EDTA) with Ultra Turrax. The homogenate was centrifuged for 5 min at 6000 g and 4°C. The supernatant was filtered through a Whatman GF/A glass fiber disc with a vacuum filtration system and stored at -70°C [15].

Superoxide dismutase (SOD EC l. 15. l. l)

SOD activity was determined according to Beyer and Fridovich [16]. The reaction mixture (3 mL) contained potassium phosphate buffer (pH 8, 0.025% Triton X-100 and 0.1 mM EDTA), enzyme extract, 12 mM

L-methionine, 75 µM nitroblue tetrazolium chloride (NBT) and 2 µM riboflavin. It was kept under fluorescent light for 10 min at 25°C. One SOD unit was described as the amount of enzyme where the NBT reduction ratio was 50%. NBT reduction ratios were measured with a spectrophotometer adjusted to 550 nm. Activity of Mn SOD was measured after addition of potassium cyanide (2mM) to the assay solution. Activity of Cu/Zn SOD and of Fe SOD was calculated by subtracting Mn SOD activity from the total SOD activity. Fe SOD activity was not separately determined.

Glutathione reductase (GR, EC l. 6. 4. 2)

GR activity was assayed at 25 ºC in a 3 mL reaction volume containing 1.5 mL potassium phosphate buffer (0.1 M, pH 7), 150 µL GSSG (20 mM), 200 µL enzyme extract, l mL bidistilled water and 150 µL NADPH2 (2 mM, dissolved in Tris-HCl buffer, pH 7). GR activity was measured according to Carlberg and Mannervik [17] by following the oxidation of NADPH2 with a spectrophotometer at 340 nm.

Catalase (CAT, EC 1. 11. 1. 6)

CAT activity was assayed at 20□C in a 3 ml reaction volume containing 2.8 ml 50 mM potassium phosphate buffer (pH=7 not containing EDTA), 120 □l enzyme extract and 80 □l 0.5 M H2O2. Activity was determined by UV spectrophotometer at 240 nm, measuring the decrease in absorbance for 30 s [18].

Statistics

Individual samples were analyzed at least in triplicate. Data were indicated as arithmetic means and standard deviations (± SD) in table and figures. The significance levels of differences between leaf age and plant species and species – leaf age interactions were analyzed by ANOVA. Results of statistics analyses were shown with P values and significance levels in figure legends.

RESULTS

The dry weight percentage in young sink leaves which not full expanded was found to be high in four species. In these four species, as the leaves aged, dry weight percentage regularly decreased. The decrease in leaf dry weight percentage of older leaves indicated that the water content was higher in aged leaves than young leaves. In C. sativus species, dry weight percentage was found to be low in young leaves, while it was found to be high in senescent leaves (Table 1). There is a negative correlation between dry weight percentages of the leaves and corresponding RWC. The highest RWC in leaves of four species were measured in senescent leaves. In C. sativus species, RWC values decreased depending on leaf age (Table 1).

Chlorophyll a values were high in young leaves of L. esculentum and C. annuum. These values decreased steadily according to leaf age. The highest chlorophyll a value in S. melongena, L. sativa and C. sativus species was measured in full expanded leaves. Chlorophyll a values decreased significantly depending on senescence in all species (Table 1); however, senescence-dependent chlorophyll destruction ratios of source leaves varied between species (L. esculentum 53%, S. melongena 56%, C. annuum 25%, L. sativa 39%, and C. sativus 42%). In chlorophyll b values, the changes depending on leaf development phases are similar to the changes in chlorophyll a values (Table 1). In addition to significant differences between species ($P<0.05$), chlorophyll a and b values also varied significantly depending on development phases ($P<0.01$). Although chlorophyll a/b ratios varied according to leaf development phases, this difference was not found to be statistically significant. Among the analyzed species, the chlorophyll a/b ratio decreased with senescence in S. melongena and C. annuum while slightly increased in other species (Table 1).

Table 1. Dry weight percentage, relative water content (RWC) chlorophyll a and b contents (µg g-1 FW) and chlorophyll a/b ratio in sink, source and senescent leaves of five crop plants. Data represent the means and ± standard deviation (*P≤0.05 and **P≤0.01).

Species	Phase	DW (%)	RWC	Chl-a (µg g^{-1} FW)	Chl-b (µg g^{-1} FW)	Chl a/b
Lycopersicum	Sink	15.2 ± 3.5	82 ± 7	1541 ± 80	602 ± 54	2,6
esculentum	Source	13.3 ± 2.7	83 ± 8	1253 ± 73	468 ± 58	2,7
	Senescent	12.6 ± 2.0	90 ± 8	558 ± 46	209 ± 25	2,7
Solanum	Sink	16.2 ± 2.4	83 ± 5	1107 ± 68	394 ± 37	2,8
melongena	Source	10.6 ± 1.3	93 ± 9	1146 ± 63	404 ± 34	2,8
	Senescent	7.8 ± 1.1	94 ± 7	496 ± 37	184 ± 17	2,7
Capsicum	Sink	15.3 ± 2.4	89 ± 8	1306 ± 85	493 ± 48	2,7
annuum	Source	14.1 ± 2.2	91 ± 7	1042 ± 54	394 ± 27	2,6
	Senescent	9.5 ± 1.3	92 ±10	801 ± 52	317 ± 40	2,5
Lactuca	Sink	9.7 ± 0.8	75 ± 4	369 ± 40	176 ± 20	2,1
sativa	Source	6.6 ± 0.4	85 ± 7	519 ± 32	181 ± 16	2,9
	Senescent	6.1 ± 0.7	87 ± 6	309 ± 23	104 ± 13	3
Cucumis	Sink	11.7 ± 1.6	86 ± 7	768 ± 60	312 ± 32	2,5
sativus	Source	12.1 ± 1.8	81 ± 9	1100 ± 72	431 ± 37	2,6
	Senescent	17.2 ± 2.2	80 ± 7	644 ± 48	239 ± 26	2,7
Statistics		Sp X phase** N=5	Sp X phase** N=5	Species* Phase* Sp X phase** N=3	Species* Phase* Sp X phase** N=3	

The values found for β-carotene and xantophyll, which are two species of carotenoid pigments, were similar. Although there was a relationship between leaf developmental phases and carotenoid values, this relationship varied between the species. L. sativa and C. sativus leaves had very low carotenoid values in all developmental phases. In contrast, C. annuum showed high carotenoid values in all development phases. It was found that there was no significant changes of carotenoid values depend on development phases and carotenoids were preserved during the senescence process (Figure 1). Chlorophyll / carotenoid (chl / car) ratios were higher in sink leaves than source leaves in four species except C. sativus. Lower chl / car ratios were found in senescent leaves of investigated species (Figure 1).

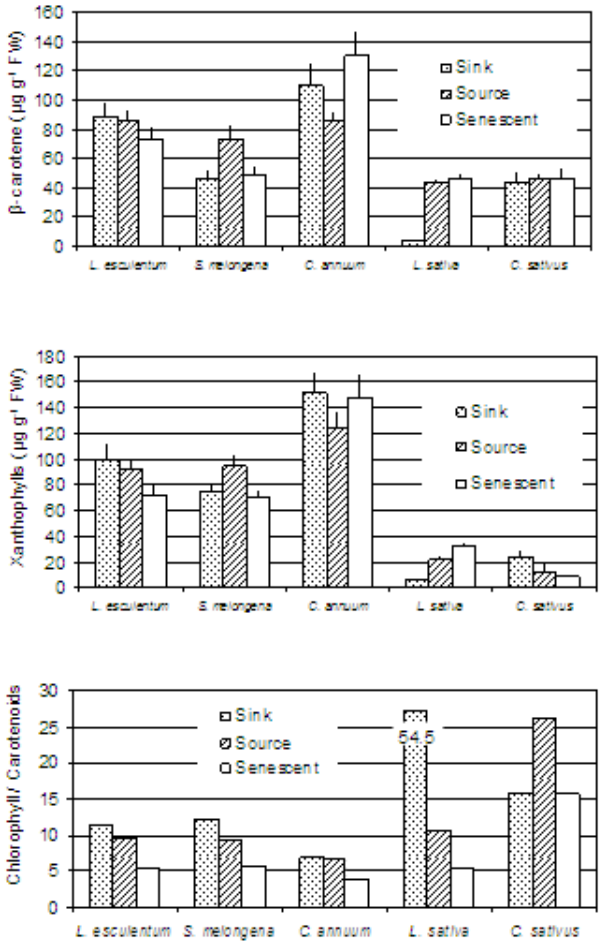

Figure 1. β-Carotene (A) and xanthophyll (B) and chl / car ratio (chl a + chl b / β-carotene + xanthophyll) (C) values in sink, source and senescent leaves of five crop plants. Statistics: β-carotene: species**, species X phase**; xanthophyll: species**, species X phase**; chl / car ratio: species*, phase**, species X phase** (*P≤0.05 and **P≤0.01).

Ascorbate content was found to be high in sink and source leaves of C. annuum, and low in all developmental phases of L. sativa and C. sativus species (Figure 2). Young leaves with sink character had a high ascorbate content. While the full expanded leaves with source character retained high ascorbate concentrations, ascorbate values in senescent leaves decreased significantly. However, ascorbate decrease dependent on senescence was not detected in S. melongena species (Figure 2).

Sink leaves of L. esculentum were found to have the highest α-tocopherol content. In other species, the highest α-tocopherol content was found in source leaves. Low α-tocopherol levels were measured in young leaves of L. sativa and C. sativus species. The α-tocopherol content decreased significantly in all species, depending on senescence (Figure 2).

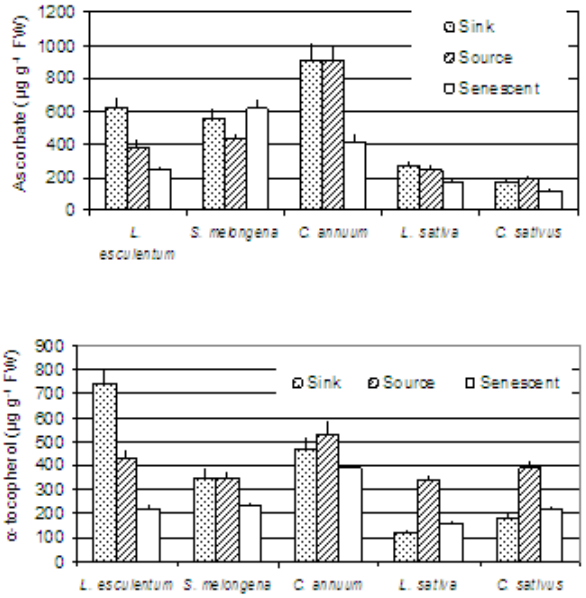

Figure 2. Ascorbate (A) and α-tocopherol (B) values in sink, source and senescent leaves of five crop plants. Statistics: ascorbate: species**, species X phase**; α-tocopherol: species X phase** (*P≤0.05 and **P≤0.01).

Measurements of catalase activity showed significant variations according to species. It was found that the sink leaves in four species except S. melongena had high catalase activity. While catalase activity increased in full expanded source leaves of S. melongena and C. annuum, it began to decline in source leaves of other species. Catalase activity rapidly decreased in the senescent leaves of all species (Figure 3).

Glutathione reductase activity was found to be high in young sink leaves of all species. It was determined that, during source and senescent phases, GR activity of leaves significantly decreased. In senescent leaves, GR activity ceased in S. melongena and C. annuum, while it was preserved in L. esculentum and C. sativus (Figure 3).

The highest SOD activity was measured in young leaves. It was found that SOD activity regularly decreased in all species depending on developmental phase. Significantly high SOD activities can be retained even in senescent leaves (Figure 3).

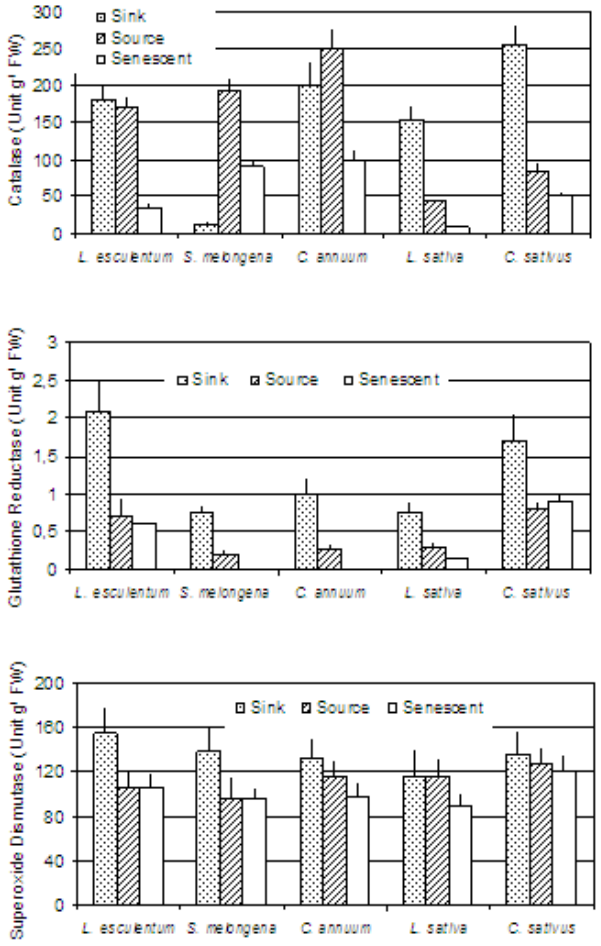

Figure 3. Catalase (A), glutathione reductase (B) and superoxide dismutase (C) enzyme activities in sink, source and senescent leaves of five crop plants. Statistics: catalase: species X phase**; glutathione reductase: species**, phase**, species X phase**; superoxide dismutase: phases**, species X phases** (*P≤0.05 and **P≤0.01).

DISCUSSION

The present study analyzed the changes in antioxidant compound concentrations and antioxidant enzyme activities of the leaves during different developmental phases in five crop plants. The findings indicate that molecular and enzymatic elements of antioxidant defense system in sink, source and senescent leaves varied significantly according to plant species. Experimental studies can depict the interaction and the mode of action of the various metabolic and biochemical parameters measured during leaf aging and the transformation from sink to source [19]. The main finding of this study is that antioxidant defense requirements of plants growing under similar conditions are determined by their morphologic genetic and physiological properties. While young leaves of L. sativa and L. melongena do not require carotenoid preservation in the sink phase, L. esculentum and C. annuum require high carotenoid content. In all analyzed species, while young leaves in the sink phase are preserved with high levels of ascorbate, SOD and GR, the need for the antioxidant defense system decreases in senescent leaves [4].

There is some proof that ROS triggers leaf senescence [8]. In addition, in some plants, the loss of oxidative capacity during the onset of senescence was reported [9, 10]. It was found that flower and leaf life spans were significantly consistent with oxidative stress resistance in A. thaliana [20]. However, the relationship between plant life span and oxidative stress tolerance has not yet been fully explained. The antioxidant defense system of plant species analyzed in this study had different levels of senescence-dependent weakening.

Inadequacies in removal of ROS cause to oxidative stress and cell death. This may result from excessive production of ROS or a decrease of antioxidants [21]. ROS increase in the cell has two major effects: damage to various cell components and activation of special signaling pathways. Both effects have a significant role in initiation and progress of the senescence process [22].

The first visible symptom of senescence is the loss of chlorophyll [23]. During dark induced senescence, the chlorophyll of barley leaves was completely destructed in 8 days and a peak was observed in chlorophyllase activity between 4 and 6 days. In the same study, there was a significant increase in chlorophyll a/b ratio after the 3rd day. This indicates that chlorophyll b is more rapidly destroyed than chlorophyll a [24]. The findings of our study indicate that variations in chlorophyll a and b content were similar during leaf development phase. The destruction rate of chlorophyll a and b varied significantly according to plant species. It has been suggested that the change in chlorophyll a/b ratio was different in natural senescence and dark induced senescence and that chlorophyll b reductase had a role in initiating dark induced senescence [24]. Although it is suggested that chlorophyll was enzymatically destroyed during the process of senescence, the role of free radicals in chlorophyll loss has not been fully explained [23].

In a previous research [23] authors found that, in some leaves, β-carotene content decreased depending on age and that in summer, β-carotene content was much lower. In the present study, although it was found that β-carotene decreased during senescence, in C. annuum species, the carotenoids were well preserved until the advanced phases of senescence. The lower level of senescence-dependent chlorophyll loss (25%) in this species can be explained by the protective effect of carotenoids [7]. Due to the close relationship between chlorophyll functions and the protective role of carotenoids, the chl / car ratio is an important indicator of photosynthetic effectiveness [25, 26]. The rapid reduction of the chl / car ratio during senescence, indicates that chlorophyll loss induced by the increase in chlorophyll-destroying enzyme activities rather than oxidative stress damage [27].

The effect of oxidative conditions formed during senescence is reduced by the ascorbate-glutathione cycle [28]. The effectiveness of the cycle depends on sufficient amounts of water-soluble antioxidants such as ascorbate and glutathione [8]. Ascorbate production capacity of the leaves decreased with aging [9, 30]. It was reported that the reduced glutathione concentration during leaf senescence decreased in mitochondria and chloroplast [9, 31]. The decrease in ascorbate content during leaf senescence was supported by the findings obtained from all species in the present study, except S. melongena. The preservation of ascorbate concentration in S. melongena species even during senescence indicated that ascorbate loss during senescence may not be a general characteristic for the plants.

It has been shown that α-tocopherol plays an important role in protection of cell membranes against oxidative stress [32]. Lipid oxidation analyses indicated that lipid catabolism in chloroplasts was higher in mature plants than in young plants [23]. Munne-Bosch et al. [6] found that membrane-dependent antioxidants such as β-carotene and α-tocopherol were significantly destroyed during chloroplast senescence. Based on this finding, the researchers suggested that single oxygen (1O2), but not H2O2, may be responsible for chloroplast senescence. The increase in lipid peroxidation may be caused by a senescence-dependent decrease in α- tocopherol levels of cell membranes.

Antioxidant enzymes have a critical role in elimination of ROS. Transforming highly reactive superoxide anions into H2O2, SOD initiated oxidative defence. The next step was the elimination of H2O2 by catalase and peroxidases [29, 32]. Antioxidant enzyme activities stimulated by oxidative stress decreased with senescence. Cassano et al. [32] found that activity of SOD under photo-oxidative stress was significantly higher in young barley leaves than in senescent ones. Based on these findings, the researchers suggested that SOD activity was regulated by developmental phases in addition to stress. These findings are consistent with the findings of the present study. Some studies indicate that antioxidant enzyme content in senescence processes is regulated differently in mitochondria, chloroplast and peroxisomes [9, 29, 33]. Activities of MnSOD, APX and GR enzymes in mitochondria significantly decreased while peroxisomal enzymes increased with dark induced senescence [9]. It was suggested that different responses of mitochondria and peroxisomes to senescence may be due to earlier effects of damage of H2O2 when compared to peroxisomes [33]. In pea leaves, peroxisomal CAT and APX activities decreased with senescence, while DHAR and GR activities increased [30]. In the same study, it was found that chloroplast GR activity reduced. The results of the present study, based on analysis of five species, indicate that CAT activity decreased in senescence phases. Although SOD activity decreased in all of the analyzed species when compared to young sink leaves, it was significantly preserved during the senescence process. It may be suggested that the senescence-dependent decrease observed in GR activity is not general for plant species. It was found that the high GR activity of young sink leaves was significantly reduced in full expanded source leaves.

Piacentini et al. [3] suggested that the delayed cotyledon senescence in C. sativus seedlings caused by a magnetic field effect was related to antioxidant potential. Study results indicated that combined high levels of SOD, CAT and GR enzymes delayed senescence and lengthened cotyledon life span. While the relatively high SOD activity continued, the fact that CAT and GR enzyme activities (which served in reactions eliminating H2O2) decreased to very low values, creates a suitable environment for the destructive reactions of the senescence process. After the initiation of the senescence process, it was clear that the need for the antioxidant defense system was eliminated and, as a result, the activity of enzymatic and non-enzymatic elements of the system declined during the senescence process. These findings indicate that, there is relationship between antioxidant defense system and developmental phases show significant variations according to plant species.

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