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The role of ascorbic acid in the preservation or degradation of chlorophyll in oat leaves

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SUMMARY

In oat leaf segments incubated in water, chlorophyll breakdown occurs faster in darkness than under light conditions.
Ascorbic acid addition to the incubation medium reversed this behaviour inducing a delay of chlorophyll breakdown in darkness but an increase of the pigment degradation in light. The accelerating effect on the chlorophyll loss in light is accompanied by an increase of malondialdehyde and membrane permeability. This effect seems to be mediated by oxygen free radicals, mainly hydroxyl radical (·OH), a fact supported by the in vitro breakdown of isolated chlorophyll by Fenton's reagents and the benzoate prevention of the chlorophyll loss.
Ascorbic acid in darkness prevents chlorophyll degradation connected with guaiacol peroxidase inactivation, a result also observed in the presence of the inhibitor of protein synthesis cycloheximide.
Results suggest that the acceleration of chlorophyll loss in light caused by ascorbate may be due to the increase of photooxidative processes and the effect of ascorbate in darkness may result from the inactivation of the guaiacol peroxidase activity.

Key words: antioxidant system, ascorbic acid, *Avena sativa* L., chlorophyll bleaching, free radical, senescence.

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RESUMEN

Cuando segmentos de hojas de avena son incubados en agua, se verifica una mayor pérdida de clorofilas en condiciones de oscuridad que en luz.
La adición de ácido ascórbico al medio de incubación invierte este comportamiento, induciendo una menor degradación de clorofilas en oscuridad y un incremento del blanqueo en condiciones de luz. El efecto en luz es acompañado por un aumento en la producción de malondialdehido y en la permeabilidad de membranas, lo que sugiere que la mayor pérdida de clorofilas en luz, inducida por ascorbato, podría estar mediada por radicales libres del oxígeno. La destrucción de clorofilas aisladas incubadas en presencia de los reactivos de Fenton y

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su prevención con benzoato sugiere que el radical hidroxilo (OH) puede ser el radical implicado.
La adición de cicloheximida al medio de incubación impide el blanqueo, sugiriendo la existencia de una vía enzimática de degradación del pigmento La menor pérdida de clorofilas en oscuridad producida por ascórbico es acompañada por la inactivación de la fenol peroxidasa, enzima que fuera implicada en la destrucción del pigmento.
Los resultados sugieren que la aceleración de la pérdida de clorofilas en luz, causada por ácido ascórbico, estaría relacionada al incremento de procesos fotooxidativos y el efecto en oscuridad a la inactivación de enzima/s capaces de degradar clorofilas como la guaiacol peroxidasa.

Palabras clave: sistema antioxidante, ácido ascórbico, *Avena sativa* L., blanqueo de clorofilas, radicales libres, senescencia.

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INTRODUCTION

Chlorophyll breakdown is the most important phenomenon within the process of leaf senescence; however, knowledge of their degradative pattern is limited (Hendry *et al.*, 1987). The cleavage of the macrocyclic ring system and subsequent bleaching of chlorophyll could be due to the action of free oxygen radicals (Harbour & Bolton, 1978; Feierabend & Winkelhüsener, 1982; Sakaki *et al.*, 1983) or to other enzymatically generated radicals (Holden, 1965; Martinoia *et al.*, 1982; Kato & Shimizu, 1985, 1986). A complex antioxidant system involving ascorbic acid constitutes the defence mechanism against these radicals in the chloroplasts (Cadenas, 1985; Sies, 1985). In accordance with these findings, Sarkar & Choudhuri (1981) observe that ascorbic acid can preserve chlorophylls in sunflower leaves in darkness. However, it has also been noted that ascorbic acid increased chlorophyll degradation in maize (Pjon, 1981) and rye leaves (Feierabend & Winkelhüsener, 1982) in light. Similar antioxidant and prooxidant effects of vitamin C and flavonoids on biological molecules have been reported by Bonet *et al.* (1996) and Yen *et al* (1997).

The causes distinct effects of the ascorbic acid on chlorophyll loss, depending on conditions of light or darkness, are not clear. We hypothesize that the explanation is related to the capacities of ascorbic acid to act either as prooxidant or antioxidant depending on internal and external conditions as suggested by Kitts (1997). Its prooxidant capacity could be related to its ability to inhibit catalase (Davison *et al.*, 1986) and peroxidase (Palmieri & Giovinazzi, 1982) activity, to induce decarboxilation of fatty acids (Khan & Kolattukudy, 1974), and to increase linoleate peroxidation in the presence of heavy metals (Haase & Dunkley 1969; Wills, 1985). This last effect is particularly important as many authors suggest that there is a close relationship between lipid peroxidation and membrane alteration with the increase of chlorophyll breakdown (Feierabend &Winkelhüsener, 1982; Sakaki *et al.*, 1983). The antioxidant effects of ascorbic acid, on the other hand, could be related to its capacity to reduce superoxide ions (Halliwell, 1978) and hydrogen peroxide (Nakano & Asada, 1981), to regenerate reduced tocopherol (Leung *et al.*, 1981), and to scavenge singlet oxygen (Rooney, 1983) and aminophenoxy radicals (Chen & Asada, 1990).

The aim of the present work was to study some of the mechanisms involved in chlorophyll degradation in oat leaves when incubated in the presence of ascorbic acid under conditions of light and darkness.

MATERIALS AND METHODS

Plant material

Seeds of *Avena sativa* L. cv. Suregrain were sown in vermiculite, and seedlings were grown under continuous white light ($40Wm^{-2}$) at 23 °C. Seven days after planting, a subapical 3-cm segment was cut from the first leaf of each plant and groups of ten segments were floated on 10 ml of distilled water or test solution (Ascorbic 50 mM, 50 ppm Cycloheximide and Ascorbic 50 mM plus 50 ppm Cyclohex-

imide) in Petri dishes, and were exposed to light ($40Wm^{-2}$) or darkness treatments, all at 23 °C. All solutions were stabilized at pH 7.

Chlorophyll and Malondialdehyde (MDA) determination

Ethanol extracts were obtained from leaf segments and used to measure chlorophyll levels (Tetley & Thimann, 1974) and MDA (Heath & Packer, 1968). Total content of malondialdehyde was obtained by adding the values for leaf segments to those for the incubation medium. Measurements were made at 0, 24, 48 and 72 h.

Membrane permeability

The treated leaf segments were washed and transferred to 5 ml of distilled water and kept for 2 h in darkness. The change in incubation medium conductivity, resulting from electrolyte efflux from the tissue, was determined by means of a conductivity meter (type 1484 pr Horizon Ecology Co.) Results were expressed as a percentage of conductivity, with 100% conductivity reached upon boiling the segments in their incubation medium for 15 min.

Measurements were made at 0 and 48 h.

Enzyme assays

Five segments were homogenized in 1 ml of 50 mM Tris-Cl buffer pH 7, containing 1% polyvinylpolypyrrolidone. The extracts were centrifuged at 16000 xg for 30 min and the supernatants were used to assay enzyme activity. The reaction mixture contained 100 mM Tris-Cl buffer (pH 7), 30 mM H_2O_2, 60 mM guaiacol and 200 ul of the enzyme extract. Guaiacol peroxidase activity was determined by the increase in absorbency at 470 nm as guaiacol was oxidized, as described by Chance & Maehly (1955).

Measurements were made at 0 and 48 h.

Isolated chlorophyll bleaching

The assay mixture contained 30 mM K phosphate buffer, pH 7; 1 mM H_2O_2;.2 mM ascorbic acid; 0.1 mM CuSO4 and 100 ul of isolated chlorophyll in a final assay volume of 1.5 ml. The initial absorbency was .110. The reaction was initiated by adding H_2O_2 and followed spectrophotometrically at 654 nm.

All the experiments were repeated three times, and the data presented are averages of the measurements. Standard deviations of the mean are indicated.

RESULTS AND DISCUSSION

Changes in chlorophyll content in oat leaves incubated in water indicated that chlorophyll loss was higher under dark than under light conditions (Fig.1). Such dark-induced chlorophyll degradation has also been observed in *Hydrangea macrophylla* (Pjon, 1981) and *Spinacia oleracea* L. leaves, but not in *Hydrilla* leaves (Kar & Choudhuri, 1987). This suggests that different effects of light or dark conditions on chlorophyll loss could result from genotypic variations.

Ascorbic acid effect in light

When the leaves were incubated in ascorbic acid (Fig. 1), the effects of light or darkness on chlorophyll loss were inverted: Chlorophyll bleaching increased in conditions of light but delayed in darkness as compared to control. The higher chlorophyll breakdown by ascorbate in leaf segments in light as compared with the control in water (Fig. 1), is in agreement with previous results obtained in maize (Pjon, 1981) and rye (Feierabend & Wilkelhüsener, 1982).

The effect of ascorbate on MDA content in the leaves, in the medium and total MDA, is shown in Fig. 2 . Ascorbate increased the MDA total content in the illuminated leaves, whereas little increase in MDA was observed in water or dark treatments

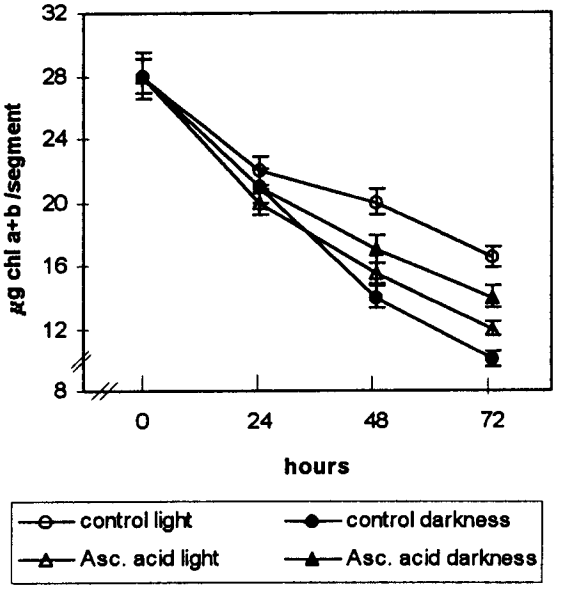

Figure 1. Effect of 50 mM ascorbic acid on the chlorophyll content in oat leaf segments under continuous light (40 Wm-2) and in darkness. Measurements were made at 0, 24, 48 and 72 h and represent the average values of three experiments. Bars represent standard errors.

(Fig. 2). The higher lipid peroxidation in the ascorbate treatments could be related to the fact that ascorbate can induce direct linoleate oxidation (Haase & Dunkley, 1969; Wills, 1985) or, indirectly, release reduced Fe from ferritine. The subsequent reaction of Fe with hydrogen peroxide produces hidroxyl radical (·OH) (Asada & Takahashi, 1987), a reactive species that produces lipid peroxidation in biological systems (Kappus, 1985). This supposition is supported by the the fact that the experimental conditions of continuous light favor the accumulation of H_2O_2 in connection with photosynthesis (Patterson & Myers, 1973).

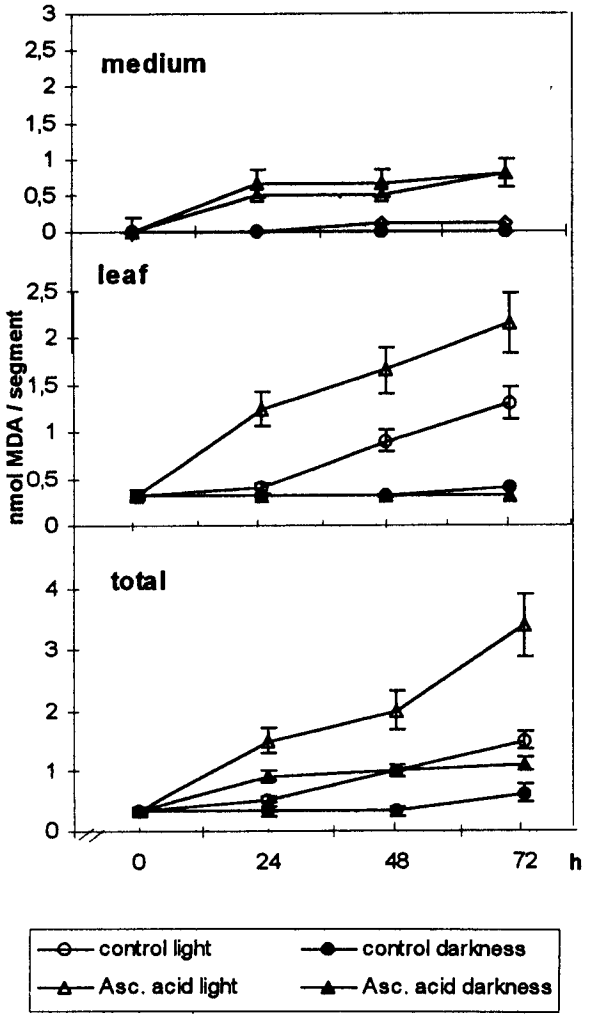

Figure 2. Effect of 50 mM ascorbic acid on the MDA content in the incubation medium , in the leaf segments and total (in the medium plus the segments) in oat leaves incubated under continuous light (40 Wm-2) and in darkness. Measurements were made at 0, 24, 48 and 72 h and represent the average of three experiments. Bars represent standard errors.

Table 1. Effect of Ascorbate on membrane permeability in oat leaf segments under light and darkness conditions.

Treatment (48 h)	conductivity (%)
control light	3
control darkness	3
ascorbic light	11
ascorbic darkness	13

The oat leaf segments were incubated with 50 mM ascorbic acid under continuous light (40 Wm^{-2}) and in darkness. Measurements were made at 48 h and represent the average values of three experiments. Initial value (0 h) was 3.

An increase in conductivity (Table 1), in both light and dark ascorbate-treated leaves, was observed in the treatments with ascorbic acid suggesting that ascorbate increased membrane permeability in leaves. Synthesis of our results showed that, in illuminated leaf segments, the ascorbate in the incubation medium caused enhacement of MDA, permeability and chlorophyll loss (Figs. 1 and 2, table 1). Consequently, the higher chlorophyll loss in ascorbate-treated leaves in light could have been caused by an increment in lipid peroxidation with the subsequent thylakoid membrane alteration. The membrane degradation and the loosening of thylakoid-bound pigments may be the major cause of chlorophyll destruction given the fact that these pigments are stable in bound state, while free pigments are labile to oxygen- and light-dependent photosensitizer reactions (Asada & Takahashi, 1987).

In order to verify the possibility of a direct ·OH -effect induced by the ascorbic acid on the chlorophyll molecules, an *in vitro* experiment was carried out in which isolated chlorophylls were incubated in the presence of Fenton's reagents (Table 2). The reaction mixture containing Cu^+, ascorbate, and H_2O_2 was effective in peroxidative chlorophyll breakdown compared with incubation with Cu^+, ascorbate, or H_2O_2 alone. The ·OH, scavenger benzoate (Harbour & Bolton, 1978) inhibited chlorophyll breakdown, increasing with its concentration in the reaction mixture. These results suggest that the ·OH radical played a direct role in *in vitro* chlorophyll bleaching and support the previous postulations on its participation *in vivo*. Similar results concerning the pro-oxidant effects of ascorbate were obtained by Yen *et al.* (1997) and Bonet *et al* (1996) in

Table 2. Incubation of isolated chlorophylls in the presence of Fenton's reagents.

Reaction mixture	isolated chlorophyll breakdown (pgr. min^{-1})
control	1 ±
$Cu^{+2} + H_2O_2$ + Asc	115 ±
Cu^{+2}	13 ±
Asc	5 ±
H_2O_2	3 ±
$Cu^{+2} + H_2O_2$ + Asc + 10 mM Benzoate	115 ±
$Cu^{+2} + H_2O_2$ + Asc + 40 mM Benzoate	73 ±
$Cu^{+2} + H_2O_2$ + Asc + 80 mM Benzoate	45 ±

The assay mixture contained 30 mM potassium phosphate buffer (pH 7); 1 mM H_2O_2; 2 mM ascorbic acid; 0.1 mM $CuSO_4$; sodium benzoate and 100 µl of isolated chlorophylls in a final assay volume of 1.5 ml. The reaction was initiated by adding H_2O_2 and followed spectrophotometrically at 654 nm during 6 min. in dark. The partial reaction mixture contained the reagents shown in the table. The total volume was completed with potassium phosphate buffer pH 7.

studies of the antioxidant properties of flavonoids and vitamin C. The results presented here also support the proposal that both direct and indirect ·OH -effects on chlorophyll breakdown could be simultaneous and complementary given the fact that, under conditions of thylakoid membrane disarrangement, the chlorophyll-hydroxyl radical interaction is more probable.

Variations in pigment levels and the activity of guaiacol peroxidase, an enzyme involved in chlorophyll degradation (Kato & Shimizu, 1985,1986; Kar & Choudhuri, 1987), were measured in relation to the ascorbic acid effect on the enzymatic pattern of chlorophyll breakdown. The leaves were incubated for 48 hs in the presence of ascorbate, cycloheximide (an inhibitor of eukariotic protein synthesis) or ascorbate plus cycloheximide. The presence of cycloheximide in the incubation medium slowed down chlorophyll loss in light (Fig. 3A) and inhibited the activity of the guaiacol peroxidase enzyme (Fig. 3B). These results suggest that chlorophyll loss was influenced by the activity of a cytoplasm-synthesized enzyme, coinciding with findings for maize (Pjon, 1981) and *Hydrilla* (Kar & Choudhuri, 1987). However, there are conflicting results related to this last observation in that ascorbate diminished guaiacol peroxidase activity (Fig. 3B), but increased chlorophyll loss with respect to the control (Fig. 3A) in light conditions. In order to explain these phenomena, it is important to consider that, in the presence of light, there are two chlorophyll-breakdown pathways: one enzymatic and the other an O_2-mediated free radical. Ascorbic acid has a specific effect on each of these; the results obtained here reflect a combination of these two effects. Given the fact that the ascorbic acid inhibits the guaiacol peroxidase, this enzymatic pathway for chlorophyll breakdown, highlighted by Kar & Choudhuri (1987), does not explain the increase in ascorbate-mediated chlorophyll loss observed in the present study under light conditions. Based on these results, it can be assumed that chlorophyll loss under the conditions tested must be attributed to the prooxidant effects of the ascorbic acid previously mentioned.

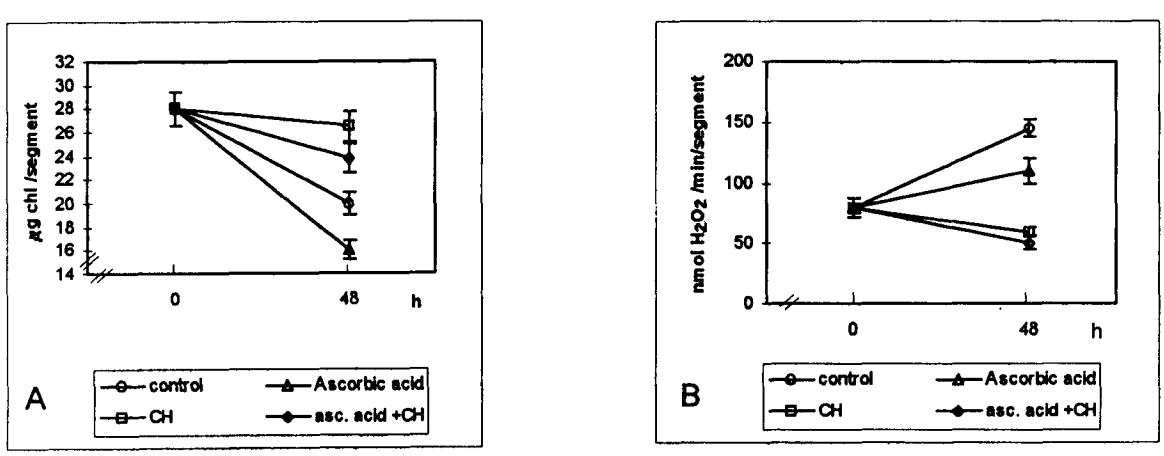

Figure 3. Effect of 50 mM ascorbic acid, 50 ppm cycloheximide (CH) and 50 mM ascorbic acid plus 50 ppm cycloheximide, on chlorophyll content (A) and phenol peroxidase activity (B) in oat leaf segments incubated for 48 h under continuous light (40 Wm-2) conditions

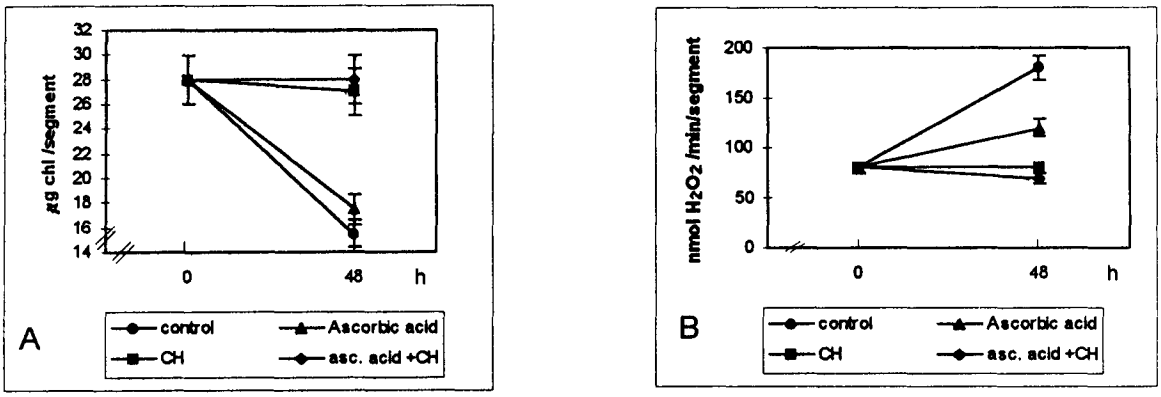

Figure 4. Effect of 50 mM ascorbic acid , 50 ppm cycloheximide (CH) and 50 mM ascorbic acid plus 50 ppm cycloheximide, on chlorophyll content (A) and phenol peroxidase activity (B) in oat leaf segments incubated for 48 h under continuous dark conditions.

Ascorbic acid effect in darkness

Ascorbic acid prevented chlorophyll loss in oat leaf segments in the dark as compared with water control (Fig 1), concurring with observations of other authors in sunflower leaves (Sarkar & Choudhuri, 1981). This is associated with the increase in MDA and membrane permeability (Fig. 2 and table 1). However, unlike the observations in light conditions, the enzymatic pathway for the production of chlorophyll-bleaching radicals should prevail in darkness, considering that the generation of O_2 active species is light-dependent. Incubation of the leaves with cycloheximide maintained the initial values for guaiacol peroxidase activity and almost completely prevented pigment degradation (Fig. 4 A, B). On the other hand, the ascorbic acid treatment induced a decrease in guaiacol peroxidase activity as well as chlorophyll degradation compared to the control in water (Fig. 4A, B). The results, therefore, suggest that the guaiacol peroxidase was involved in chlorophyll bleaching and that the protection of the chlorophylls induced by ascorbic acid in darkness could be related, at least partly, to inhibited guaiacol peroxidase activity under these conditions.

Ascorbic acid could act on this enzymatic pathway on two levels; first, by inhibiting the enzyme's activity as already mentioned; and secondly, by reducing the phenoxy radicals responsible for pigment bleaching. These radicals are the primary product of the one-electron oxidation of phenol by hydrogen peroxide catalysed by phenol peroxidase; it has been shown that ascorbate is capable of deactivating and reducing the radicals (Chen & Asada, 1990). This interpretation is supported by the findings of others (Kato & Shimizu, 1985) who were able to completely inhibit the loss of purified chlorophylls mediated by peroxidase activity by including ascorbate in the reaction medium.

In conclusion, our results may be explained by the existence of at least two mechanisms of regulation of chlorophyll loss by ascorbate; one, light-dependent possibly due to the increase in lipid peroxidation, membrane alteration and ·OH production and its direct or indirect effects on the pigment. The other antioxidant effect, would depend on both guaiacol peroxidase regulation and a direct role of ascorbate as a free radical scavenger . The promotion of the first pathway in light and the inhibition of the guaiacol peroxidase activity in darkness would explain the above-noted inversion of chlorophyll performance in the presence of ascorbate.

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