Response of Chloroplast Structure to Photodynamic Herbicides and High Oxygen

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Response of chloroplast on the structural level to photodynamic herbicides and high oxygen concentration was compared with symptoms of chloroplast senescence. Based on the present results and those known from the literature a general pattern of response was proposed: the action of most of environmental factors induces the oxidative stress and often gives similar symptoms of structural damage and dysfunctions independent of the primary stressing factor. These alterations consist mostly in swelling of thylakoids, disruption of chloroplast membranes, intensive plastoglobuli accumulation, photodestruction of pigments and inhibition of photosynthesis.

Introduction

Various environmental factors affect plants leading to different kind of responses. Any unfavorable condition, caused by natural or anthropogenic factors, that influences plant's metabolism, growth, or development is regarded as stress (Lichtenthaler, 1996, 1998). External factors such as intense light, UV radiation, herbicides, drought and others can initiate oxidative stress, i.e. stress caused by excess of the reactive oxygen species overwhelming the system of natural defense. Reactive oxygen species cause damage on the molecular level, give different cellular effects, some physiological dysfunctions, and in extreme conditions can lead to the death of cell (Scandalios, 1993).

Chloroplasts are potentially the most powerful source of oxidants in plant because of the high internal O_2 concentration (Halliwell, 1991), in particular inside thylakoid. All reactive oxygen species: superoxide radical, hydroxyl radical, hydrogen peroxide and singlet oxygen are produced by illuminated chloroplasts (Scandalios, 1993; Foyer and Hall, 1980). In normal conditions enzymic and non-enzymic defense mechanisms protect chloroplasts against oxidative injuries by detoxifying and eliminating reactive oxygen species. The role of enzymic antioxidants, especially those that detoxify hydrogen peroxide in the ascorbate-glutathione cycle (Halliwell-Asada pathway) is well known (Halliwell, 1991; Bowler *et al.*, 1992; Foyer *et al.*, 1994; Miyake and Asada, 1994).

The present investigation was performed to elucidate the response of chloroplasts on the structural level to photodynamic herbicides and high oxygen concentration and compare it with symptoms of chloroplast senescence.

Photodynamic herbicides or bleaching herbicides are compounds that induce accumulation of tetrapyrrole chlorophyll precursors in the darkness and cause photooxidative damage of plants after exposure to light (Böger and Sandmann, 1993; Rebeiz *et al.*, 1984). Herbicides such as 1.10-phenanthroline (Phe) or 2.2′-bipyridyl were known to affect plant tissues via generation of singlet oxygen and free radical reaction (Rebeiz *et al.*, 1984). In etiolated pea seedlings, treated with one of these herbicides and subsequently exposed to light, inhibition of grana formation and of chlorophyll accumulation and also dilation of ER cisternae was observed (Mostowska *et al.*, 1991b; Mostowska and Siedlecka, 1995), and in green plants, with already differentiated chloroplasts, a photodynamic damage was registered (Mostowska *et al.*, 1991a; Mostowska *et al.*, 1996).

High oxygen concentration is another factor affecting photosynthetic function. It causes inhibition of photosynthesis (Warburg effect). In etiolated plants it induces low chlorophyll content and distribution of photosynthetic products including a decrease of starch content (Wróblewska *et al.*,

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1994). High oxygen concentration can also destroy photosynthetic pigments and induce photodynamic action caused by toxic effects of its reactive products (Halliwell, 1981).

Senescence is yet another process during which a release of reactive oxygen species occurs (Scandalios, 1993). There is probably shared biochemistry between senescence and environmental stress responses in plants (Nooden, 1988).

In the present paper the pattern of response of chloroplast structure caused by a photodynamic herbicide, 1.10-phenantroline (Phe), was compared with response on high oxygen concentration during development of plastids. The responses caused by these factors were also compared with structural changes of senescencing chloroplasts and with response of chloroplasts to environmental factors, such as strong and weak light conditions (Lichtenthaler, 1984) and high and low temperature (Velitchkova *et al.*, 1989; Starck, *et al.*, 1993; Wise and Naylor, 1987) and with response of chloroplasts to stress factors such as UV-radiation (Brandle *et al.*, 1977), heavy metals (Baszynski *et al.*, 1988) and air pollutants (Miyake *et al.*, 1989; Schiffgens-Grüber and Lütz, 1992; Ebel *et al.*, 1990), known from literature. Common aspects of response of chloroplasts were presented in this paper.

Material and Methods

Photodynamic herbicides

Eigth day-old pea seedlings (*Pisum sativum* L. var. De Grace) grown in the growth chamber and illuminated 11 h per day (with irradiance 82 μmol m^{-2} s^{-1}) were sprayed with solutions of Phe (in 0.05% Tween 20) at concentrations of 2, 10 and 20 mM or with 0.05% Tween (control plants). Four ml of one of these solutions was used for each sample of 10 plants. After spraying, the plants were placed overnight for 13 h in darkness to accumulate tetrapyrrole intermediates and then subsequently exposed to white light with the same intensity as before for 9 h.

High oxygen concentration

Eleven day-old, etiolated seedlings of bean (*Phaseolus vulgaris* L. var. Golden Saxa) were placed in a plexiglass chamber, which was placed in a close circulation system filled either with air or with pure oxygen, and illuminated with irradiance 920 μmol m^{-2} s^{-1} white light for 24 h.

When the chamber was filled with air we considered the O_2 concentration to be 21%, and when filled with pure oxygen, as 100%. O_2 concentration was estimated using a Clark-type oxygen electrode (Oxi 39, WTW, G. M. B.H., D 812). The accuracy of the determination of oxygen concentration was about 1%. After each 2 h period the chamber was flushed for 10 min with air or oxygen with a flow rate of 1.5 l/min. At the beginning of the experiment and after each 2 h period 400 ppm of CO_2 were added to the system filled with 100% O_2 to maintain the same concentration of CO_2 as in the air.

Seedlings were illuminated with white light with 2300 μmol m^{-2} s^{-1} photoflood lamps for 24 h. A water filter was used to reduce infrared radiation.

Tobacco seedlings

Tobacco seedlings (*Nicotiana tabacum*, var. Petit Havana SR1) were grown in growth chamber, illuminated 16 h per day with the irradiance 161 μmol m^{-2} s^{-1} and at a temperature of 24 °C during the day and 22 °C at night. The following classes of leaves were taken for analysis: 10 cm-long, fully green leaves from approximately one month-old seedlings, 12.5 cm-long, green-yellowish leaves from 1.5 month-old seedlings and 16 cm-long yellow leaves from approximately 4 month-old seedlings.

Electron microscopy

Samples for electron microscopy were taken always from the second leaf of pea, bean and tobacco plants. Pieces about 1–4 mm^2 in area were cut from the broadest part of this leaf near the main vein. The material was fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer at pH 7.4 for 2 h, washed in buffer and placed overnight in cold 2% OsO_4 in the same buffer. The specimens, dehydrated in a graded acetone series, were embedded in a low viscosity epoxy resin (Spurr, 1969) and cut on a LKB ultramicrotome. Sections stained with uranyl acetate and lead citrate (Reynolds, 1963) were examined with a Tesla BS 500 EM.

Leaf samples for all experiments were taken from 5 independent experiments.

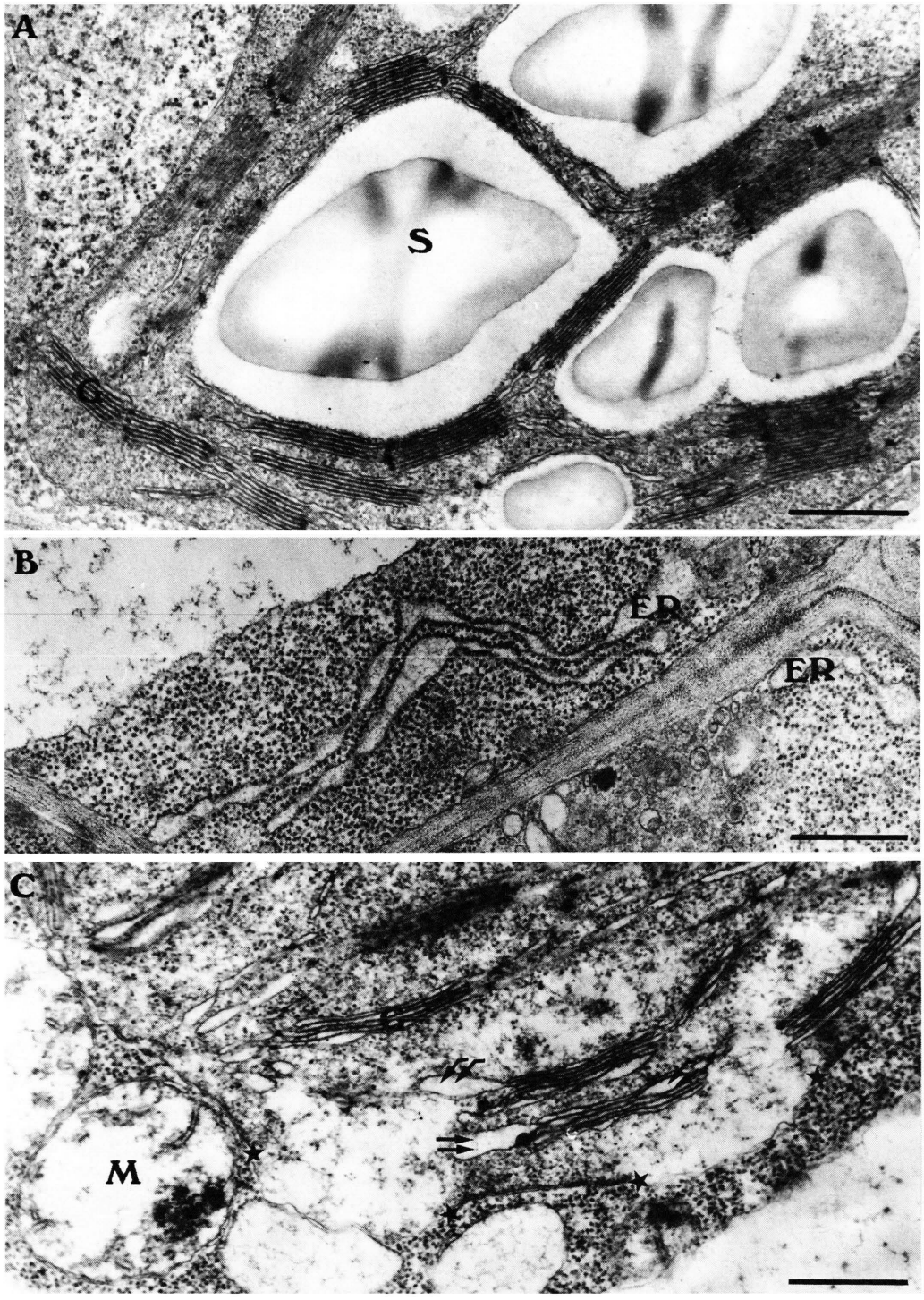

Fig. 1. Portions of mesophyll cells of green pea seedlings A) untreated (control plants), B) and C) treated with 20 mM Phe, placed overnight for 13 hours in darkness and subsequently exposed to white light for 9 h. ER, endoplasmic reticulum; G, granum; M, mitochondrium; S, starch grain; T, thylakoid; double arrows indicate dilations of thylakoids; stars indicate disrupted chloroplast envelope. The markers in all micrographs represent 0.5 μm.

Results and Discussion

Response of chloroplast structure caused by the photodynamic herbicide 1.10-phenantroline (Phe) (green leaves)

Thylakoid, membranes of chloroplast envelope and endoplasmic reticulm (ER) cisternae in mesophyll cells of control pea seedlings did not form any dilations or swellings (Fig. 1A). It was quite different in the case of these membranes in Phe-treated plants and subsequently exposed to light (Figs.1B, C). Ultrastructural changes were manifested in ER cisternae (Fig. 1B), thylakoid swellings and in disruption of the chloroplast envelope (Fig. 1C). Damage to the chloroplasts' ultrastructure, clearly increasing with illumination period, was accompanied by a decreased level of pigments and inhibition of photosynthesis (Mostowska *et al.*, 1991a, 1996) which is probably due to tetrapyrrole-dependent photooxidative mechanisms (Re-

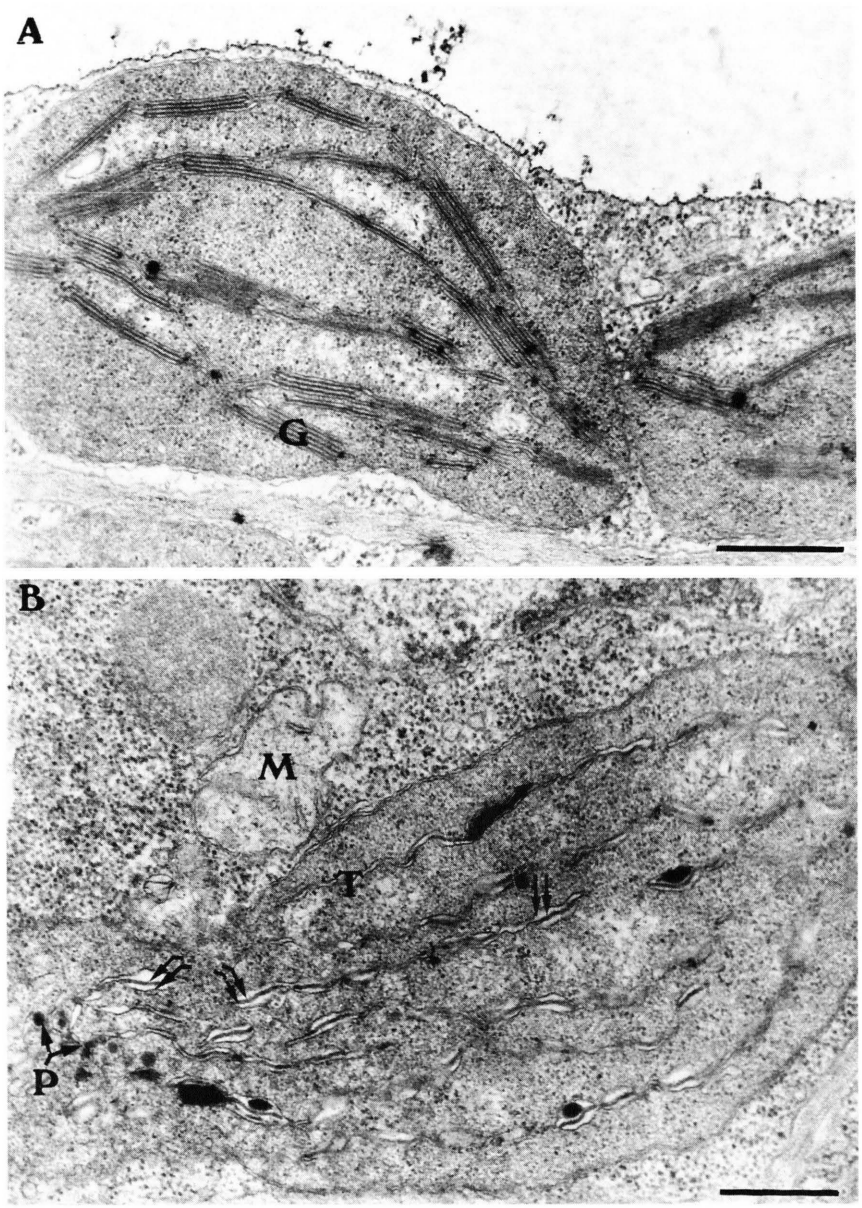

Fig. 2. Chloroplasts of etiolated bean seedlings illuminated with white light A) in normal (21%) oxygen atmosphere, B) in 100% oxygen atmosphere. G, granum; P, plastoglobuli; T, thylakoid; double arrows indicate dilatations of thylakoids. The markers in all micrographs represent 0.5 μm.

beiz *et al.*, 1984). Accumulated porphyrins generate the formation of singlet oxygen which oxidizes the lipoprotein components of cellular membranes that eventually leads to membrane destruction. Because of a similar damage of thylakoid, ER cisternae membranes and also the membranes of the plastid envelope, this mode of action is probably not specific for Phe treatment but may be similar to other herbicides (Böger and Sandmann, 1993) that induce the accumulation of photosensitizing porphyrins .

Response of chloroplast ultrastructure to high oxygen concentration (greening leaves)

Development of plastids in atmosphere of 21% O_2 (normal atmosphere) revealed that after 24 h of greening the chloroplasts contained numerous grana with narrow thylakoids (Fig. 2A). Neither membranes of the chloroplast envelope nor ER cisternae formed any dilations or swellings (Fig. 2A).

High oxygen concentration (100% O_2) not only inhibited the chloroplast development (decay of prolamellar bodies, grana formation, starch grain formation), but similarly to photodynamic herbicides caused also a dilation of thylakoids (Fig. 2B) and swellings of ER cisternae. Chloroplast thylakoid destruction caused by high oxygen concentration was accompanied by pigment photooxidation (Frank and Schmid, 1985; Halliwell, 1981).

We compared the response of the chloroplast structure caused by photodynamic herbicides and high oxygen concentration with symptoms of chloroplast senescence (aging leaves).

Chloroplasts from green, fully developed leaves had only narrow thylakoids in grana (Fig. 3A). Older, green- yellowish leaves represented intermediate stage of chloroplast senescence with swollen thylakoids and increased number of plastoglobuli (Fig. 3B). Chloroplasts from old yellow leaves exhibited the last stage of chloroplast degeneration (Fig. 3C). Chloroplasts from this stage were nearly filled with large plastoglobuli orginating from the destroyed thylakoids. Some rudimentary, swollen thylakoids were visible on the periphery of plastoglobuli (Fig. 3C).

Swelling of the thylakoids and the increased accumulation of plastoglobuli were observed during senescence of chloroplasts in radish seedlings (Meier and Lichtenthaler, 1982).

Results presented above showed different stages of chloroplast response: from swelling and dilations of all kind of membranes in chloroplasts and in mesophyll cells, through their disruption, to particularly large accumulation of plastoglobules originating from the destroyed membranes. Mechanism of membrane swelling is probably the following: reactive oxygen species oxidize the lipoprotein components of thylakoid and other membranes in the cell, that cause membrane damage and leackage of membranes, i.e. an increase in ion permeability.

Change in irradiance is the largest environmental factor modyfying chloroplast ultrastructure. High-light adaptation response resulting in formation of sun- type chloroplasts exibits a lower stacking degree, a high starch content, and a greater number and larger size of plastoglobuli as compared to low-light adaptation response resulting in formation of shade-type (Lichtenhaler *et al.*, 1984). Long-term exposure of chloroplasts to strong light leads to production of reactive oxygen species, which causes the photo-oxidation of pigment and chloroplast photodestruction (Powles, 1984).

Ultrastructural alterations to chilling and high light were observed in pea and cucumber chloroplasts (Wise and Naylor, 1987). Pea chloroplasts affected by low temperature had fairly normal thylakoid system but dilated thylakoids after longer illumination. The cucumber chloroplasts showed a more rapid decline in ultrastructure; they had dilated thylakoids and completely destroyed chloroplast membranes after longer illumination. In the development of chilling injury in light, photoperoxidative processes were shown to play the main role (Wise and Naylor, 1987).

Similar structural changes were observed in chloroplasts exposed to a heat stress. Pea chloroplasts had swollen thylakoids and a disorganized structure (Velitchkova *et al.*, 1989). Tomato chloroplasts had a desintegrated thylakoid network, a disrupted chloroplast envelope and a large amount of plastoglobuli (Starck, *et al.*, 1993).

Ultrastructural changes of pea cells exposed for a few hours to the ultraviolet B-radiation consisted mainly in swelling of thylakoid, disruption of chloroplast envelope and of both granal and stromal thylakoid network (Brandle *et al.*, 1977). Damage in ultrastructure of chloroplast membrane showed

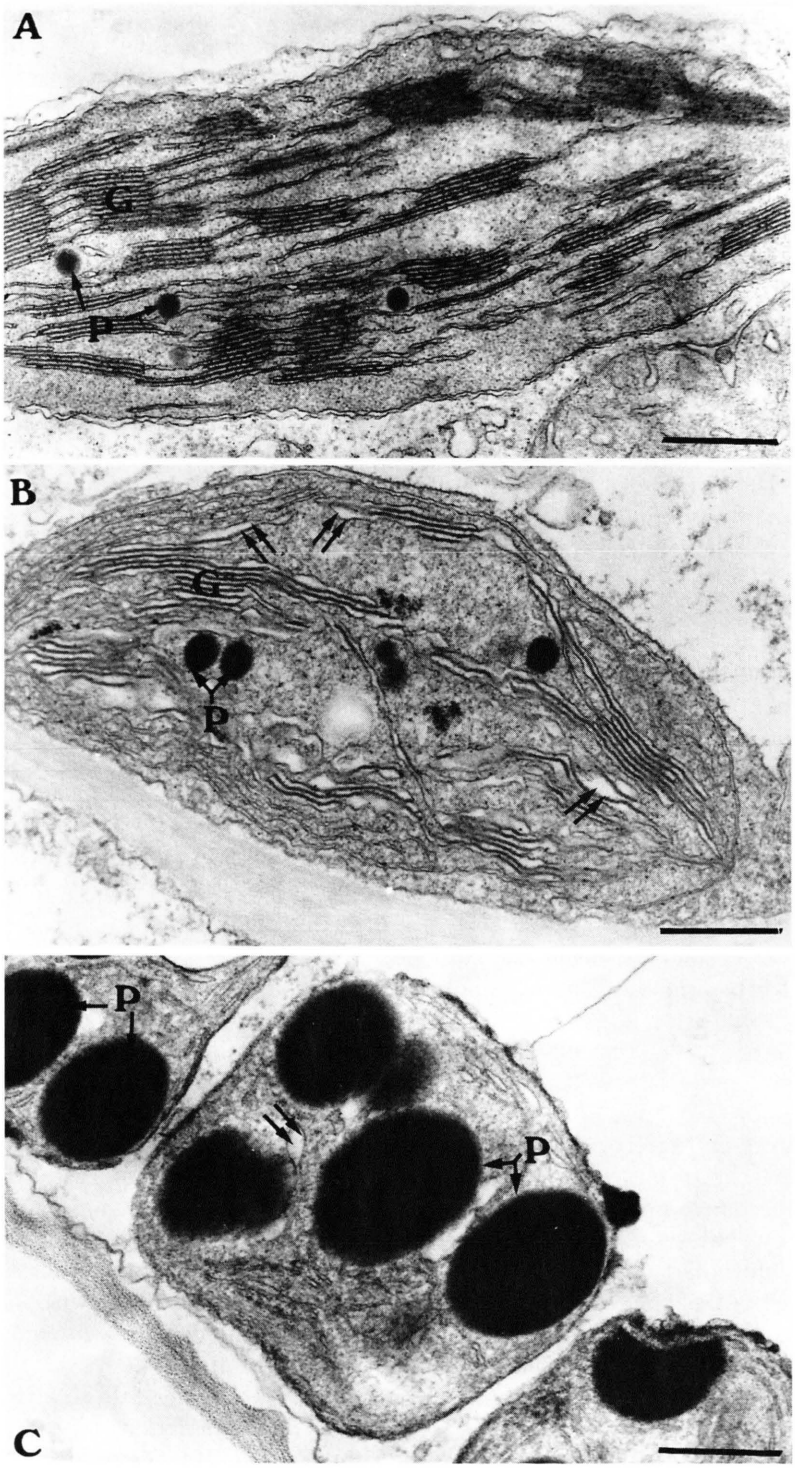

Fig. 3. Chloroplasts of tobacco seedlings A) from 1 month-old, green leaf, B) from 1.5 month-old, green-yellowish leaf, C) from 4 month-old, yellow leaf. G, granum; P, plastoglobuli; arrows indicate dilations of thylakoids. The markers in all micrographs represent 0.5 μm.

typical oxidative changes, and was often visible after longer time due to the cumulative character of the UV-B radiation action (Brandle *et al.*, 1977).

Heavy metals at high concentrations also generate the toxic oxygen species and thus cause peroxidative breakdown of membrane lipids. Spinach chloroplasts treated with excess copper, at an intermediate stage of degeneration contained an increased number of plastoglubuli built-up probably from the lipid material that originated from thylakoid breakdown. At the late stage of degeneration the chloroplasts contained degradated thylakoids and disrupted chloroplast envelope (Baszynski *et al.*, 1988).

Changes in the structure of mesophyll cell chloroplasts of radish leaves (Miyake *et al.*, 1989) and spruce needles (Schiffgens-Grüber and Lütz, 1992; Ebel *et al.*, 1990) caused by ozone consisted mainly in swelling and breaking of thylakoids and a massive accumulation of plastoglobuli (Miyake *et al.*, 1989; Schiffgens-Grüber and Lütz, 1992; Ebel *et al.*, 1990). Some lipids available from the breakdown of the thylakoids are deposited in plastoglobuli (Lichtenthaler, 1968).

The response of plant on the cellular level induced by other external factors, such as drought-stress, salt-stress, high light, gives a very similar pattern of changes to those already presented and is accompanied by lower chlorophyll levels and lower photosynthesis rates (see e.g.: Powles, 1984; Tuba *et al.*, 1993; Gosset *et al.*, 1994).

An outlook of the responses of the chloroplast structure and function to various stress factors, both natural and anthropogenic, leads to general conclusion presented in Fig. 4. Most of these factors induces an oxidative stress and often gives similar symptoms of structural damage and dysfunction independent of the primary stressing factor: swelling of thylakoids, intensive plastoglobuli accumulation, disruption of the chloroplast envelope, photodestruction of pigments and inhibition of photosynthesis.

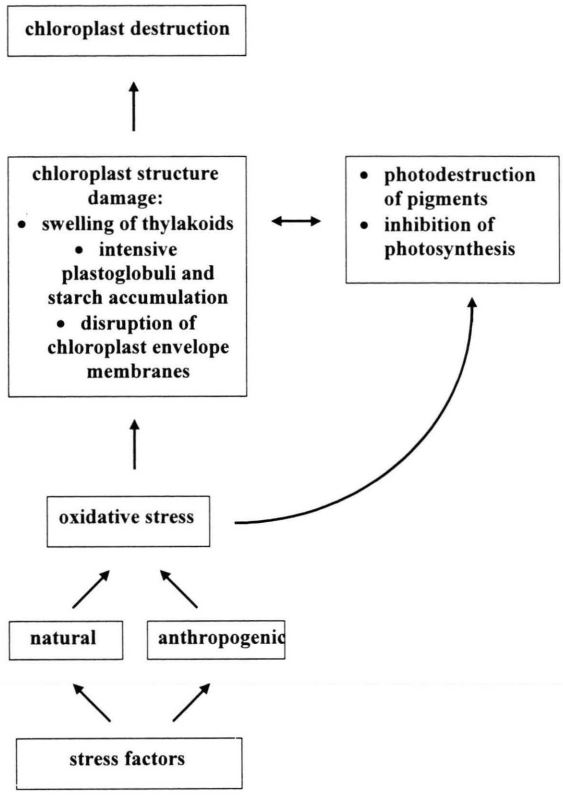

Fig. 4. Stress factors inducing oxidative stress leading to changes in chloroplast structure and function.

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