Determination and Involvement of Aqueous Reducing Compounds in Oxidative Defense Systems of Various Senescing Leaves†

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A method for determining different types of reducing activity in aqueous leaf extracts of various edible herb species [watercress (*Rorippa nasturtium-aquaticum*), parsley (*Petroselinum crispum*), basil (*Ocimum basilicum*), and sage (*Salvia officinalis* L.)] was developed. This method, based on kinetics of the reduction of Fe(III) to Fe(II), was employed to study the correlation between leaf senescence rates under darkness and their relative reducing capacity in aqueous extracts, which represents an oxidative defense mechanism. Evidence is presented showing that the total reducing capacity is negatively correlated with the relative senescence rate of these four herb species and thereby provides a means to predict their storage potential. Also, the initiation of senescence processes was associated with a weakening of the oxidative defense systems. Exogenous application of active reducing agents (morin, ferulic acid, kaempferol, and glutathione) inhibited significantly chlorophyll degradation of detached parsley leaves to an extent related to their *in vitro* effectiveness of reducing activity. The results further emphasize the correlation between decreasing senescence rate and increasing reducing activity, thereby confirming the possible involvement of reducing compounds in oxidative defense systems of various edible senescing leaves.

Keywords: *Antioxidants; basil; leaves; Ocimum basilicum; oxidative defense; parsley; Petroselinum crispum; phenolic compounds; reductants; Rorippa nasturtium-aquaticum; sage; Salvia officinalis L.; senescence; watercress*

INTRODUCTION

Senescence appears to be correlated with increases in lipid peroxidation and membrane permeability (Strother, 1988). The involvement of free radicals in the process of leaf senescence has been well established (Leshem, 1988; Strother, 1988). It has been found that senescence-associated lipid peroxidation is induced by free radicals and singlet oxygen (Dhindsa et al., 1982). On the basis of accumulated evidence, it has been suggested that the life span of an organism may be connected with the peroxidative breakdown in lipid metabolism and thus, indirectly, with lipid turnover as well as with the cellular concentration of antioxidants. Hence, leaf senescence may be controlled to some extent by the relation between oxidative and antioxidative potentials in the cell (Dhindsa et al., 1982; Thomas, 1986; Leshem, 1988). The extent of age-related peroxidative damage in cells seems to be controlled by the potency of antioxidative systems (Kunert and Ederer, 1985).

The biologically important and most intensively investigated oxy-free radical species include the superoxide radical anion ($O^{\bullet -}_2$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($HO^{\bullet}$), singlet oxygen ($^1O_2$), and the organic free radicals ($RO^{\bullet}$, $ROO^{\bullet}$), all of which may participate directly or indirectly in lipid peroxidation (Elstner, 1987; Kanner et al., 1987; Leshem, 1988). The main PUFA of plant membranes are linoleic (18:2) and linolenic (18:3) acids, both of which have been found to be the target of oxidative attacks by oxygen radicals [see reviews of Elstner (1987) and Kanner et al. (1987)]. These metabolites are of great significance in the regulation of membrane structure, function, and turnover in the green tissue of higher plants (Thomas, 1986). Higher plants are equipped with means to combat free radicals, either by direct scavengers or through incipient preventive mechanisms against radical formation (Leshem, 1988). While superoxide ($O^{\bullet -}_2$) and H_2O_2 generated in higher plants can be scavenged enzymatically by superoxide dismutase (SOD), catalase, and peroxidases, no specific enzyme catalysis of hydroxyl radical ($HO^{\bullet}$) or singlet oxygen ($^1O_2$) detoxification has been shown. The detoxification of these reactive species more or less concerns the biosynthesis of scavengers and quenchers within the critical compartments (Elstner, 1987). Electron donor reducing compounds, such as ascorbic acid (AA), reduced glutathione (GSH), plant phenolics, and flavonoids, can act as $O^{\bullet -}_2$ and $HO^{\bullet}$ scavengers, as well as $^1O_2$ quenchers (Torel et al., 1986; Elstner, 1987; Husain et al., 1987; Larson, 1988; Puppo, 1992), and therefore could be involved in the oxidative defense mechanism of plant leaves. Flavonoids, which occur widely in plants and are particularly common in leaves and flowering tissues, have been reported to have antioxidant properties, and their inhibitory effects on nonenzymatic lipid peroxidation in various systems have been described (Husain et al., 1987; Puppo, 1992).

In the present work, we have developed further and modified a method for assessing the reducing capacity of various edible herb leaf extracts, based on the kinetics of the reduction of Fe(III) to Fe(II). This method has been employed before for measuring reductive iron release by plant ferritin (Boyer et al., 1988, 1990;

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Laulhere and Briat, 1993) or for determining the reducing capacity of muscle food (Kanner et al., 1991). Recently we have adopted this method for comparing various oxidative defense systems of various edible herb species (Philosoph-Hadas et al., 1994) and further developed it in the present study for determining different types of reducing activity in aqueous leaf extracts. By employing this method, studies were conducted of the correlation between leaf senescence rates of various herb species and their endogenous reducing capacities in aqueous extracts, which represent an oxidative defense mechanism. The changes in the different types of endogenous reducing activity were subsequently examined in the course of the rapid senescence of detached watercress leaves. Results obtained with the endogenous reducing capacities of the various herbs were further confirmed by examining the effects of exogenously applied active reducing agents on the rate of Chl degradation in detached parsley leaves. This herb was chosen as a model system that exhibits a moderate senescence rate.

MATERIALS AND METHODS

Materials. Ascorbic acid (AA) and trichloroacetic acid (TCA) were purchased from Merck (Darmstadt, Germany). NADPH, ammonium acetate, sodium acetate, Ferrozine, neocuproine, caffeic acid (CA), ferulic acid (FA), glutathione (GSH), kaempferol, and morin (MO) were from Sigma Chemical Co. (St. Louis, MO). $FeCl_3 \cdot 6H_2O$ was from Riedel-de Haen (Seelze, Holland) and Tween 20 from Aldrich (Milwaukee, WI). The Ferrozine reagent was composed of ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine], neocuproine, and concentrated HCl (Stookey, 1970).

Plant Material and Extraction of Reducing Compounds. Experiments were performed with mature, green, fully expanded leaves of watercress (*Rorippa nasturtium-aquaticum*), parsley (*Petroselinum crispum*), sage (*Salvia officinalis* L.), and basil (*Ocimum basilicum*) that had been freshly harvested from the field. Total reducing compounds were extracted from the various leaf tissues by homogenizing 1 g of leaves in 100 mL of 0.2 M acetate buffer, pH 3, to protect reductants from oxidation by PPO. After a 10-min centrifugation at 18000*g*, the amount of reductants in the supernatant was assayed by determination of its capacity to reduce Fe(III) to Fe(II).

Reduction Assay. The kinetic assay developed for measuring the activity of the reductants uses Ferrozine as a chromophoric, high-affinity chelator for the product Fe(II). The standard assay for Fe(II) release consisted of 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. The rate of Fe(III) reduction by leaf 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) and Philosoph-Hadas et al. (1994).

Determination of Chl. Chl was extracted from samples of cut leaf segments (0.5 g) of watercress or parsley 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 quantitated by determination of the absorbance at 645 and 663 nm, according to the procedure of Arnon (1949).

Application of Exogenous Reducing Agents. Parsley bunches were dipped for 10 min in either of the following solutions (containing 0.05% Tween 20): 1 mM MO, 0.1 mM KF, 1 mM FA, and 1 mM GSH. After dipping, the bunches were rinsed with distilled water (to remove the surfactant) and dried. The leaves were then detached from the bunches and placed in Petri dishes (six to eight leaves/dish) on filter papers (Whatman No. 1) moistened with 1 mL of distilled water containing chloramphenicol (50 μg/mL) to avoid contamination. The treated leaves incubated in the Petri dishes were allowed to senesce in darkness at 20 °C for 9–11 days, and their Chl content was assayed periodically.

RESULTS

Development of a Method for Determination of Reducing Capacity in Leaf Tissues. The total reducing potential of leaf extracts depends on the amount and activity of their reductants, which is determined by their capacity to reduce *in vitro* Fe(III) to Fe(II). The kinetic assay developed for measuring the activity of the reductants uses Ferrozine as a chromophoric, high-affinity chelator for the product Fe(II) (Stookey, 1970). This compound was chosen because it forms a stable, highly colored complex with Fe(II), and it has very low affinity for Fe(III) (Boyer et al., 1988).

The extraction procedure for reducing compounds was performed at pH 3 to protect the extractable reductants from oxidation by endogenous antioxidants and to inhibit activity of polyphenol oxidase (PPO) (Tremolieres and Bieth, 1984). However, extraction at pH 4.5 gave similar results (Philosoph-Hadas et al., 1994), suggesting that PPO was already inactivated and did not affect the extracted reductants. The validity of the method was further confirmed by recovery experiments of two defined reductants, with known quantities of AA (3–10 mg) and GSH (30–100 mg) added to parsley leaf extracts. A 97–100% recovery was obtained in these experiments for both the rapid and prolonged reduction reactions. The rate of Fe(III) reduction by the leaf reductants was quantified by spectrophotometric measurements of the $Fe(Ferrozine)^{2+}$ complex, which absorbs maximally at 562 nm. The reduction rate depends on reductant concentration but is independent of Ferrozine concentration in the range 0.2–1.5 mM or of Fe(III) concentration in the range 1–10 mM (data not shown). This indicates that neither Ferrozine nor Fe(III) is involved in the rate-limiting step (Boyer et al., 1988).

Detailed kinetics of the reducing activity of the various leaf extracts tested (Figure 1) exhibited three characteristic phases: (1) a very rapid increase that lasted for 2–10 min (Figure 1A), with 85% of the reduction being obtained within the initial 2 min (watercress, parsley, sage, and basil leaves); (2) a linear increase of reduction rate which lasted for 0.5–6 h (in watercress and parsley leaves) or for 1–5 h (in sage and basil leaves) (Figure 1B); (3) a gradual decrease of reduction rate, which was monitored for up to 26 h (watercress, parsley, sage, and basil leaves) (Figure 1B) and continued for up to 72 h (data not shown). Additionally, it was found that the rate during the second reduction phase was positively correlated with the rate obtained in the initial rapid phase (data not shown). Therefore, to compare the reducing capacities of various leaf extracts, the reaction was performed with the appropriate dilutions of the extracts, to obtain similar initial reducing activities (0.4 OD at 562 nm) during the first 10 min (Figure 1A). The reduction rate during this period increased linearly with increasing extract concentration (data not shown). Results depicted in Figure 1 show that to get an equal initial reduction rate (0.4 OD), the sage leaf extract had to be diluted 5-fold as compared with the watercress leaf extract. These results suggest that sage leaves exhibited the highest reducing activity among the four herb species tested. Hence, the relative order of reducing capacity of the various leaf extracts was sage > basil > parsley > watercress.

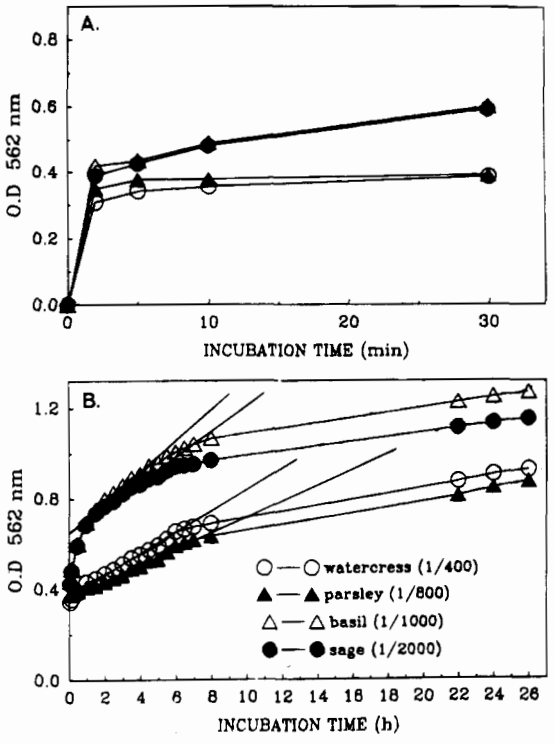

Figure 1. Kinetics of changes in total reducing capacity of fresh watercress, parsley, basil, and sage leaf extracts, during incubation of 30 min (A) and 26 h (B) with Fe(III). Numbers in parentheses represent the dilution ratios of the various leaf extracts required to obtain an initial (10 min at the rapid phase) OD value of 0.4 unit at 562 nm. Data represent means of four replicates with SD smaller than 2%. The initial point in graph B was measured after incubation of 5 min.

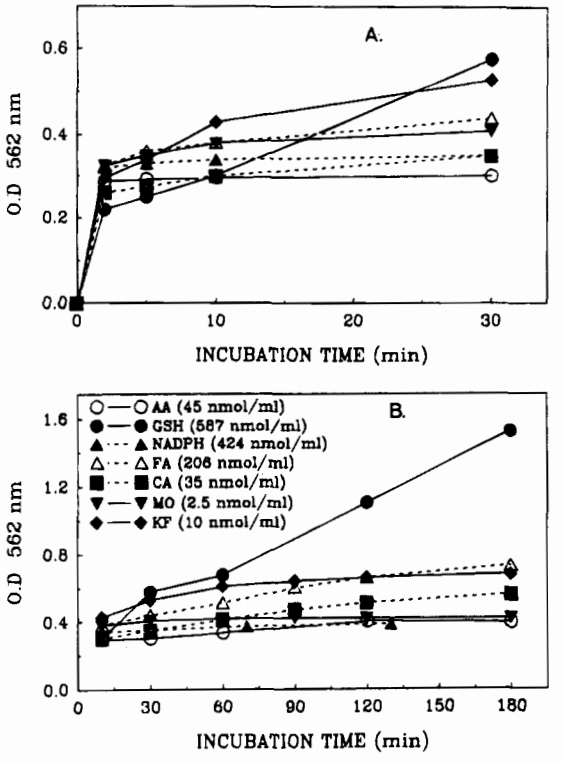

Figure 2. Time course of the Fe(III) reducing capacity of various reductants during incubation of 30 (A) and 180 min (B) with Fe(III). The numbers beside the different reducing compounds indicate the concentrations required to give an absorbance of 0.4 OD during the initial 10 min. Data represent means of three replicates with SD smaller than 2%.

Several water-soluble plant reducing agents, such as AA, GSH, NADPH, and phenolic compounds, which could potentially simulate these three phases of reducing activity in leaf extracts, were tested in the Fe(III) reduction assay. The various reducing compounds were assayed at different concentrations which gave similar initial rates (in the range of 0.28–0.4 OD) (Figure 2A), and their reducing kinetics was evaluated during the first 30 (Figure 2A) and 180 min of reaction (Figure 2B). The reductants assayed could be divided, according to their reducing behavior, into two groups: The first group, which includes AA, NADPH, and MO, exhibited mainly a fast reducing reaction (terminated within 10 min) with a concentration-dependent rate (Figure 2A). The second group, which includes GSH, FA, CA, and KF, exhibited, in addition to the fast phase, a prolonged, linear reducing reaction (0.5–4 h), with a rate that was both time- and concentration-dependent (Figure 2B).

The standard curves of the fast reducing reactions (10 min) of the seven reducing agents tested were plotted (Figure 3) to calibrate their relative reactivities. The reducing rate of FA is illustrated as a function of both the low-range (Figure 3A) and the high-range (Figure 3B) concentrations, for comparison of the two scales (0–100 and 0–1000 nmol/mL) with the same reference compound. Results show that the reducing activity increased linearly with increasing concentrations in the assay, and the relative order of reductant reactivity was MO > KF > CA > AA > FA > NADPH > GSH. This relative order confirms the relative order of increasing concentrations of these agents, which was required to obtain an initial reducing activity of OD = 0.4 at 562 nm (Figure 2A). Thus, a concentration of 2.5 nmol/mL of the most reactive compound (MO) and a concentration

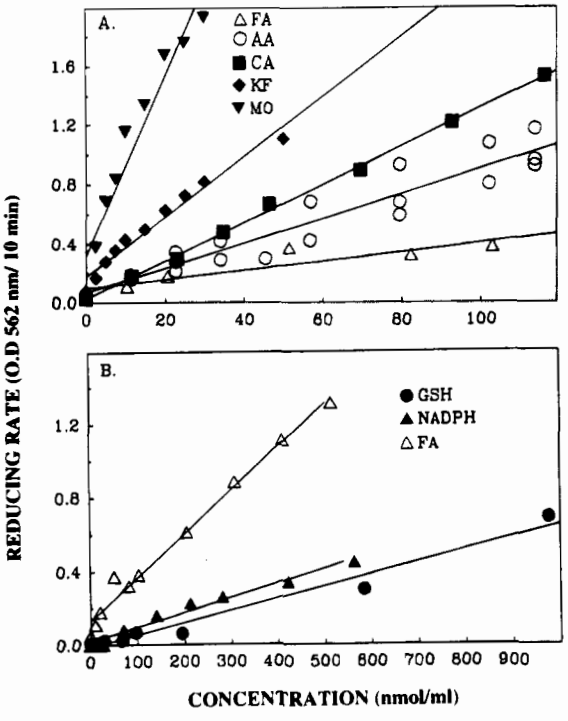

Figure 3. Standard curves of Fe(III) reduction rates by various reductants during the initial 10 min, as a function of their low-range (A) or high-range (B) concentrations. Data represent means of three replicates with SD smaller than 2%.

of 587 nmol/mL of the least reactive compound (GSH) were required to obtain the same reducing capacity (Figure 2A).

The rates of the prolonged reducing reactions (0.5–3 h), represented by the slopes, were also positively

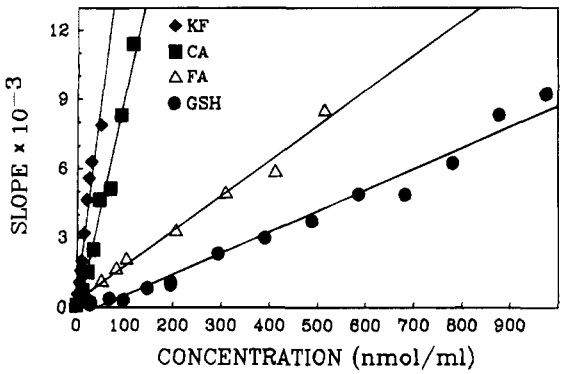

Figure 4. Standard curves of Fe(III) reduction rates by various reductants during 30–180 min as a function of their concentrations in the assay. The reaction rate is represented by the slope of the curves, calculated as the ratio of ΔOD at 562 nm and Δtime (150 min). Data represent means of three replicates with SD smaller than 2%.

correlated with the concentrations of the reducing agents of the second group (Figure 4). Since the rate in this type of reducing reaction was both time- and concentration-dependent, it was represented by the slopes of the curves, calculated as the ratio of ΔOD (at 562 nm)/Δtime (150 min) (data not shown). The relative order of reductant reactivity in this group was KF > CA > FA > GSH (Figure 4).

On the basis of these results, which show a significant difference between the reducing reactions of AA and GSH (Figure 2), we have developed a method for determining the three types of reducing activity in the leaf extracts: (1) AA equivalent reducing activity (mg of AA equiv/g of FW), which represents the fraction of the fast reductants and which was determined after a 10-min incubation of the extract with Fe(III). AA equivalents were estimated from a standard curve of AA concentrations (Figure 3A). (2) GSH equivalent reducing capacity (mg of GSH equiv/g of FW), which represents the fraction of the slow reductants and which was determined, at 30-min intervals, during the linear phase of a 4-h incubation of the extract with Fe(III). GSH equivalents were estimated from a standard curve of GSH slopes (Figure 4). (3) Total reducing capacity (units/g of FW), which represents the combination of both the fast and slow types of reductants. The total reducing capacity was determined after a 24-h incubation of the extract with Fe(III) (see Figure 1B). A unit was defined as the reducing capacity of the tissue that yielded an absorbance of 1 OD at 562 nm (Figure 1). It should be emphasized that the terms of AA or GSH equivalents are assigned to express in general the two types of reducing activity, rather than indicating the endogenous levels of these two specific reductants. Therefore, these terms represent similar activities of other reducing compounds extracted in the aqueous phase and exhibiting AA- or GSH-like reducing kinetics.

Correlation between Leaf Senescence Rate and Reducing Potential for Various Herb Species. The method described above was employed for conducting correlation studies between the leaf senescence rates of various herb species (watercress, parsley, basil, and sage) and their reducing potential. The total reducing capacity of leaf extracts depends on the amount and activity of their reductants, which is determined by measuring their capacity to reduce Fe(III) to Fe(II) (Figure 1). The senescence rates of the various species were determined according to the time required to degrade 50% of their initial Chl content (Table 1). These two parameters are inversely related, when the highest number of days to obtain 50% Chl represents the slowest senescence rate. The decreasing relative order of leaf senescence rate, based on this parameter, was watercress > parsley > basil > sage (Table 1). This order also represents the increasing relative order of reducing capacity, since the results of Table 1 show that sage leaves, which senesced at the slowest rate, exhibited the highest reducing activity. Thus, the AA equivalent type activity of sage leaves was 3-fold higher, the GSH equivalent type activity was 5-fold higher, and the total reducing capacity was 6-fold higher than the corresponding reducing activities of watercress leaves. Parsley leaves, which senesce at a moderate rate, showed only a 2-fold increase in all types of reducing activities as compared with watercress leaves, whereas basil leaves, which senesce at a relatively slow rate, showed a 3-fold increase in their reducing capacity (Table 1). These results suggest that the total reducing capacity is negatively correlated with the relative senescence rate for these four herb species.

For further examination of this possible correlation, the changes in the three types of reducing activity in senescing detached watercress leaves were monitored during 4 days of dark senescence. This herb was chosen for this study to relate its rapid senescence to the possible decrease in its reducing capacity (Table 1). The senescing watercress leaves exhibited a moderate Chl loss during the first day, followed by a phase of rapid Chl degradation during the next 3 days (Figure 5A). Thus, the leaves lost about 70% of their initial Chl content within 4 days. A sharp decline of about 25% in the reducing activity of type 1 compounds (AA equivalents) was observed during the first 10 h of incubation, followed by a gradual moderate increase later on (Figure 5B). Consequently, this type of reducing compound reached, at the end of incubation, a level lower by 11% from that of fresh leaves (Figure 5B). The reducing activity of type 2 compounds (GSH equivalents) was characterized by a general trend of moderate decrease during the whole incubation period. Total reducing capacity of the leaves decreased sharply by 26% during the first 10 h of incubation and then leveled off (Figure 5B).

Similar results were obtained when Chl and reducing compounds were extracted from frozen and lyophilized watercress leaves rather than from fresh ones. In this experiment, the decrease in the reducing activity of type 1 compounds (AA equivalents) during senescence was similar to the pattern described in Figure 5B, and the reducing activity of type 2 compounds (GSH equivalents) decreased by 40% during the initial 10 h of senescence and then leveled off (data not shown). The results of these two experiments, performed with fresh and lyophilized leaves, indicate that a significant decrease in the total reducing capacity, which occurs at an early stage of leaf senescence, precedes the rapid phase of Chl degradation.

Effect of Application of Exogenous Reducing Agents on Chl Degradation. The negative correlation obtained between reducing capacity of leaf extracts and their relative senescence rates (Table 1) and the significant decrease in reducing activity which preceded Chl degradation (Figure 5) suggest that a high reducing capacity of detached leaves at harvest retarded their Chl degradation during dark senescence. To confirm this suggestion further, the effect of exogenously applied reductants, which would be expected to enrich the tissue

Table 1. Reducing Potential of Leaves of Various Herb Species, As Related to Their Senescence Rates[a]

herb species	reducing activity of extracts: type 1 (mmol of AA equiv/g of FW)	reducing activity of extracts: type 2 (mmol of GSH equiv/g of FW)	total reducing capacity (units/g of FW)	Chl degradation rates (days to 50% Chl loss)
watercress	16.43 ± 0.98 (100%)	246 ± 12 (100%)	363 ± 14 (100%)	1.5–2
parsley	36.11 ± 2.41 (220%)	307 ± 15 (124%)	679 ± 64 (187%)	5–6
basil	51.71 ± 2.35 (317%)	746 ± 23 (303%)	1250 ± 26 (344%)	8–9
sage	57.84 ± 4.75 (352%)	1229 ± 80 (499%)	2270 ± 58 (625%)	>11

[a] Experiments were performed with fresh leaves of watercress, parsley, basil, and sage. The three types of reducing activity in the various leaf extracts were determined with the appropriate dilutions which gave an initial OD value of 0.4 unit at 562 nm after 10 min of reaction. The rate of Chl degradation was estimated as the time required to obtain 50% of the initial Chl content in detached leaves kept in darkness at 20 °C. Data represent the means of three to six replicates ± SD, and numbers in parentheses represent activities as percentage of the activities of watercress leaves.

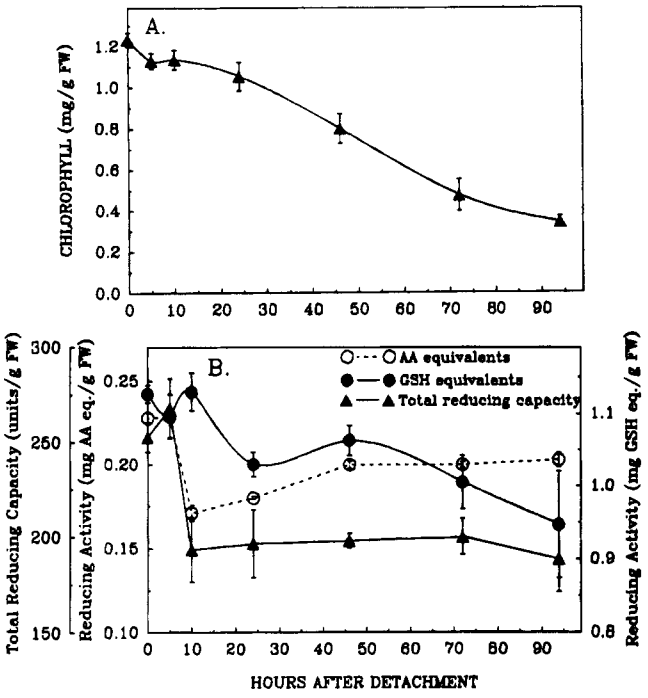

Figure 5. Time course of changes in Chl content (A) and in the three types of reducing activities (B) during dark senescence of detached watercress leaves incubated at 20 °C. The bars represent SD of four replicates.

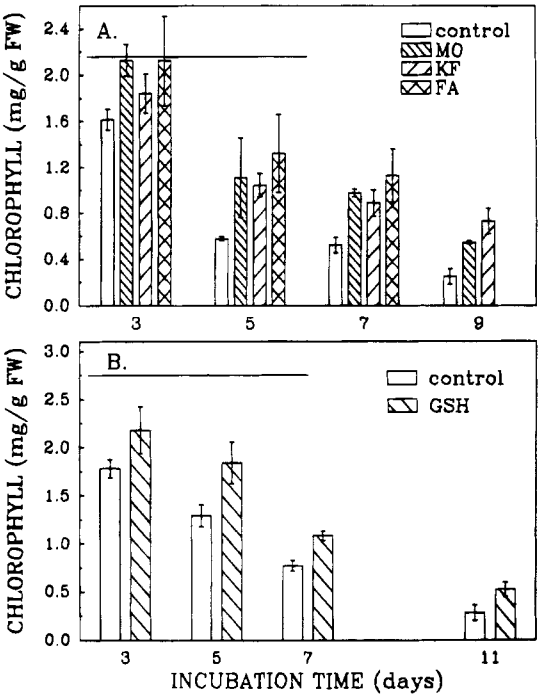

Figure 6. Effect of various antioxidants (A) and GSH (B) on Chl content of detached parsley leaves during dark senescence at 20 °C. Leaves were dipped for 10 min in 1 mM solutions of either MO, GSH, or FA or in 0.1 mM KF, rinsed with distilled water, and placed on moistened filter paper in Petri dishes for 9–11 days of dark incubation. Chl content of the treated leaves was determined at the indicated periods. The horizontal lines in the graphs represent the initial level of Chl immediately after detachment. The bars represent SD of four replicates.

with reducing compounds, on the rate of Chl degradation in parsley leaves was examined. Parsley was chosen for this assay since it exhibits a moderate senescence rate (Table 1) that can be significantly manipulated in the presence of senescence retardants or stimulators (Philosoph-Hadas et al., 1989). Dipping parsley bunches in 1 mM AA or CA did not affect the rate of their Chl degradation (data not shown). However, dipping the bunches in 1 mM MO, FA (Figure 6A) or GSH (Figure 6B), or in 0.1 mM KF (Figure 6A) resulted in significant inhibition of Chl degradation during 9 days of dark senescence. Hence, the reductant-treated leaves remained green after 7 days of incubation and had a Chl level of 0.9 mg/g of FW, whereas control leaves turned yellow and retained 50% lower Chl levels (Figure 6). Dipping of parsley leaves in the various reductants was assayed for several periods of time (1, 5, 10, and 30 min). The senescence-retarding effect of the reductants increased with dipping duration up to 10 min, but a 30-min dipping period did not improve their retarding effect (data not shown). Although the relative absorption of each compound was not measured directly, dipping for 10 min was chosen as the optimal period in which most of the compounds were readily absorbed. It should be noted that although applied in a concentration 10 times lower than the other agents, KF was the most active compound and showed the highest capacity for retaining high Chl levels after at least 7 days of incubation (Figure 6A). This high capacity of KF to retard Chl degradation could be attributed to the high reducing activity it exhibited in both the rapid (Figure 3A) and the slow (Figure 4) phases of the reducing reaction.

DISCUSSION

The extent of senescence-related peroxidative damage is controlled by the potency of antioxidative systems in the tissue. Various secondary compounds of higher plants, either water- or lipid-soluble, have been demonstrated in *in vitro* experiments to protect the tissue against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species (Larson, 1988). Current ideas suggest the possible involvement of naturally occurring reductants in oxidative defense mechanisms of plants. Hence, some of the water-soluble antioxidants such as, AA, GSH, phenolic acids, and

flavonoids, which are considered to be electron-donor reductants, play a major role in driving biochemical reactions. This includes also iron redox cycle-dependent lipid peroxidation. Among these compounds, a special emphasis was given to the role of AA and GSH and their related enzymes (such as ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase) in leaf senescence (Foyer and Halliwell, 1976; Dhindsa et al., 1982; Pauls and Thompson, 1984; Kunert and Ederer, 1985; Strother, 1988). Additionally, phenolic acids and flavonoids, acting as strong scavengers of $O^{\bullet-}_2$, $^1O_2$, and $HO^{\bullet}$ (Torel et al., 1986), may play an important role in protecting the leaves against senescence-associated oxidative processes. Therefore, the total reducing capacity in the leaf tissue may serve as a significant indicator of its potential antioxidants, the activity of which is related to their ability for electron transfer.

The stable oxidation states of iron in aqueous systems are exclusively the ferric Fe(III) and ferrous Fe(II) forms (Kanner et al., 1987). The reducing capacity was studied *in vitro* by using Ferrozine, which is a high-affinity Fe(II) ligand that does not exist naturally in plants. Because no absorbance increase at 540 nm was observed, the ferrous–Ferrozine complex must be more stable than the ferrous complex with the reductants (Boyer et al., 1990). According to the reaction conditions, excess Fe(III) and Ferrozine favored the formation of the stable Fe(II)–ferrozine complex, thereby eliminating any free Fe(II) in equilibrium. This situation excludes the possibility of Fe(II) oxidizing reactions that could compete with Ferrozine for Fe(II). Consequently, the time-dependent absorbance increase at 562 nm, depicted in Figures 1–4, was directly related to the reducing capacity of the reductants. The prolonged time frame for reduction *in vitro* (Figures 1B and 2B), which resembles kinetics of reversible reactions, may reflect formation over time of incubation of regenerated reducing compounds that can further reduce Fe(III), until it is depleted from the reaction mixture.

The method developed in this study for estimation of the reducing capacity of leaf extracts seems appropriate for determining the potential of the water-soluble reducing activity of various leaves, using either fresh or dry tissues. The kinetics of the reducing activity of the various reducing agents tested *in vitro* was similar to the kinetics of reducing activities exhibited by leaf extracts of various herb species, showing a very rapid increasing phase that lasted for 2–10 min (Figure 1A) and a second phase of a continuous slower reaction that lasted for several hours (Figure 1B). When the kinetics of Fe(III) reduction by various reducing agents was examined, several compounds showed only the rapid reducing phase, whereas others exhibited both types of reducing reactions (Figure 2). It seems, therefore, that all reducing agents present in the leaf extract participate in the rapid reducing phase but only part of the reductants participate in the slow and continuous phase. The reducing activity of reductants of both types was found to be concentration-dependent (Figures 3 and 4). On the basis of these results, the reducing activity of leaf extracts could be quantified as equivalents of AA (mg of AA equiv/g of FW) that represent the fraction of the fast reductants (type 1) or as GSH equivalents (mg of GSH equiv/g of FW) that represent the fraction of the slow reductants (type 2) (Table 1). The literature suggests that the reducing activity of leaf extracts arises largely from AA and GSH (Foyer and Halliwell, 1976; Pauls and Thompson, 1984). Analysis of watercress leaf extracts showed that the combined endogenous content of both reducing agents, AA and GSH, was less than 10% of the corresponding equivalents of reducing activity (data not shown). This indicates that besides AA and GSH, the leaf extract contains many additional reducing agents, which together account for most of the reducing potential of the tissue. Therefore, the reduction kinetics demonstrated here do not measure the titer of the reducing compounds but rather their reducing capacity (expressed in equivalents) under *in vitro* conditions.

The phenolic acids and flavonoids tested occur widely in plants and are common in leaves (Larson, 1988; Le Tutour and Guedon, 1992; Markham et al., 1992; Okamura et al., 1993). The strong antioxidative activity of the flavonoids, MO and KF, as singlet oxygen quenchers and as superoxide and hydroxyl radical scavengers, compared with other phenols and flavonoids, has been demonstrated previously in various systems (Torel et al., 1986; Husain et al., 1987; Puppo, 1992). Similarly, also in reducing Fe(III) to Fe(II), these agents showed the highest activity: MO and KF in the rapid phase of the reducing reaction (Figure 3A) and KF in the slow reducing phase (Figure 4). It should be noted that the importance of flavonoids, acting as $HO^{\bullet}$ scavengers, may be much more pronounced in aging cells, where a decline in the reducing power can occur. This is due to reducing agents present in normal cells, which precludes the activity of flavonoids in redox cycling the iron, thus preventing any stimulation of $HO^{\bullet}$ formation (Puppo, 1992).

The physiological significance of our findings implies that, in addition to other defense systems, several reducing agents present in plants are able to protect leaf tissues against free radical formation, thereby retarding their senescence. This conclusion is based on the following observations: (a) the negative correlation found between the senescence rate of various herb species and their reducing capacity (Table 1); (b) the significant decrease in reducing capacity of the tissue, detected prior to the rapid phase of Chl degradation (Figure 5); and (c) the retardation of leaf senescence obtained by exogenous application of various reducing agents, which enhanced the reducing capacity of the leaves (Figure 6). Recently we have reported that the total reducing capacity is negatively correlated with the relative senescence rate of three herb species, and of five oxidative defense systems examined, only the system of total reducing capacity correlated well with their relative order of Chl degradation (Philosoph-Hadas et al., 1994). Additionally, a positive correlation was obtained in watercress and sage leaves between total reducing capacity and their antioxidant capacity for lipid peroxidation, measured as the induction time required for β-carotene oxidation (Philosoph-Hadas et al., 1994). These results, together with the findings of the present study, further emphasize the importance of determining the reducing capacity of edible leaves as a means to predict their short-term storage quality and potential. Similarly, an age-dependent decline in the concentration ratio of vitamin C (AA) and vitamin E (α-tocopherol), which determines the potency of the antioxidative system consisting of both vitamins, was found in beech leaves and fir needles (Kunert and Ederer, 1985). This further implies the importance of both water- (AA) and lipid-(α-tocopherol) soluble reductants in antioxidative defense.

Cells normally possess balances in the form of enzyme scavengers and antioxidants to control the titer of free radicals, but any relaxation of these defense mechanisms could initiate tissue degradation. In watercress leaves, which exhibited the fastest senescence rate (Table 1), it would appear that one consequence of this relaxation is the rapid decline in their total reducing capacity, observed just 10 h following detachment (Figure 5B). Hence, the parameters developed in this study for estimating the total reducing capacity of the tissue may be used for both assessment of the senescence rate and estimating the tissue resistance to various stresses associated with intensified oxidative processes. Additional work is required to characterize the various reducing compounds in the different tissues, their compartmentalization in the cell, and the variations of their contents in the course of senescence.

ABBREVIATIONS USED

AA, ascorbic acid; CA, caffeic acid; Chl, chlorophyll; FA, ferulic acid; GSH, glutathione; KF, kaempferol; MO, morin; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); PUFA, polyunsaturated fatty acids.

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