Correlations among Attributes of Senescence and Antioxidative Status of Leaf Discs during Epiphyllous Bud Differentiation in *Kalanchoe pinnata* Lam. (Pers.)

Sarita Jaiswal[a,b,*], Raman Chawla[c], and Sudhir Sawhney[a]

[a] Department of Botany, University of Delhi, Delhi - 110 007, India
[b] Department of Plant Sciences, 51 Campus Drive, College of Agriculture and Bioresources University of Saskatchewan, Saskatoon, Saskatchewan - S7N 5A8, Canada. E-mail: sarita782000@gmail.com
[c] Division of CBRN Defence, Institute of Nuclear Medicine and Allied Sciences, Brig SK Mazumdar Marg, Delhi - 110 054, India

* Author for correspondence and reprint requests

Z. Naturforsch. **67 c**, 418–428 (2012); received September 3, 2011/March 21, 2012

Leaf detachment is a common signal that triggers both the differentiation of dormant epiphyllous buds as well as the onset of foliar senescence in *Kalanchoe pinnata* Lam. (Pers.). The present study looked for any probable correlations among selected attributes of foliar senescence, *e.g.* soluble proteins, chlorophylls a and b (Chl_{a+b}), and membrane stability index (MSI), and the antioxidative status, *e.g.* phenolics, ferric reducing ability in plasma equivalence ($FRAP_{eq}$), and membrane protection index (MPI), during epiphyllous bud differentiation. The experimental system comprised 0.75-cm leaf discs, with or without a dormant epiphyllous bud, cultured *in vitro* and exposed for ten days to continuous light or dark. A steady depletion of soluble proteins and Chl_{a+b}, and lowering of MSI in the leaf discs were observed, the decline being relatively faster and of higher magnitude in discs exposed to dark rather than to light. The pigment loss in discs with differentiating epiphyllous buds was greater and faster than in those lacking buds, a somewhat reverse situation was observed in case of soluble proteins. Simultaneously, a time-dependent decrease in the level of phenolics was also observed. Their content was found to be lower in discs exposed to dark as compared to light, pointing to a relationship with a higher rate of senescence-related degradative processes in the dark. The change in the content of Chl_{a+b} was found to be significantly correlated with the variation in the level of phenolics. The average $FRAP_{eq}$ after ten days was one half that of the initial level, which could be correlated with the decreasing levels of phenolics (intra-correlation) and maximally correlated with variations in Chl_{a+b} and protein contents (inter-correlation). Aqueous alcohol foliar extracts significantly ($p < 0.05$) protected membranes against peroxidative stress, although the pattern was not found to be in line with that of the phenolics content or $FRAP_{eq}$. The diminishing Chl_{a+b} content was found to be maximally correlated with alterations in the membrane protection.

Key words: Epiphyllous Bud Differentiation, Leaf Senescence, Antioxidant Potential

Introduction

Epiphylly is one of the survival-cum-proliferation strategies that is adopted by the crassulacean succulents and especially so in the genus *Kalanchoe* exhibiting regeneration in response to damage. The epiphyllous bud primordia, also called foliar embryos, are lodged in the notches along the leaf margins that exhibit at least two different developmental patterns in a species-specific manner. In the monophasic types, bud differentiation is a continuous process from primordium initiation to plantlet formation, *e.g.* in *Kalanchoe daigremontiana.* In case of the biphasic types, the first phase extends from initiation to the *in situ* development of morphologically mature but physiologically dormant buds, with the second phase leading to plantlet formation, triggered normally by leaf detachment, *e.g.* in *Kalanchoe pinnata* (Jaiswal and Sawhney, 2006). Leaf detachment in the latter case not only acts as a trigger for epiphyllous bud dif-

Abbreviations: Chl_{a+b}, chlorophylls a and b content; $FRAP_{eq}$, ferric reducing ability in plasma equivalence; MSI, membrane stability index; MPI, membrane protection index; TEM, transmission electron microscopy; v/v, volume/volume.

ferentiation but also accelerates foliar senescence, which appear to be correlative developmental phenomena (Jaiswal and Sawhney, 2006). The two developmental responses appear to be linked in the manner of a source-sink duo, especially during the early establishment phase of epiphyllous plantlets (Jaiswal and Sawhney, 2006). In any case, senescence is characterized by a programmed recycling of nutrients (Hörtensteiner and Feller, 2001; Lim *et al.*, 2007; Gregerson *et al.*, 2008; Guiboileau *et al.*, 2010).

Leaf senescence in general is characterized by diminishing photosynthesis accompanied by disintegration of organelle structures, degradative loss of chlorophyll and proteins, disruption of the microtubular network, and a dramatic increase in lipid peroxidation as well as membrane leakiness (Dhindsa *et al.*, 1981; Thompson *et al.*, 1987; Leshem, 1988; Halliwell and Gutteridge, 2006; Schelbert *et al.*, 2009; Keech *et al.*, 2010). Generally, the first visible sign of senescence is the onset of chloroplast degradation (Thomas and Stoddart, 1980; Smart, 1994), concurrent with a decrease of the chlorophyll content, while the catabolic products are transported to the vacuole. It also acts as a recycler of nutrients from senescing cells to young leaves (Hörtensteiner and Feller, 2001; Lim *et al.*, 2007). *In vitro* experimentation on epiphyllous bud differentiation has been shown to create conditions similar to senescence in the subtending mother leaf disc that nourishes the growing plantlet (Jaiswal and Sawhney, 2006). Removal of the bud from the mother disc enhances the life span of the disc by several months (unpublished data), which could be related to a strong enzymatic and non-enzymatic antioxidative status prevailing in its tissues. During foliar senescence, the load of oxidative stress increases due to the formation and accumulation of different reactive oxygen species (ROS) as well as to the weakening of the antioxidant protection. In addition, the integrity of cellular membranes and proteins also contributes towards delaying senescence (Cheour *et al.*, 1992). *Kalanchoe pinnata* has been identified as a rare plant with potential anticancer and insecticidal activity (Joseph *et al.*, 2011). In light of the above observations, it was considered of interest to analyse selected attributes of foliar senescence [soluble protein content, chlorophylls a and b (Chl_{a+b}) content, and membrane stability index (MSI)] and antioxidant status [total phenolics content, ferric reducing ability in plasma equivalence ($FRAP_{eq}$), and membrane protection index (MPI)] and evaluate their probable correlations in the leaf discs during epiphyllous bud differentiation in *K. pinnata.*

Material and Methods

Preparation of leaf discs and aqueous alcoholic extract

Leaf discs of 0.75 cm in diameter were made from freshly harvested $3^{rd}/4^{th}$ pairs of leaves of garden-grown vegetative plants of *Kalanchoe pinnata* (Lam.) Pers. Harvested leaves were surface-sterilized with ethanol. The crenate leaves of this species possess dormant epiphyllous buds all along their margins. Two types of discs were made. Each marginal leaf disc had a bud harboured in the notch flanked by crenations whereas the sub-marginal discs were budless. The discs were inoculated abaxially in Petri plates, containing 0.8% agar under sterile conditions (Fig. 1), and cultured in a growth chamber maintained at (25 ± 2) °C. On days 0, 2, 4, 6, 8, and 10 from the start of the experiment, 30 discs were placed on the medium for each variable, *i.e*. budded or budless, in continuous light or in the dark. All leaf discs were concurrently harvested on the 10^{th} day, for a uniform comparative analysis. The leaf discs of each treatment were pooled into 3 batches and processed separately to determine the status of foliar senescence by quantifying the soluble protein and Chl_{a+b} content, and MSI.

For preparation of the extract, 30 leaf discs (0.75 cm in diameter) were homogenized at room temperature (25 °C) in 5 ml of 50% ethanol (v/v) using a pestle and mortar. The crushed fine slurry

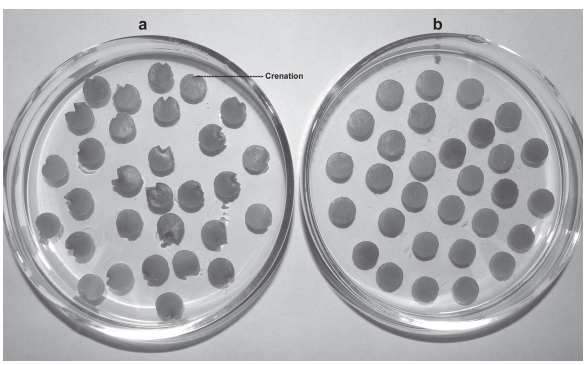

Fig. 1. Experimental set up showing (a) budded and (b) budless discs cultured on 0.8% agar medium.

was filtered through a 0.2-μm Millipore filter immediately prior to use. This parent extract is referred to as aqueous alcoholic extract. For various *in vitro* studies, PBS (phosphate-buffered saline) was used to dilute the parent extract. The antioxidative status was evaluated by analysing the total phenolics content, $FRAP_{eq}$, and MPI.

Evaluation of early senescence characteristics

Protein extraction and quantitation

Leaf discs dipped in liquid nitrogen were homogenized in chilled extraction buffer, Tris-HCl (50 mM, pH 8.5), containing EDTA (1 mM), phenylmethylsulfonyl fluoride (1 mM), and polyethylene glycol 20,000 (2% w/v). The protein quantitation was done according to Bradford (1976).

Chlorophyll content (Chl_{a+b})

Chlorophyll was extracted using dimethyl sulfoxide, and its content was calculated by the formula: total Chl_{a+b} ($\mu g\ ml^{-1}$) $= 6.45 \cdot A_{663} + 17.72 \cdot A_{645}$, where A_{663} and A_{645} represent absorbance at 663 and 645 nm (Hiscox and Israelstam 1979). Total content was expressed on dry weight (DW) basis.

Membrane stability index (MSI)

The MSI was determined by recording the electrical conductivity of leaf disc leachates in double distilled water following the method of Premchandra *et al.* (1990), as modified by Sairam *et al.* (2002). The membrane stability was calculated using the formula: $MSI = [1 - (C1/C2)] \cdot 100$, where $C1$ and $C2$ represent electrical conductivity at 25 °C after exposing leaf discs for 30 min at 40 °C and 15 min at 100 °C (causing release of all electrolytes), respectively.

Evaluation of antioxidant properties

Phenolics content

The total phenolics content in aqueous alcoholic extracts was estimated by the method of Singleton and Rossi (1965), with quercetin as standard.

Ferric reducing ability in plasma equivalence ($FRAP_{eq}$)

The kinetic analysis of $FRAP_{eq}$ proposed by Benzie and Strain (1996) and modified by Firuzi *et al.* (2005) as a FRAP microassay was used, with rutin as standard. FRAP solution was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), $FeCl_3 \cdot 6\ H_2O$ (20 mM) and 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ 10 ml dissolved in 40 mM HCl) in a 10:1:1 ratio. Twenty five μl of extract at suitable dilution (1:25 v/v as determined experimentally from the parent extract, *i.e.*, 5 ml extract of 30 leaf discs in 50% ethanol) were mixed with 175 μl of FRAP solution (warmed at 37 °C), and absorbance (A) was recorded at 595 nm using a microplate reader. Observations were recorded at time intervals for up to 150 min. $FRAP_{eq}$ was calculated as: $FRAP_{eq\ (time\ =\ 300\ s)} = \Delta A_{595\ (time\ =\ 300\ s)} \cdot$ standard constant factor$_{(rutin\ 1\ -\ 25\ \mu g\ ml^{-1})}$.

Membrane protection index (MPI)

The MPI of the leaf discs extract was determined using artificial soy lecithin/cholesterol membranes prepared by the method of New *et al.* (1990), and Fenton reagents were used to induce peroxidative stress (Caillet *et al.*, 2007). Liposomal membranes were prepared using an 1:1 molar ratio of lecithin/cholesterol. Then they were subjected to sodium tungstic acid negative staining and observed under a transmission electron microscope for their uniform size distribution. The reaction mixture containing 0.1 ml of parent extract (in appropriate dilution, *i.e.*, 1:10, 1:25, 1:50 diluted with PBS), 0.5 ml liposomal dispersion, and 0.4 ml Fenton reagent was placed at 37 °C for 30 min. The peroxidative stress was evaluated using thiobarbituric acid (TBA), and the chromogen was extracted into *n*-butanol/pyridine (15:1 v/v). The MPI of the extracts was compared on the basis of percent inhibition of degeneration (decrease in absorbance) using the formula: percent membrane protection = [control – test/control] · 100, where control is absorbance of the reaction mixture without any extract while test refers to absorbance of samples containing varied dilutions of extract.

Intra-/inter-correlations and statistical analysis

An analysis of correlations among different parameters of either senescence or antioxidative status (intra-correlations; Tables I, II) as well as among those for both of them (inter-correlations; Tables III–V) was made using Pearson's correlation coefficient estimation.

The experiment was repeated three times under identical conditions and results were expressed as mean ± SE. One-way ANOVA analysis followed by Dunnet's t-test was employed to analyse the variation with respect to different parameters.

Table I. Intra-correlation among senescence attributes (MSI, soluble proteins, Chl_{a+b}) of leaf discs (budded, budless) exposed to continuous light (L) and dark (D) conditions.

Parameter	Budded (L) MSI	Budless (L) MSI	Budded (D) MSI	Budless (D) MSI
Budded (L) protein	0.994*	0.856*	0.692	0.701
Budless (L) protein	0.956*	0.926*	0.781	0.846*
Budded (D) protein	0.807	0.977*	0.826*	0.949*
Budless (D) protein	0.804	0.958*	0.858*	0.970*
Budded (L) Chl_{a+b}	0.899*	0.937*	0.81	0.784
Budless (L) Chl_{a+b}	0.845*	0.872*	0.568	0.558
Budded (D) Chl_{a+b}	0.924*	0.894*	0.781	0.733
Budless (D) Chl_{a+b}	0.974*	0.887*	0.739	0.710
Parameter	Budded (L) Chl_{a+b}	Budless (L) Chl_{a+b}	Budded (D) Chl_{a+b}	Budless (D) Chl_{a+b}
Budded (L) protein	0.940*	0.893*	0.958*	0.991*
Budless (L) protein	0.931*	0.831*	0.923*	0.958*
Budded (D) protein	0.918*	0.787	0.877*	0.863*
Budless (D) protein	0.901*	0.741	0.861*	0.850*

* Significant level at $p < 0.05$.

Table II. Intra-correlation among antioxidative attributes (MPI, phenolics, $FRAP_{eq}$) of leaf discs (budded, budless) exposed to continuous light (L) and dark (D) conditions.

Parameter	Budded (L) MPI	Budless (L) MPI	Budded (D) MPI	Budless (D) MPI
Budded (L) phenolics	0.773	0.975*	0.971*	0.036
Budless (L) phenolics	0.781	0.961*	0.99*	-0.017
Budded (D) phenolics	0.808	0.953*	0.982*	0.121
Budless (D) phenolics	0.792	0.899*	0.959*	0.135
Budded (L) $FRAP_{eq}$	0.820*	0.967*	0.981*	0.002
Budless (L) $FRAP_{eq}$	0.939*	0.883*	0.921*	0.350
Budded (D) $FRAP_{eq}$	0.917*	0.890*	0.915*	0.363
Budless (D) $FRAP_{eq}$	0.917*	0.890*	0.915*	0.363
Parameter	Budded (L) $FRAP_{eq}$	Budless (L) $FRAP_{eq}$	Budded (D) $FRAP_{eq}$	Budless (D) $FRAP_{eq}$
Budded (L) phenolics	0.925*	0.888*	0.932*	0.932*
Budless (L) phenolics	0.990*	0.927*	0.894*	0.894*
Budded (D) phenolics	0.943*	0.940*	0.955*	0.955*
Budless (D) phenolics	0.949*	0.953*	0.932*	0.932*

* Significant level at $p < 0.05$.

The intra-correlation and inter-correlation analysis of the senescence and antioxidative parameters were also carried out using SPSS-10 software at the $p < 0.05$ level of significance.

Results and Discussion

Evaluation of early senescence characteristics

A detached young leaf may remain healthy for quite some time, particularly in case of succulents, though the initiation/acceleration of senescence is inevitable. This process is characterized by the enhancement of several degradative processes including the loss of proteins (Woo *et al.*, 2001; Hörtensteiner and Feller, 2001), photosynthetic pigments (Fang *et al.*, 1998; Hörtensteiner, 2006), and membrane integrity (Buchanan-Wollaston, 1997; Lim *et al.*, 2007). The observations recorded in the present investigation showed a steady and persistent depletion from the leaf discs of soluble proteins and Chl_{a+b} and lowering of MSI, soon after leaf detachment.

Table III. Inter-correlation between protein content and antioxidative attributes (phenolics, $FRAP_{eq}$, MPI) of leaf discs (budded, budless) exposed to continuous light (L) and dark (D) conditions.

Parameter	Budded (L) protein	Budless (L) protein	Budded (D) protein	Budless (D) protein
Budded (L) phenolics	0.824*	0.713	0.686	0.641
Budless (L) phenolics	0.929*	0.837*	0.733	0.691
Budded (D) phenolics	0.905*	0.807	0.748	0.709
Budless (D) phenolics	0.972*	0.886*	0.765	0.740
Budded (L) $FRAP_{eq}$	0.927*	0.855*	0.770	0.732
Budless (L) $FRAP_{eq}$	0.966*	0.951*	0.92*	0.900*
Budded (D) $FRAP_{eq}$	0.918*	0.951*	0.877*	0.857*
Budless (D) $FRAP_{eq}$	0.918*	0.951*	0.877*	0.857*
Budded (L) MPI	0.843*	0.951*	0.987*	0.972*
Budless (L) MPI	0.828*	0.730	0.694	0.642
Budded (D) MPI	0.896*	0.794	0.719	0.673
Budless (D) MPI	0.256	0.439	0.611	0.655

* Significant level at $p < 0.05$.

Table IV. Inter-correlation between chlorophyll content and antioxidative attributes (phenolics, $FRAP_{eq}$, MPI) of leaf discs (budded, budless) exposed to continuous light (L) and dark (D) conditions.

Parameter	Budded (L) Chl_{a+b}	Budless (L) Chl_{a+b}	Budded (D) Chl_{a+b}	Budless (D) Chl_{a+b}
Budded (L) phenolics	0.89*	0.936*	0.913*	0.858*
Budless (L) phenolics	0.906*	0.98*	0.926*	0.942*
Budded (D) phenolics	0.913*	0.946*	0.938*	0.914*
Budless (D) phenolics	0.917*	0.926*	0.948*	0.965*
Budded (L) $FRAP_{eq}$	0.937*	0.991*	0.947*	0.955*
Budless (L) $FRAP_{eq}$	0.984*	0.932*	0.984*	0.976*
Budded (D) $FRAP_{eq}$	0.973*	0.903*	0.98*	0.936*
Budless (D) $FRAP_{eq}$	0.973*	0.903*	0.98*	0.936*
Budded (L) MPI	0.947*	0.846*	0.908*	0.873*
Budless (L) MPI	0.891*	0.981*	0.902*	0.870*
Budded (D) MPI	0.907*	0.982*	0.928*	0.917*
Budless (D) MPI	0.325	0.028	0.276	0.229

* Significant level at $p < 0.05$.

Discs cut from leaves immediately after their detachment from the mother plant had an overall soluble protein content of (17 ± 1.0) μg mg^{-1} DW. It was observed that the protein content of discs decreased gradually but steadily in all cases. However, initially the decrease was steeper in the budless as compared to the budded discs, and it was more pronounced in either case under dark as compared to light condition. By the 6^{th} day, the protein content was reduced to a similar level in both light and dark conditions, although the budded discs always maintained a higher content than the budless ones (Fig. 2a). The factorial univariate analysis of the data revealed significant alterations in the protein content response to experimental variables of leaf discs being budded or budless ($F_{1,20} = 36.31$; $p < 0.05$) and maintained under continuous light or in the dark ($F_{1,20} = 235.22$; $p < 0.05$), over a period of 10 days. Of the different factors, the influence of the light/dark condition was found to be significant ($F_{4,20} = 13.72$; $p < 0.05$) with respect to increasing time of culture, while the other interaction effects were found to be non-significant.

The analysis of changes in the levels of Chl_{a+b} revealed that at the onset of the experiment the leaves had a total Chl_{a+b} of (1.9 ± 0.03) μg mg^{-1} DW. Whereas the chlorophyll content in both budded and budless discs was maintained more or less at the same level for 10 days under light,

Table V. Inter-correlation between MSI and antioxidative attributes (phenolics, $FRAP_{eq}$, MPI) of leaf discs (budded, budless) exposed to continuous light (L) and dark (D) conditions.

Parameter	Budded (L) MSI	Budless (L) MSI	Budded (D) MSI	Budless (D) MSI
Budded (L) phenolics	0.770	0.743	0.527	0.471
Budless (L) phenolics	0.900*	0.809	0.510	0.497
Budded (D) phenolics	0.866*	0.787	0.528	0.542
Budless (D) phenolics	0.957*	0.794	0.569	0.575
Budded (L) $FRAP_{eq}$	0.891*	0.85*	0.597	0.547
Budless (L) $FRAP_{eq}$	0.933*	0.931*	0.732	0.778
Budded (D) $FRAP_{eq}$	0.875*	0.874*	0.737	0.744
Budless (D) $FRAP_{eq}$	0.875*	0.874*	0.737	0.744
Budded (L) MPI	0.791	0.983*	0.809	0.904*
Budless (L) MPI	0.773	0.787	0.505	0.450
Budded (D) MPI	0.856*	0.793	0.506	0.482
Budless (D) MPI	0.247	0.444	0.558	0.807

* Significant level at $p < 0.05$.

it fell steadily and significantly in those exposed to dark condition, and slightly more so in the budded ones (Fig. 2b). The factorial univariate analysis of the data revealed significant alterations in the chlorophyll content in response to experimental variables, *i.e.* for budded or budless leaf discs ($F_{1,20} = 41.35$; $p < 0.05$), as well as when maintained under continuous light or in the dark ($F_{1,20} = 685.32$; $p < 0.05$), over a period of 10 days. The interaction effects of light/dark and budded/budless conditions ($F_{1,20} = 10.12$; $p < 0.05$) as well as light/dark and the days of exposure ($F_{4,20} = 43.60$; $p < 0.05$) were also significant. In short, the initial decline in protein and Chl_{a+b} content was faster in leaf discs in the dark as compared to light, which indicated more stressful conditions in the dark and hence a higher rate of degradation. This is also in agreement with other reports (Noodén, 1988; Hörtensteiner, 2006; Guiboileau *et al.*, 2010). The pigment loss in discs with differentiating epiphyllous buds was faster than in those lacking buds. This could be explained on the basis of a source-sink type of relationship that exists between disc and the developing plantlet, as shown earlier (Jaiswal and Sawhney, 2006), resulting in a faster senescence in budded discs as compared to those without a bud. However, a somewhat reverse situation was observed in case of soluble proteins, for which a greater loss was recorded in budless discs.

The MSI of leaf discs displayed a steep fall within 2 days in the dark regardless of the presence or absence of an epiphyllous bud. On the other hand, the illuminated discs did not show any significant loss during this period. After 4 days, while the MSI in budded discs did not alter, it fell in the budless ones and recovered after 6 days, to a greater extent in the light than in the dark. However, a further fall in the MSI was seen in the budded discs after 6 days of illumination and after 8 days in the dark. The attained lower levels in such cases were maintained until the 10^{th} day (Fig. 2c). The factorial univariate analysis revealed significant alterations in the MSI in response to experimental variables of the days of culture ($F_{4,20} = 87.88$; $p < 0.01$), leaf discs being budded or budless ($F_{1,20} = 20.32$; $p < 0.01$), as well as when maintained under continuous light or in the dark ($F_{1,20} = 1198.52$; $p < 0.01$), over a period of 10 days. The interaction effects of light/dark ($F_{4,20} = 7.43$; $p < 0.05$) and budded/budless ($F_{4,20} = 43.93$; $p < 0.05$) conditions with respect to the time variable were also significant.

Evaluation of antioxidant properties

To evaluate any probable correlation between early senescence and antioxidative parameters, the total phenolics content, $FRAP_{eq}$, and MPI were estimated in the leaf disc aqueous ethanolic extracts. On day 0, the leaf discs had a total phenolics content of (22.4 ± 2) $\mu g\ mg^{-1}$ DW. The content remained more or less unaltered for the first 4 days in both the budded and budless leaf discs exposed to light or dark conditions. Thereafter, the content fell steadily, the decline initially being slightly steeper in the dark in both types of discs. After 10 days, however, all leaf discs, irre-

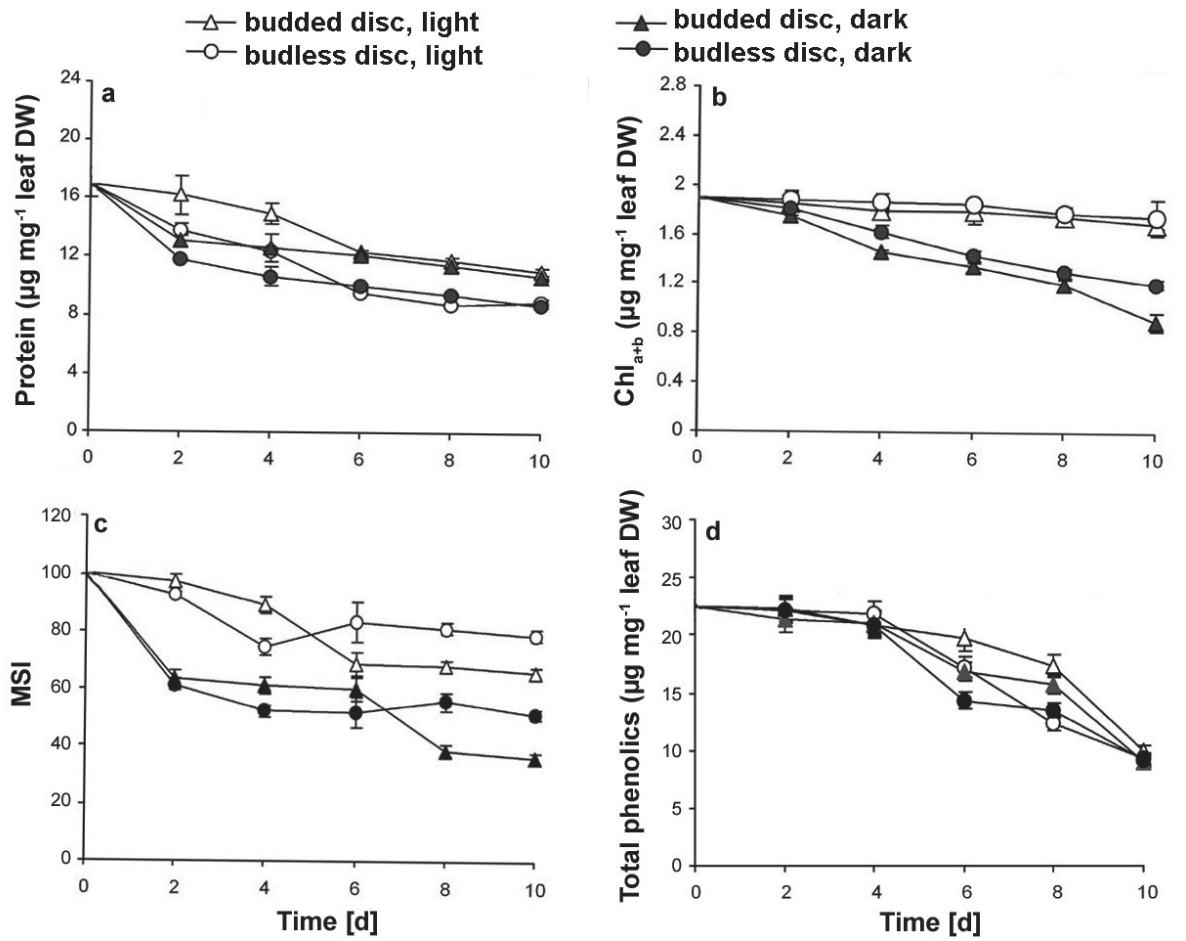

Fig. 2. Changes in (a) soluble protein content, (b) Chl_{a+b} content, (c) MSI, and (d) total phenolics content in leaf discs with or without an epiphyllous bud and exposed to continuous light or dark conditions.

spective of the treatment, had an identical phenolics content of about 9 μg mg^{-1} DW (Fig. 2d).The factorial univariate analysis of the data revealed significant alterations in the phenolics content in response to experimental variables, *i.e.* the number of days of culture ($F_{4,20}$ = 678.27; $p < 0.05$), leaf discs being budded or budless ($F_{1,20}$ = 41.38; $p < 0.05$), as well as maintained in the light or in the dark ($F_{1,20}$ = 28.73; $p < 0.05$), over a period of 10 days. The interaction effects of light/dark ($F_{4,20}$ = 7.52; $p < 0.05$) and budded/budless ($F_{4,20}$ = 20.58; $p < 0.05$) conditions with respect to time were also significant. A time-dependent decrease in the level of phenolics was observed during the 10-days period following leaf detachment (Fig. 2d). The content was found to be lower in discs in the dark as compared to those in the light, indicating a relationship with the higher rate of senescence-related degradative processes in the dark. This was further confirmed by a correlation analysis where Chl_{a+b} was found to be significantly correlated with the level of phenolics (Table IV).

FRAP is an established method to evaluate the total antioxidative status of a system. The value of ΔA_{595} rose in a time-dependent manner for both types of discs, *i.e.* budded or budless, upon exposure to either light or dark conditions (Fig. 3a). A period of 5 min (time segment exhibiting maximum linearity with respect to ΔA_{595}) was used to evaluate $FRAP_{eq}$, with rutin as standard. The maximal $FRAP_{eq}$ of 5.01 was observed in discs at

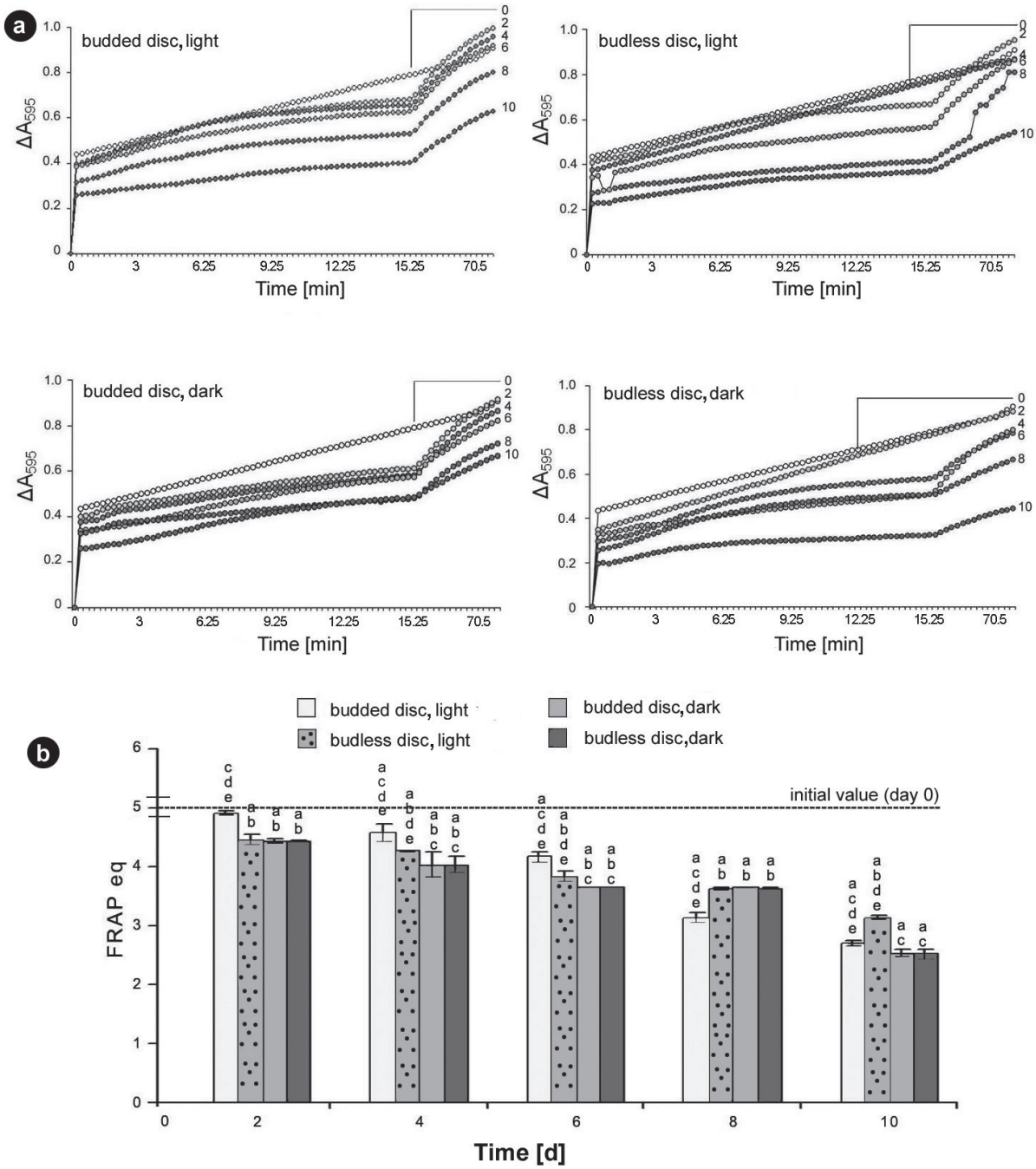

Fig. 3. (a) Comparative evaluation of absorbance change over a period of 70.5 min (in FRAP microassay) among different sets of leaf discs with or without an epiphyllous bud and exposed to continuous light or dark conditions. Labels 0 – 10 indicate days after detachment. (b) $FRAP_{eq}$ of leaf discs (n = 30) receiving different treatments. Different letters indicate significant differences $p < 0.05$) among treatments for Tukey's post-hoc comparison.

the beginning of the experiment, a value that diminished gradually but significantly so until day 10 of culture (Fig. 3b). The factorial univariate analysis of the data obtained revealed significant alterations in $FRAP_{eq}$ in response to experimental variables, *i.e.* light/dark condition ($F_{1,20} = 79.16$; $p < 0.05$), over a period of 10 days. The individual interactive effect of light/dark ($F_{4,20} = 24.46$; $p < 0.05$) and budded/budless ($F_{4,20} = 16.16$; $p < 0.05$) conditions with respect to increasing period of culture were significant. However, both pairs of variables varied independently. The interaction effect of all three experimental variables taken together was found to be significant ($F_{4,20} = 16.87$; $p < 0.05$). Based upon this micro-FRAP assay, the average $FRAP_{eq}$ on the 10^{th} day of leaf detachment was found to be 54% of the initial level on day 0. This was found to be correlated to the decreasing pattern observed for phenolics (intra-correlation; Table II) and maximally correlated with variations in Chl_{a+b} (Table IV) and protein contents (inter-correlation; Table III). This indicated a probable role played by phenolics in the overall antioxidant activity of the leaf discs. It is known from an earlier report that due to their oxidant-stabilizing ability, flavonoids, like quercetin and rutin, possess significant ($p < 0.05$) FRAP values (Nilsson *et al.*, 2005). A comparative analysis of $FRAP_{eq}$ of discs exposed to light and dark conditions during 2 to 6 days of detachment exhibited no significant difference, indicating the presence of some light-insensitive constituents other than phenolics contributing synergistically in the overall activity.

Further, Fenton reagent-mediated OH°-induced degradation of artificial soy lecithin/cholesterol membranes was analysed, and membrane protection in terms of percent inhibition of membrane degradation over the control was estimated and is referred to as MPI. The budded leaf discs under continuous light exhibited a more than 40% decrease in activity in the first 2 days, with an additional 20% reduction of activity recorded over the next 8 days. In case of budless discs under similar conditions, the overall reduction of activity was 30% after 8 days. No appreciable effect was observed in both budded and budless discs in continuous dark (Fig. 4). These main effects, as mentioned above, varied significantly individually; however, factorial univariate analysis revealed that there were no significant interactive effects between these experimental variables. In case of budded discs exposed to continuous light, membrane protection increased with an increase in dilution factor, and a decreasing trend over a time period was similar at all dilutions, *i.e.* 1:10, 1:25, and 1:50 (parent extract diluted with PBS). The substantial increase in dilution factor could be attributed towards an interplay of various constituents playing antagonistically at higher concentration but synergistically at lower concentration, *i.e.* at appropriate dilution on which it achieves optimal balance. Further dilution did not exhibit any increase in activity, exhibiting a biphasic activity. A similar biphasic effect of the variation of activity has been reported in case of the *in vitro* evaluation of fractions of an endophyte, *Entrophosphora infrequens* (Arora *et al.*, 2010). However, in case of budless discs exposed to continuous light, the effect of dilution was observed only at the 8^{th} and 10^{th} day. In case of budded/budless discs in the dark, such significant changes in membrane protection with respect to dilution were not observed (Fig. 4).

Intra-/inter-correlation analysis

Intra-correlation among leaf senescence parameters (Chl_{a+b}, protein, and MSI) was as follows: Chl_{a+b} and protein contents were maximally correlated, followed by protein content and MSI, and finally Chl_{a+b} content and MSI under continuous light (Table I) while there was low correlation in the dark. Among various antioxidative parameters (phenolics, $FRAP_{eq}$, and MPI), $FRAP_{eq}$ was maximally correlated with the phenolics content, while MPI did not exhibit such pattern (Table II). $FRAP_{eq}$ and MPI also exhibited a significant correlation pattern, except in budless discs under dark condition. In this context, the level of responsiveness (senescence and antioxidant parameters) under different experimental variables can be arranged in the following decreasing order: budless discs/light > budded discs/light > budded discs/dark > budless discs/dark.

Inter-correlation analysis revealed that $FRAP_{eq}$ was maximally correlated with the Chl_{a+b} content. The level of significance from the highest to the lowest ranking for correlation among pairs of different parameters, regardless of them being components of foliar senescence or of antioxidative status, were in the following order: $FRAP_{eq}$-Chl_{a+b} > phenolics-Chl_{a+b} > $FRAP_{eq}$-protein > MPI-Chl_{a+b} > $FRAP_{eq}$-MSI > MPI-protein > phenolics-protein

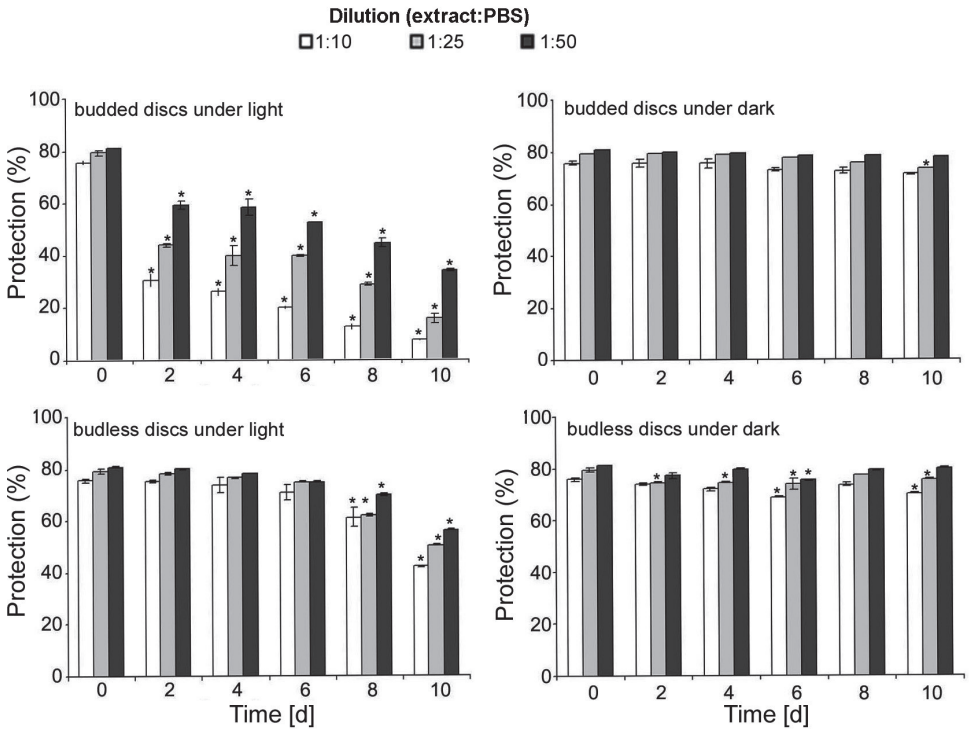

Fig. 4. Percent membrane protection against Fenton-mediated OH°-induced stress in liposomes by tissue extracts of leaf discs with or without an epiphyllous bud and exposed to continuous light or dark conditions. Membrane protection in terms of percent inhibition of membrane degradation over control was estimated using one-sided Dunnet's t-test [single control (0 day) and five test samples, *i.e.*, 2, 4, 6, 8, and 10 days]. *Significance at $p < 0.05$ with respect to control.

> MPI-MSI > phenolics-MSI (Tables III–V). In this context, the order of responsiveness for different experimental variables was budded discs/light > budless discs/light > budded discs/dark > budless discs/dark.

The aqueous ethanolic leaf disc extracts exhibited a significant ($p < 0.05$) membrane protection ability against peroxidative stress, although the pattern was not found to be in line with that of phenolics content or $FRAP_{eq}$. The probable reason for the difference in the pattern of activity could be the dominance of lipophilic constituents in dark- as compared to light-exposed discs. This might be attributed to the synergistic and/or antagonistic action of phenolics with other bioactive constituents. Further, the decreasing trend of chlorophyll content was found to be maximally correlated with alterations in the membrane protection, which indicated a probable loss of lipophilic constituents in the proximity of intra-cellular membranes like those of chloroplasts.

Conclusion

The patterns of diminishing $FRAP_{eq}$ (aqueous phase) and phenolics indicated that with the progression of senescence the gross secondary metabolites level initially available in the leaf tissue got reduced, thereby resulting in a temporal loss of antioxidant potential. A strong correlation between $FRAP_{eq}$ and Chl_{a+b} content also affirms a linkage of such loss of diminishing antioxidant potential with foliar senescence.

Arora R., Chawla R., Dhaker A. S., Adhikari M., Sharma J., Jaiswal S., Gupta D., Amna T., Puri S. C., Kumar R., Sharma A., Sharma R. K., and Tripathi R. P. (2010), Pro-antioxidant activities of fractions of a novel camptothecin-producing endophyte (*Entrophosphora infrequens*). Trakia J. Sci. **8**, 1–15.

Benzie I. F. F. and Strain J. J. (1996), The ferric reducing ability of plasma FRAP as a measure of "antioxidant power": The FRAP assay. Anal. Biochem. **239**, 70–76.

Bradford M. M. (1976), A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem. **72**, 248–254.

Buchanan-Wollaston V. (1997), The molecular biology of leaf senescence. J. Exp. Bot. **48**, 181–199.

Caillet S., Yu H., Lessard S., Lamoureux G., Ajdukovic D., and Lacroix M. (2007), Fenton reaction applied for screening natural antioxidants. Food Chem. **100**, 542–552.

Cheour F., Joseph A., Joseph M., and Willemot C. (1992), Delay of lipid degradation by calcium treatment during cabbage leaf senescence. Plant Physiol. **100**, 1656–1660.

Dhindsa R. S., Plumb-Dhindsa P., and Thorpe T. A. (1981), Leaf senescence: correlation with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J. Exp. Bot. **32**, 93–101.

Fang Z., Bouwkamp J., and Solomos T. (1998), Chlorophyllase activities and chlorophyll degradation during leaf senescence in non-yellowing mutant and wild type of *Phaseolus vulgaris* L. J. Exp. Bot. **49**, 503–510.

Firuzi O., Lacanna A., Petrucci R., Marrosu G., and Saso L. (2005), Evaluation of the antioxidant activity of flavonoids by "ferric reducing antioxidant power" assay and cyclic voltammetry. Biochim. Biophys. Acta **1721**, 174–184.

Gregersen P. L., Holm P. B., and Krupinska K. (2008), Leaf senescence and nutrient mobilization in barley and wheat. Plant Biol. **10**, 37–49.

Guiboileau A., Sormani R., Meyer C., and Daubresse M. C. (2010), Senescence and death of plant organs: nutrient recycling and developmental regulation. CR Biol. **333**, 382–391.

Halliwell B. and Gutteridge J. M. C. (eds.) (2006), Free Radicals in Biology and Medicine, 4th ed. Clarendon Press, Oxford, UK.

Hiscox T. D. and Israelstam G. F. (1979), A method for extraction of chlorophyll from leaf tissue without maceration. Can. J. Bot. **57**, 1332–1334.

Hörtensteiner S. (2006), Chlorophyll degradation during senescence. Ann. Rev. Plant Biol. **57**, 55–77.

Hörtensteiner S. and Feller U. (2001), Nitrogen metabolism and remobilization during senescence. J. Exp. Bot. **53**, 927–937.

Jaiswal S. and Sawhney S. (2006), Correlation of epiphyllous bud differentiation with foliar senescence in crassulacean succulent *Kalanchoe pinnata* as revealed by thidiazuron and ethrel application. J. Plant Physiol. **163**, 717–722.

Joseph B., Sridhar S., Sankarganesh, Justinraj, and Edwin B. T. (2011), Rare medicinal plant – *Kalanchoe pinnata*. Res. J. Microbiol. **6**, 322–327.

Keech O., Pesquet E., Gutierrez L., Ahad A., Bellini C., Smith S. M., and Gardeström P. (2010), Leaf senescence is accompanied by an early disruption of the microtubule network in *Arabidopsis*. Plant Physiol. **154**, 1710–1720.

Leshem Y. Y. (1988), Plant senescence processes and free radicals. Free Radical Biol. Med. **5**, 39–49.

Lim P. O., Kim H. J., and Nam H. G. (2007), Leaf senescence. Annu. Rev. Plant Biol. **58**, 115–136.

New R. R. C., Rickwood D., and Hames B. D. (1990), Liposomes, a practical approach. In: Practical Approach Series (Rickwood D. and Hames B. D., eds.). IRL Press, Oxford, UK.

Nilsson J., Pillai D., Önning G., Persson C., Nilsson A., and Akesson B. (2005), Comparison of the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) methods to assess the total antioxidant capacity in extracts of fruit and vegetables. Mol. Nutr. Food Res. **49**, 239–246.

Noodén L. D. (1988), Whole plant senescence. In: Senescence and Aging in Plants (Noodén L. D. and Leopold A. C., eds.). Academic Press, San Diego, pp. 391–439.

Premchandra G. S., Soneoka H., and Ogata S. (1990), Cell membrane stability, an indicator of drought tolerance as affected by applied nitrogen in soybean. J. Agric. Sci. **115**, 63–66.

Sairam R. K., Rao K. V., and Srivastava G. C. (2002), Differential response of wheat genotypes to long term salinity stress in relation to oxidative stress, antioxidant activity and osmolyte concentration. Plant Sci. **163**, 1037–1046.

Schelbert S., Aubry S., Burla B., Agne B., Kessler F., Krupinska K., and Hörtensteiner S. (2009), Pheophytin pheophorbide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in *Arabidopsis*. Plant Cell **21**, 767–785.

Singleton V. L. and Rossi J. A. (1965), Colorimetry of total phenolics with phosphomolybdic- phosphotungstic acid reagents. Am. J. Enol. Vitic. **16**, 144–158.

Smart C. (1994), Gene expression during leaf senescence. New Phytol. **126**, 419–448.

Thomas H. and Stoddart J. L. (1980), Leaf senescence. Annu. Rev. Plant Physiol. **31**, 83–111.

Thompson J. E., Paliyath G., Brown J. H., and Duxbury C. L. (1987), The involvement of activated oxygen in membrane deterioration during senescence. In: Plant Senescence: Its Biochemistry and Physiology (Thomson W. W., Nothnagel E. A., and Huffaker R. C., eds.). The American Society of Plant Physiologists, Rockville, MD, pp. 146–155.

Woo H. R., Chung K. M., Park J. H., Oh S. A., Ahn T., Hong S. H., Jang S. K., and Nam H. G. (2001), ORE9, an F-box protein that regulates leaf senescence in *Arabidopsis*. Plant Cell **13**, 1779–1790.