Oxidative Defense Systems in Leaves of Three Edible Herb Species in Relation to Their Senescence Rates†

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Correlation studies between leaf senescence rates of three edible herb species, watercress (*Rorippa nasturtium-aquaticum*), parsley (*Petroselinum crispum*), and sage (*Salvia officinalis* L.), and their oxidative defense systems were conducted with detached leaves under simulated shelf-life storage conditions. The relative order of leaf senescence rate, based on their rate of chlorophyll (Chl) degradation and malondialdehyde accumulation, was watercress > parsley > sage. However, all three herb species showed high proteolysis rates from the first day of incubation. Of five oxidative defense systems examined in the three herb species, only the system of total reducing capacity correlated well with their relative order of Chl degradation and could therefore predict their storage potential. The results indicate that each herb species has developed specific oxidative defense systems, which may also prevent rapid Chl loss but do not affect proteolysis. It seems, therefore, that among the various components of the senescence syndrome, Chl breakdown is closely linked to lipid oxidation, while proteolysis seems to proceed independently of these two senescence-associated processes.

Keywords: *Chlorophyll loss; proteolysis; lipid oxidation; leaf senescence; fresh herbs; oxidative defense system*

INTRODUCTION

Plant senescence is a multifactoral syndrome in which free radical metabolism seems to play an important role (Leshem, 1988). In leaves, senescence is often followed by changes in the most easily assessed parameter, chlorophyll (Chl), as well as by accumulation of amino acids and lipid peroxidation products (Takegami, 1975; Thomas and Stoddart, 1975; Dhindsa et al., 1981; Kunert and Ederer, 1985; De Kok and Graham, 1989; Philosoph-Hadas et al., 1991; Meir et al., 1992). Senescence is often investigated in light or darkness, using convenient model systems such as detached leaves (Peterson and Huffaker, 1975; Philosoph-Hadas et al., 1991), leaf disks (Takegami, 1975), or isolated chloroplasts (Choe and Thimann, 1975; McRae and Thompson, 1983; Casano and Trippi, 1992). These model systems are also quite important for the simulation of shelf-life storage of several agricultural materials, such as leafy edible herbs and spices.

Activated oxygen species have been implicated in the development of many stresses in leaves, following drought (Mittler et al., 1991), herbicide treatments (Askira et al., 1991), photooxidation (Leshem, 1988), and air pollution (Elstner and Hippeli, 1991). The chloroplast is one of the main cellular locations where such reactive oxygen species can be generated. The main cellular components susceptible to damage by free radicals are polyunsaturated fatty acids (PUFA) in membranes, proteins, carbohydrates, nucleic acids, and pigments such as Chl or carotenoids (Leshem, 1988).

Plant cells and chloroplasts have especially developed defense systems to prevent the formation of or to scavenge oxygen active species. These systems include enzymes such as superoxide dismutase (SOD), peroxidases, and catalase (Kalir et al., 1984) or antioxidants such as ascorbic acid, glutathione, and polyphenolic compounds (Rabinowitch and Fridovich, 1983; Kunert and Ederer, 1985; Asada and Takahashi, 1987; Pastori and Trippi, 1992). More recently, it has been suggested that detachment of leaves and continuous darkness cause an abrupt switch from predominantly anabolic mechanisms to catabolic breakdown (Matile, 1993). Hence, the presence of such defense systems may determine the senescence rate of detached leaves incubated in darkness under simulated storage conditions. The aim of the present study was to evaluate the defense systems against oxidation in leaves of three edible herb species, which vary significantly in their senescence rates, in an attempt to assess their storage potential.

MATERIALS AND METHODS

Materials. Ascorbic acid, trichloroacetic acid (TCA), and hydrogen peroxide (30%, for synthesis) were purchased from Merck (Darmstadt, Germany). NADPH, ammonium acetate, sodium acetate, Ferrozine, thiobarbituric acid (TBA), neocuproine, DL-α-tocopherol, β-carotene, and linoleic acid were from Sigma Chemical Co. (St. Louis, MO). $FeCl_3 \cdot 6H_2O$ was from Riedel-de Haen (Seelze, Holland) and adenosine 5′-diphosphate (ADP) salt from Boehringer-Mannheim, GmbH (Mannheim, Germany).

Plant Material and Senescence Treatments. Experiments were performed with mature, green, fully expanded leaves of watercress (*Rorippa nasturtium-aquaticum*), parsley (*Petroselinum crispum*), and sage (*Salvia officinalis* L.), which had been freshly harvested from the field. The bunches were rinsed with distilled water and dried; the leaves were subsequently detached from the bunches and placed in Petri dishes (5–8 leaves/dish) on filter papers (Whatman No. 1) moistened with 1.5 mL of distilled water and chloramphenicol (50 μg/mL). Four Petri dishes were employed for each incubation

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period. The various leaves incubated in the Petri dishes were allowed to senesce in darkness at 22 °C for 5–11 days, and their Chl, amino acid, and MDA contents were assayed periodically.

Chlorophyll and Amino Acid Determination. Chl and amino acids were extracted from samples of cut leaf segments (0.5 g) of watercress, parsley, or sage by boiling them for 30 min in 10 mL of 80% (v/v) ethanol, as previously described (Philosoph-Hadas et al., 1991; Meir et al., 1992). Chl was subsequently quantified by determining the absorbance at 645 and 663 nm, according to the method of Arnon (1949), and expressed as milligrams of Chl per gram of fresh weight (g FW).

The ethanolic extract was then further used for determination of amino acids by the ninhydrin method (Yemm and Cocking, 1954), adapted to microquantities suitable for ELISA plates (Philosoph-Hadas et al., 1991). A standard methionine curve was included in every plate, and each point was repeated three or four times. This modified procedure enabled a rapid and accurate quantification of amino acids in a large number of samples.

Extraction and Determination of MDA. TBA-reactive substances were extracted as described previously (Meir et al., 1992) by homogenization of 0.5 g of tissue in 5 mL of solution containing 20% TCA plus 1.5 mM ethylenediaminetetraacetic acid (EDTA). TBA-reactive substances were assayed according to the TBA test (Kosugi and Kikugawa, 1985), as modified by Meir et al. (1992). Briefly, 1 mL of 0.67% TBA was added to 3-mL aliquots of the supernatant, and the solution was incubated at 100 °C for 1 h. The solution was then cooled and centrifuged for 10 min at 8000 rpm. The volume of the resultant supernatant was made up to 10 mL with distilled water, and the absorbance of the colored reaction product was determined at 532 nm (for MDA). For calculations of the MDA content, an extinction coefficient (ϵ) of 1.56×10^5 $M \cdot cm^{-1}$ was used (Kosugi and Kikugawa, 1985).

Extraction and Determination of Reducing Compounds. Total reducing compounds were extracted from the various leaf tissues by homogenizing 1 g of fresh leaves in 100 mL of 0.2 M acetate buffer, pH 4.5. After a 10-min centrifugation at 18000*g*, the amount of reductants in the supernatant was assayed by determining its capacity to reduce Fe(III) to Fe(II), using Ferrozine as a chromophoric, high-affinity chelator for the product Fe(II). The standard assay for Fe(II) release consisted of 1 mL of the herb extract in various dilutions, 0.2 M acetate buffer, pH 5.5, 1 mM $FeCl_3$, 11.3% TCA, 0.34 M ammonium acetate, and 1 mM Ferrozine reagent, in a total volume of 3 mL. The Ferrozine reagent was composed of ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine] plus neocuproine plus concentrated HCl (Stookey, 1970). The rate of Fe(III) reduction by the leafy reductants was quantified by measuring the initial formation rate of the ferrozine–ferrous complex, $Fe(Ferrozine)^{2+}$ at 562 nm according to the method of Stookey (1970), as modified by Kanner et al. (1991). Total reducing capacity (units/g FW) of the leaves was determined after a 24-h incubation of the leaf extracts with Fe(III) at 22 °C, in the standard assay mixture detailed above. A unit was defined as the reducing capacity of the tissue that yielded an absorbance of 1 OD at 562 nm.

Determination of Antioxidative Enzymes. *Peroxidase* activity of the various herb extracts was determined spectrophotometrically by measuring their capacity to oxidize pyrogallol to purpurogalin (PuG). The cytosolic enzyme was prepared from the fresh herbs as described previously (Kanner et al., 1983). A typical assay contained 0.1 mL of cytosolic enzyme extract, 0.1 mL of H_2O_2 (4 mM), and 0.1 mL of pyrogallol (4 mM) in 1.7 mL of sodium acetate buffer (0.05 M), pH 6.0 at 25 °C. The absorbance changes at 420 nm were recorded by a Varian double-beam spectrophotometer. For calculation of the PuG formed during the reaction, an extinction coefficient of 2470 was used.

Catalase activity of the various herb extracts was determined by monitoring the oxygen evolution resulting from breakdown of H_2O_2 by the extracted enzyme. The rate of oxygen accumulation was determined as previously described (Kanner et al., 1991), by means of an oxygen monitor (Yellow Spring Instrument Co., Model 53) equipped with a Clark electrode and a recorder. The test system contained 10 mM acetate at pH 6.5, 1 mM H_2O_2, and various amounts of the soluble, dialyzed cytosolic enzyme extracts, before and after their heating at 98 °C for 5 min.

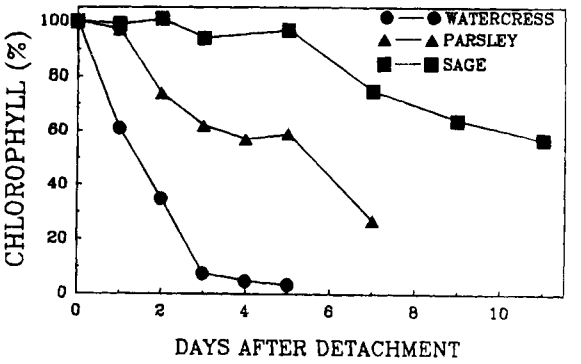

Figure 1. Comparative time-dependent changes in levels of Chl during dark senescence of detached watercress, parsley, and sage leaves incubated at 22 °C. Samples consisting of five detached watercress leaves, seven parsley leaves, or eight sage leaves were incubated on filter paper in Petri dishes with 1.5 mL of distilled water and 50 μg/mL chloramphenicol. At the indicated periods, Chl was quantified in four replicates with 0.5-g samples of leaf segments. Results are expressed as percentage of the Chl levels of each herb species at harvest. These Chl levels (100%) were 1.5 ± 0.02 mg/g FW for watercress leaves, 2.4 ± 0.03 mg/g FW for parsley leaves, and 1.6 ± 0.02 mg/g FW for sage leaves.

Determination of α-Tocopherol Content. α-Tocopherol was determined for a freeze-dried leaf powder. The raw material was prepared as previously described (Kanner et al., 1979). Tocopherols were separated by HPLC with fluorescence detection, using a column of Lichrosphere 100 RP 18 (5 μm), 125 mm (Merck), as described by Puronen et al. (1986).

Determination of β-Carotene–Linoleate. The oxidizing activity of β-carotene–linoleate was assayed colorimetrically (Kanner et al., 1992), by monitoring the decrease in absorbance at 460 nm in the cuvette of a double-beam recording spectrophotometer. The test sample contained 1.5 mL of buffered carotene–linoleate mixture at pH 7.0, 0.1–0.4 mL of active fractions, and distilled water to a final volume of 2.0 mL. The initial reaction mixture included: 14 μM β-carotene, 2 mM linoleate, 0.05% Tween 20, 0.1 M phosphate buffer, pH 7.0, and 0.5 mM diethylenetriaminepentaacetic acid (DATA). The oxidation reaction was catalyzed by addition of 5 μM myoglobin, and the induction period following the addition of the various herb extracts was calculated and presented as seconds per 0.4 mg FW.

RESULTS

The rate of leaf senescence, as indicated by Chl loss and accumulation of amino acids resulting from enhanced proteolysis, was examined in detached leaves of three herb species that vary in their senescence rates. In parallel, several lipid oxidation products, such as TBA reactants (MDA and aldehydes) and lipofuscin-like fluorescent compounds (FC), were examined (Meir et al., 1992). In general, the senescence-related changes in these three lipid peroxidation products followed similar trends for the three species tested (data not shown). Therefore, only the changes in MDA content are presented, since this parameter is most often employed to indicate the process of lipid oxidation in plants and animals (Kosugi and Kikugawa, 1985).

Results depicted in Figure 1 show that detached watercress leaves exhibited a very rapid senescence rate, as a result of which about 60% of their initial Chl content was lost within 2 days. The senescence rate of parsley leaves was much slower than that of watercress

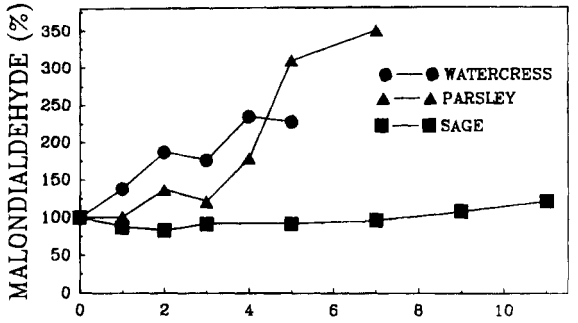

Figure 2. Comparative time-dependent changes in levels of MDA during dark senescence of detached watercress, parsley, and sage leaves incubated at 22 °C. Leaves were incubated in Petri dishes as described in Figure 1, and MDA was quantified in four replicates with 0.5-g samples of leaf segments. Results are expressed as percentage of the MDA level of each herb species at harvest. These MDA levels (100%) were 0.4 ± 0.02 μmol/g FW for watercress leaves; 0.6 ± 0.03 μmol/g FW for parsley leaves, and 1.4 ± 0.1 μmol/g FW for sage leaves.

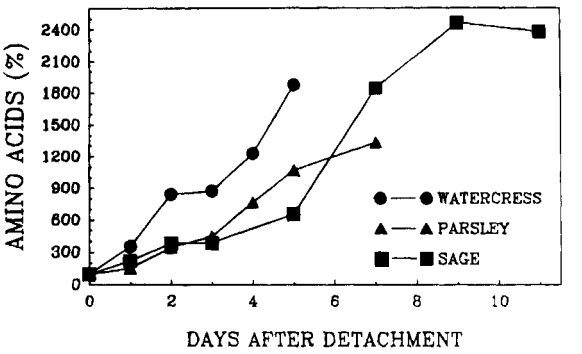

Figure 3. Comparative time-dependent changes in levels of amino acids during dark senescence of detached watercress, parsley, and sage leaves incubated at 22 °C. Leaves were incubated in Petri dishes as described in Figure 1, and amino acids were quantified in four replicates with the same 0.5-g samples of leaf segments used for Chl determination. Results are expressed as percentage of the amino acid level of each herb species at harvest. These amino acid levels (100%) were 0.1 ± 0.02 μmol/g FW for watercress leaves, 0.2 ± 0.03 μmol/g FW for parsley leaves, and 0.1 ± 0.01 μmol/g FW for sage leaves.

leaves, showing no Chl decline during the first day and a 70% loss only after 5 days of senescence. The senescence rate of detached sage leaves was much slower than those of the other two species. Results depicted in Figure 1 demonstrate that no decline in Chl level could be detected during the initial 5 days of senescence, and only a moderate reduction could be observed later on. However, even after 11 days of dark senescence, sage leaves lost only 40% of their initial Chl content.

The rapid Chl degradation of watercress leaves was accompanied by a parallel and rapid increase of 225% in MDA content during 5 days of senescence (Figure 2). The corresponding increase in MDA content of parsley leaves also paralleled their rate of Chl loss (Figure 1), showing a 350% increase in MDA after 7 days of senescence (Figure 2). In this case, the MDA increase of parsley leaves even exceeded that of watercress leaves. Similarly to these two herb species, the extremely slow Chl degradation of sage leaves was accompanied by a correspondingly very moderate increase of 125% in MDA content after 11 days of senescence (Figure 2). In spite of these differences in their rates of Chl degradation and the corresponding MDA accumulation, all three herb species showed a very high rate of proteolysis from the first day of incubation (Figure 3). Consequently, at the end of the various senescence periods, amino acid content increased in watercress leaves by 1800% after 5 days, by 1200% in parsley leaves after 7 days, and by 2400% in sage leaves after 11 days of senescence (Figure 3). Hence, sage leaves, which showed the lowest rates of Chl degradation (Figure 1) and MDA accumulation (Figure 2), exhibited the highest rate of proteolysis (Figure 3). These results indicate that in detached sage leaves the process of Chl degradation is associated with the process of lipid oxidation but is separated from the process of proteolysis. Hence, these three senescence-associated processes are not necessarily linked.

To further confirm the close correlation found between the processes of Chl loss and lipid oxidation, correlation studies between Chl breakdown rates of the three herb species and their oxidative defense systems were conducted. The relative order of leaf senescence rate, based on their rates of Chl degradation, summarized in Table 1, was watercress > parsley > sage. Among the five systems of oxidative defense tested, only the relative differences between the total reducing capacity of the leaves were directly correlated with their relative rates of Chl breakdown (Table 1). Thus, the results in Table 1 show that sage leaves, which senesced at the slowest rate, exhibited the highest reducing activity, and their total reducing capacity was 6 times greater than the corresponding reducing activity of watercress leaves. Parsley leaves, which senesced at a moderate rate, showed only a 2-fold increase in their total reducing capacity as compared with watercress leaves (Table 1). These results suggest that the system of total reducing compounds correlates well with the relative rates of Chl degradation of these three herb species and, therefore, may be used to predict their storability.

The lag times required for oxidation of β-carotene in watercress and sage leaves (but not in parsley leaves) also correlated with differences in their relative rates of Chl loss. Thus, the lag time required for preventing oxidation in sage leaves (slow Chl degradation rate) was 10 times higher than that of watercress leaves (rapid Chl degradation rate) but 160 times higher than that of parsley leaves (medium Chl degradation rate). Since the lag time required for oxidation reflects the antioxidant content of the tissue, the results suggest that sage leaves contain much more endogenous antioxidants than watercress or parsley leaves. Indeed, sage leaves are known as a good source of natural antioxidants (Loliger, 1989).

DISCUSSION

The three herb species examined in this study differed significantly in their rates of Chl breakdown and MDA accumulation during dark senescence. However, all three species exhibited very high rates of proteolysis from the first day of incubation, showing increases of amino acid content between 1200 and 2400% after 5–11 days of senescence (Figure 3). The net loss of protein has long been recognized as a dominant feature of foliar senescence (Peoples and Dalling, 1988). The decrease in protein level results mainly from the decline in the content of the enzyme RuDP carboxylase, which comprises about 50% of total mesophyll proteins (Peterson and Huffaker, 1975). As shown in our results for watercress and parsley leaves (Figures 1 and 3), in most

Table 1. Comparison of Several Oxidative Defense Mechanisms in Leaves of Various Herb Species, As Related to Their Senescence Rates[a]

herb species	α-tocopherol content (mg/g FW)	β-carotene oxidation induction time (s/0.4 mg FW)	antioxidative enzyme activity: catalase [μmol of H_2O_2 min^{-1} (mg FW)$^{-1}$]	antioxidative enzyme activity: peroxidase [mmol of PuG min^{-1} (g FW)$^{-1}$]	total reducing capacity (units/g FW)	Chl degradation rate (days to 50% Chl loss)
watercress	0.34	16	0.38	7.37	363	1.5–2
parsley	0.29	1	3940.00	294.74	679	5–6
sage	0.49	160	0.00	146.09	2270	>11

[a] Experiments were performed with fresh leaves of watercress, parsley, and sage. The rate of Chl degradation, obtained as a mean of several experiments, was estimated as the time required to obtain 50% of the initial Chl content in detached leaves kept in darkness at 22 °C.

cases the process of Chl degradation corresponds to proteolysis, as previously reported for senescing tobacco leaves (Dhindsa et al., 1981) or leaf disks (Takegami, 1975), *Arabidopsis* leaves during dark or natural senescence (De Kok and Graham, 1989), rice leaves during senescence in the light (Biswas and Choudhuri, 1978) or in darkness (Chen and Kao, 1991), barley leaves (Peterson and Huffaker, 1975), and bean leaves during natural senescence (Venkatarayappa et al., 1984). In many cases hormonal treatments that retarded or enhanced senescence affected the processes of Chl loss and proteolysis in a similar manner, as reported for the effects of benzyladenine (BA) and abscisic (ABA) on rice leaves (Biswas and Choudhuri, 1978; Chen and Kao, 1991) and the effect of BA in tobacco leaves (Takegami, 1975).

The first demonstrated separation of Chl degradation from other senescence processes in leaves was reported for leaves of the nonyellowing mutant of meadow fescue (Thomas and Stoddart, 1975). The proteolysis in this mutant followed the same pattern as that of the wild type, while its Chl level remained constant. A similar separation of Chl degradation from proteolysis has also been observed in bean (Wang et al., 1989), barley (Hall et al., 1978), and *Hydrilla* (Kar and Choudhuri, 1985) leaves. In the last case, Chl degradation corresponded to proteolysis in leaves senescing in darkness, but during senescence in the light the proteolytic enzymes were inhibited (Kar and Choudhuri, 1985). On the other hand, in leaves of the rice mutant which exhibited less Chl content, the decrease in Chl started only when 60% of the proteins had already been hydrolyzed (Hall et al., 1978), similar to the results obtained with senescing sage leaves (Figures 1 and 3).

The possible link between the processes of Chl degradation and lipid oxidation has been reported previously in several systems of rice (Lin et al., 1984) and pea (Wang et al., 1990) leaves during natural senescence, in barley and rumex leaves during dark senescence (Dhindsa et al., 1982), in oxygen-stressed leaves of spinach (Sakaki et al., 1983), oat (Trippi et al., 1989), and tobacco (Kato and Shimizu, 1987), and in isolated chloroplasts (Heath and Packer, 1968; McRae and Thompson, 1983). However, on the basis of the report showing that proteolysis of oat chloroplasts was enhanced by oxygen stress and inhibited by scavengers of hydroxyl radicals (Casano and Trippi, 1992), the link between proteolysis and oxidative processes may be also suggested. This may occur in an indirect manner, as shown for the RuDP carboxylase enzyme of citrus leaves that was inactivated in response to oxygen stress and thereby became more susceptible to proteolytic degradation (Penarrubia and Moreno, 1990).

The accumulated results, which show a positive correlation between the processes of Chl degradation and lipid oxidation, and the separation of proteolysis from these senescence processes in sage leaves (Figure 3) suggest that Chl degradation is associated with lipid oxidation rather than with proteolysis. This suggestion is supported by the results reported for natural senescence of bean leaves, showing that Chl loss, which was accompanied by accumulation of MDA, preceded the process of proteolysis (Wang et al., 1989). Similarly, our data showing the good correlation obtained in the three herb species between Chl loss and total reducing capacity (Table 1), which represents one of their oxidative defense systems, provide an additional support for this idea.

It is assumed that leaf senescence is a case of unbalanced turnover of metabolites, in which the catabolic processes prevail (Matile, 1993). These catabolic processes seem to be enhanced in detached leaves during dark-induced senescence. Initiation of senescence is supposed to occur by a cascade of deterioration processes, which also include the generation of oxygen free radicals and lipid peroxidation products (Leshem, 1988). We therefore suppose that the reducing compounds, which act as electron donors and scavengers of free radicals, may help to keep the anabolic processes in the cell and support the oxidative defense systems. Our results (Table 1) may imply a possible role for the endogenous antioxidants in defending the leaf against deteriorative senescence processes.

Apart from total reducing capacity, the other oxidative defense systems of the three herb species, such as α-tocopherol contents, antioxidative enzyme activities, and the induction time required for β-carotene oxidation, varied in an order unrelated to their Chl degradation rates (Table 1). Thus, parsley leaves, which showed the highest catalase and peroxidase activities, had the lowest induction time for β-carotene oxidation (Table 1), suggesting that each herb species has a specific defense mechanism against oxidation. Usually these defense systems predict the relative capacity of the plant to cope with oxidative stress; they may therefore determine its rate of Chl degradation. Hence, except for the β-carotene oxidation, all of the other oxidative defense systems of watercress leaves were very slow relative to those of the other leaf species (Table 1) and could, therefore, account for the fastest rate of Chl loss observed in these leaves (Figure 1). However, as shown for sage leaves, Chl breakdown, which is widely used as an index of leaf senescence, may not be an inevitable part of the aging process and may not be the only parameter to determine senescence rate. Thus, sage leaves, which showed the slowest Chl degradation rate (Figure 1) and the highest reducing capacity (Table 1), also showed the highest proteolysis rate (Figure 3). Thus, the high level of reducing compounds may protect sage leaves against rapid Chl loss but does not seem to affect their proteolysis rate. It may, therefore, be concluded that although the oxidative defense system of total reducing

activity in these three herb species correlates well with their relative rates of Chl breakdown, it may not necessarily be related to the other biochemical processes associated with their senescence process. These results indicate, as previously suggested by Thomas and Stoddart (1975), that different components of the senescence syndrome may be controlled by different genes and therefore may be susceptible to independent manipulation.

ABBREVIATIONS USED

ABA, abscisic acid; BA, benzyladenine; Chl, chlorophyll; FW, fresh weight; MDA, malondialdehyde; PUFA, polyunsaturated fatty acids; PuG, purpurogalin; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

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