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Changes in morphological and anatomical structure of cabbage (*Brassica oleracea* L.) outer leaves and in ultrastructure of their chloroplasts caused by an *in vitro* excess of nickel

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Abstract

Morphological, anatomical and ultrastructural changes, the quantity and quality of stomata, and the chlorophyll (Chl) content in primary outer leaves of cabbage plants cv. Sława from Enkhouizen were examined. The plants were grown in agar with basic MS medium containing added nickel (as $NiSO_4 \times 7\ H_2O$) in concentrations of 0, 5, 10, and 20 g m^{-3} (Ni_0, Ni_5, Ni_{10}, Ni_{20}). Reduction of leaf blade area, of succulence and of leaf density, and growth of specific leaf area were noticed in plants treated with all concentrations of Ni. In Ni-treated plants the total number of stomata and open stomata decreased, and the number of defective stomata in both adaxial and abaxial side of leaves was higher. In all Ni-treated samples the volume of spongy and palisade mesophyll cells was smaller in comparison to control, and it was decreasing when the Ni concentration was increasing; at the same time, the number of mesophyll cells on the same area of cross sections of leaves was increasing. In comparison to control, the intercellular spaces of mesophyll tissue decreased in Ni_{10} and Ni_{20} plants and increased in Ni_5 plants. In Ni_5 plants the number of chloroplasts in mesophyll cells was higher than in the Ni_0 control. Reduction of grana size and increase of number of non-appressed lamellae, which often had central arrangement, were observed. In the Ni_{10} and Ni_{20} plants, the number and size of chloroplasts decreased, and their internal membranes (especially grana) were reduced and swollen. In Ni_5 plants the concentration of Chl in leaves was slightly higher than in the control; in Ni_{10} and Ni_{20} plants it was lower than in the control.

Additional key words: cell number; chlorophyll; grana; Ni; non-appressed thylakoids.

Introduction

Heavy metals, such as nickel, have received considerable attention over the last years as a result of increased environmental pollution from agricultural and industrial

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activities and urban wastes. In mobile forms (especially in acid environment), Ni is very easily absorbed by plant roots and translocated into vegetative and generative organs (Cataldo *et al.* 1978a,b, Soon *et al.* 1980). Studies on higher plants grown in Ni-contaminated soils or media show reduction of plant growth, of their fresh and dry mass, and disturbance of metabolic and physiological processes (Mishra and Kar 1974, Hara and Sonoda 1979, Van Assche and Clijsters 1990). Photosynthesis is the most sensitive process affected by this metal. The influence of Ni on photosynthesis is multidirectional, and the mechanism of photosynthesis inhibition by Ni may be different in isolated chloroplasts and in whole plants (Clijsters and Van Assche 1985). Nickel in excessive concentration disturbs photosynthetic electron transport (Tripathy *et al.* 1981, Mohanty *et al.* 1989, Krupa *et al.* 1993) as well as CO_2 assimilation (Sheoran *et al.* 1990b). It affects the contents of Chls and carotenoids (Veeranjaneyulu and Das 1982, Sheoran *et al.* 1990a,b, Krupa *et al.* 1993), and electron transport chain intermediates (such as cytochromes *f*, b_6 and b_{559}, ferredoxin, and plastocyanin) in leaves (Veeranjaneyulu and Das 1982). In whole plants, Ni can inhibit photosynthesis by decreased stomatal conductance and decreased CO_2 assimilation rate (Sheoran *et al.* 1990a).

The disturbance of photosynthesis caused by Ni, particularly at a molecular level, has recently been studied. However, the influence of this metal on leaf morphological and anatomical structures and on the ultrastructure of their chloroplasts is less known. This is why I studied the changes of these leaf parameters important for photosynthesis under the excess of Ni in the early phase of cabbage plant growth.

Materials and methods

Plants: Seeds of cabbage (*Brassica oleracea* L.) cv. Sława from Enkhouizen were surface-sterilized in NaOCl (*ca.* 1 % active chlorine) for 5 min, rinsed in sterilized distilled water, and placed on agar medium in glass pots. In control series, the medium contained macroelements and microelements according to Murashige and Skoog (1962) and 0.8 % agar. In experimental series, the basic MS medium was supplemented with nickel added as $NiSO_4 \times 7\ H_2O$ in concentrations of 5, 10, and 20 g m^{-3} (Ni_5, Ni_{10}, and Ni_{20} plants). The pH value of the media was 5.2. Seedlings grew *in vitro* under an irradiance of 125 W m^{-2} with 16 h photoperiod for 21 d.

Start of growth of primary, outer leaves, changes in their morphological structure, colour, shape, and appearance of chlorosis and necrosis were noted. 21 d from seed germination the analysed leaves from each series were prepared for examination. Fresh and dry masses and leaf areas were measured to assess succulence, specific leaf area, and tissue density. Leaf area was measured by means of cut-and-weight method of the leaves' photocopies. Dry mass of leaves was measured by drying the material overnight at 70 °C, followed by 1 h at 105 °C.

Microscopic study: Samples from the middle area of leaf blades were hand cut and fixed in 3.5 % glutaraldehyde solution buffered in 0.05 M sodium cacodylate, pH 6.9, for 8 h at room temperature, for both the light microscopy (LM) and transmission electron microscopy (TEM). The samples were further postfixed for 1 h

in similarly buffered 2 % OsO_4, dehydrated in graded ethanol, and embedded in an *Araldit* epoxy resin. For LM, semithin sections were cut on a *LKB* ultramicrotome; they were later stained in 0.5 % toluidine blue 0 in 0.5 % borax solution, and then examined and photographed with a *Zeiss* light microscope. For TEM, ultrathin sections were stained with uranyl acetate as well as with Reynolds lead citrate solutions for 15 min. The samples were examined in a *Tesla BS 613* transmission electron microscope.

Morphometric study: The thickness of leaf blades from each series was measured by LM with an ocular micrometer. The number of stomata on the adaxial side (upper leaf surface) and abaxial side (lower leaf surface) of leaves were determined per unit area (1 mm^2) of stripped upper and lower leaf epidermis, and also on replicas of both leaf surfaces. Replicas were made by coating of thin layer of *DPX* (*BDH Chemicals*, Poole, UK) on the leaf surface. When the *DPX* had dried, the replicas were pulled off the leaf surface and observed in a LM. The quality of stomata was examined by LM and TEM. The number of chloroplasts per cell section was determined by TEM.

Leaf Chl content: 1 g of fresh leaves was homogenized in a small quantity of 80 % acetone, and centrifuged at 10 000×*g* for 15 min. The transparent supernatant was then filtered and brought to 10 cm^3 with 80 % acetone (Arnon 1949). Absorbance (*A*) was measured at 645 and 663 nm using an *LKB Ultraspec* spectrophotometer.

Results

Macroscopically, the primary, outer leaves of about 80 % of cabbage seedlings from the control series emerged 6-7 d from seed germination. In the Ni_5 samples the initiation of leaf growth was slightly accelerated (by 1-2 d), and in Ni_{10} and Ni_{20} samples it was delayed by 1-2 and 4-5 d, respectively, in comparison with the control. The 21-d-old cabbage leaves of Ni_{10} and Ni_{20} plants showed the characteristic edge chlorosis and interbundle chlorosis; the leaves of Ni_{20} plants were chlorotic from the very beginning of growth.

The Ni_5 plants showed the decrease of leaf area up to about 5.3 % as compared with control, and the Ni_{10} and Ni_{20} plants showed reduction of leaf area up to 34.2 and 64.2 %, respectively, in comparison with control (Table 1). Succulence and leaf density decreased in comparison with control in all three Ni samples; however, the degree of reduction of these indicators was different (Table 1). Together with the increase of Ni concentration, succulence was reduced more than leaf density. Specific leaf area (contrary to succulence and leaf density) increased together with the increase of Ni concentration, and it was larger than in the control in all the Ni samples (Table 1). Decrease of succulence and leaf density and increase of specific leaf area as compared to control indicated a real water deficiency in outer leaves of cabbage plants grown on the medium with the addition of nickel.

Light micrographs of cross sections of outer leaves from control and Ni-treated cabbage plants showed anatomical differences among the examined samples (Fig. 1). The thickness of leaf blade was reduced in Ni_5, Ni_{10}, and Ni_{20} plants in relation to Ni_0 plants by about 7.5, 29.9, and 23.9 %, respectively (Table 1, Fig. 1). In Ni_5

plants the size of spongy and palisade cells was a little smaller than in the control, but the intercellular spaces of mesophyll tissue were larger than in the control (Fig. 1). No differences were observed in the size of chloroplasts between the Ni_0 and Ni_5 samples, but chloroplasts were more numerous in the Ni_5 sample. The size of mesophyll cells and of intercellular spaces, the size and number of chloroplasts in Ni_{10} and namely in Ni_{20} plants were reduced in comparison to Ni_0 (Fig. 1).

Table 1. Characteristics of outer leaves of Ni-treated cabbage plants. The value represent mean ± SE, $n = 50$; *$n = 10$ (% of control in parentheses). The specific leaf area is defined as leaf area per unit fresh mass, the succulence as water content per unit area, and the leaf density as dry mass per unit area.

Parameter	Ni_0	Ni_5	Ni_{10}	Ni_{20}
Leaf area [cm^2]	10.4 ± 1.0 (100)	9.9 ± 1.1 (94.7)	6.8 ± 0.8 (65.8)	3.7 ± 1.1 (35.8)
Thickness of leaf blade * [μm]	670.6 ± 17.2 (100)	620.2 ± 12.6 (92.5)	470.0 ± 23.1 (70.1)	510.4 ± 24.0 (76.1)
Specific leaf area [$cm^2\ kg^{-1}$(FM)]	325 ± 14 (100)	340 ± 10 (104.6)	555 ± 8 (170.8)	628 ± 22 (193.2)
Succulence [$g(H_2O)\ m^{-2}$]	278.5 ± 30.4 (100)	265.9 ± 2.78 (95.5)	168.1 ± 2.43 (60.4)	134.6 ± 20.7 (48.3)
Leaf density [g(DM) m^{-2}]	29.2 ± 0.1 (100)	28.5 ± 0.1 (97.7)	24.9 ± 0.1 (85.2)	24.5 ± 0.1 (84.1)

LM micrographs of cross leaf sections also showed differences in the number of mesophyll cells located on the same area (cross sections of leaves were photographed at the same magnification). In all samples with nickel, the number of mesophyll cells on the surface of examined fragment was bigger than in the control, and it increased when the Ni concentration increased (Fig. 1).

The total number of stomata per unit leaf area on both adaxial and abaxial sides of leaves decreased with the increase in Ni concentration; however, it was smaller in all Ni-treated samples than in the control (Table 2). In the leaves of all samples with nickel, four categories of stomata were observed: normal stomata (similar to control stomata), big stomata, small stomata, and defective stomata. Two categories of defective stomata were observed: small stomata (of the size similar to undeveloped stomata), and normal stomata (of the size similar to control stomata or normal, undamaged stomata). All defective stomata were closed. Their guard cell borders were difficult to discern as compared to undeveloped and normal stomata. The guard cells of defective stomata examined by TEM were of different sizes, and their protoplasts were degenerating. The cell organelles visible in these cells were damaged, and chloroplasts were not usually noticed in them. Normal and small stomata were open to a different degree. The number of open stomata in leaves of Ni-treated plants was smaller than in the control, and it was decreasing when the Ni concentration increased (Table 2). Big stomata were open in all samples with nickel, and their aperture was much bigger than that of remaining stomata, including control stomata.

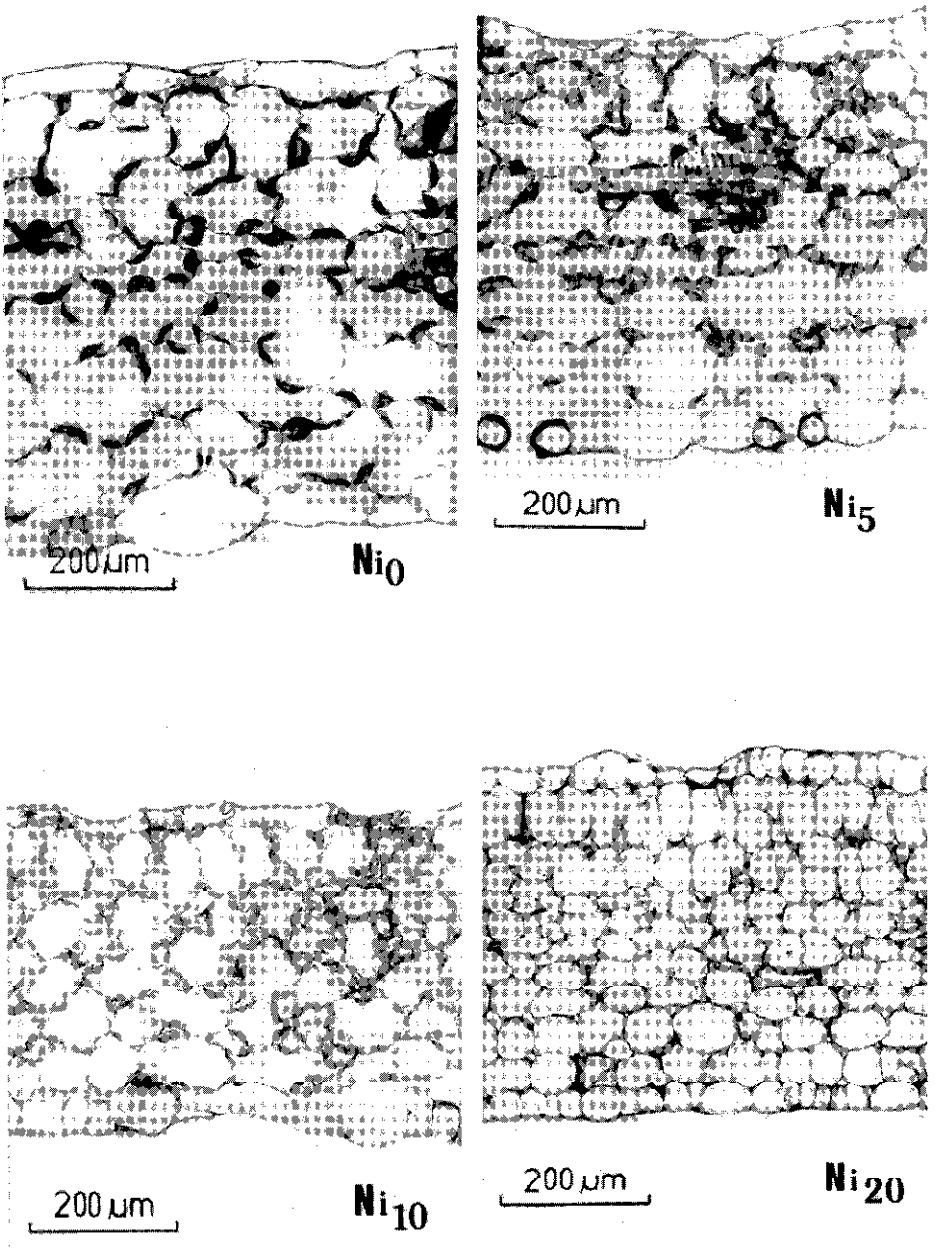

Fig. 1. Light micrographs of outer leaf cross sections from control plant (Ni_0) and from Ni-treated plants Ni_5, Ni_{10}, and Ni_{20}.

The TEM examination showed differences in number, shape, size, and internal structure of chloroplasts among the samples examined. Only in the Ni_5 sample the number of chloroplasts per cell section was similar to the Ni_0 sample; in the Ni_{10} and Ni_{20} samples it was smaller than in the control, the largest decrease being found in

Table 2. Frequency of different types of leaf stomata of Ni-treated cabbage plants. Numbers of stomata per mm^2 of leaf surface are taken for 100 %. Means ± SE of three experiments with 5 replicas in each experiment and 10 microscopic fields (×40) observed for each replica.

Surface	Characteristic	Ni_0	Ni_5	Ni_{10}	Ni_{20}
Adaxial	Number of stomata	136.2 ± 6.4	92.7 ± 6.4	47.7 ± 5.4	21.0 ± 4.2
	% of normal stomata	94.9 ± 2.3	55.8 ± 4.1	50.4 ± 3.8	44.8 ± 2.0
	% of big stomata	1.1 ± 0.2	20.5 ± 2.8	10.4 ± 2.1	6.2 ± 0.6
	% of small stomata	2.8 ± 0.7	16.1 ± 3.2	23.8 ± 2.6	31.6 ± 2.4
	% of defective stomata	1.2 ± 0.4	7.6 ± 1.1	15.4 ± 1.8	17.4 ± 2.1
	% of open stomata	85.0 ± 3.3	81.7 ± 2.4	62.6 ± 3.1	38.4 ± 3.2
Abaxial	Number of stomata	227.1 ± 5.9	105.0 ± 6.0	105.0 ± 8.2	48.7 ± 6.8
	% of normal stomata	92.6 ± 2.6	52.2 ± 5.0	43.2 ± 4.2	23.4 ± 1.8
	% of big stomata	2.3 ± 0.4	27.2 ± 3.1	11.5 ± 1.5	8.6 ± 0.8
	% of small stomata	3.4 ± 1.1	12.3 ± 1.8	26.8 ± 2.2	41.2 ± 2.5
	% of defective stomata	1.7 ± 0.3	8.3 ± 0.7	18.5 ± 1.9	26.8 ± 3.0
	% of open stomata	82.7 ± 2.8	76.4 ± 2.7	54.5 ± 3.0	27.0 ± 2.8

Table 3. Chloroplast number and chlorophyll content in outer leaves of Ni-treated plants. The values represent the mean ± SE, $n = 50$, $^*n = 5$ (% of control in parentheses).

	Ni_0	Ni_5	Ni_{10}	Ni_{20}
No. of chloroplasts per cell section	8.2 ± 1.1 (100)	9.5 ± 0.7 (115.8)	5.8 ± 1.4 (70.7)	4.1 ± 1.6 (50.0)
Chlorophyll ($a+b$) [g kg^{-1} (FM)]	0.78 ± 0.02 (100)	0.82 ± 0.02 (105.1)	0.59 ± 0.03 (75.6)	0.47 ± 0.02 (60.3)

Ni_{20} (Table 3). Most chloroplasts in the Ni_5 sample were similar to the chloroplasts in Ni_0 plants with respect to shape, size and ultrastructure (Fig. 2, *left*). In about 25-30 % of chloroplasts, the number of grana was smaller, they were shorter, irregular, and contained fewer thylakoids. The number of non-appressed lamellae was larger, and they often had central arrangement. Numerous chloroplasts of the Ni_{10} samples were round, sometimes even spherical, and they were smaller than the chloroplasts of Ni_0 plants (Fig. 2, *middle*). Their membranes were reduced (particularly appressed membranes) and often swollen. These chloroplasts usually contained large plastoglobuli (see *arrows*), and had only a small amount od starch. Numerous, electron-opaque precipitates were identified on the surface of internal membranes and in stroma (Fig. 2, *insert*); such precipitates might be induced by uranyl-acetate

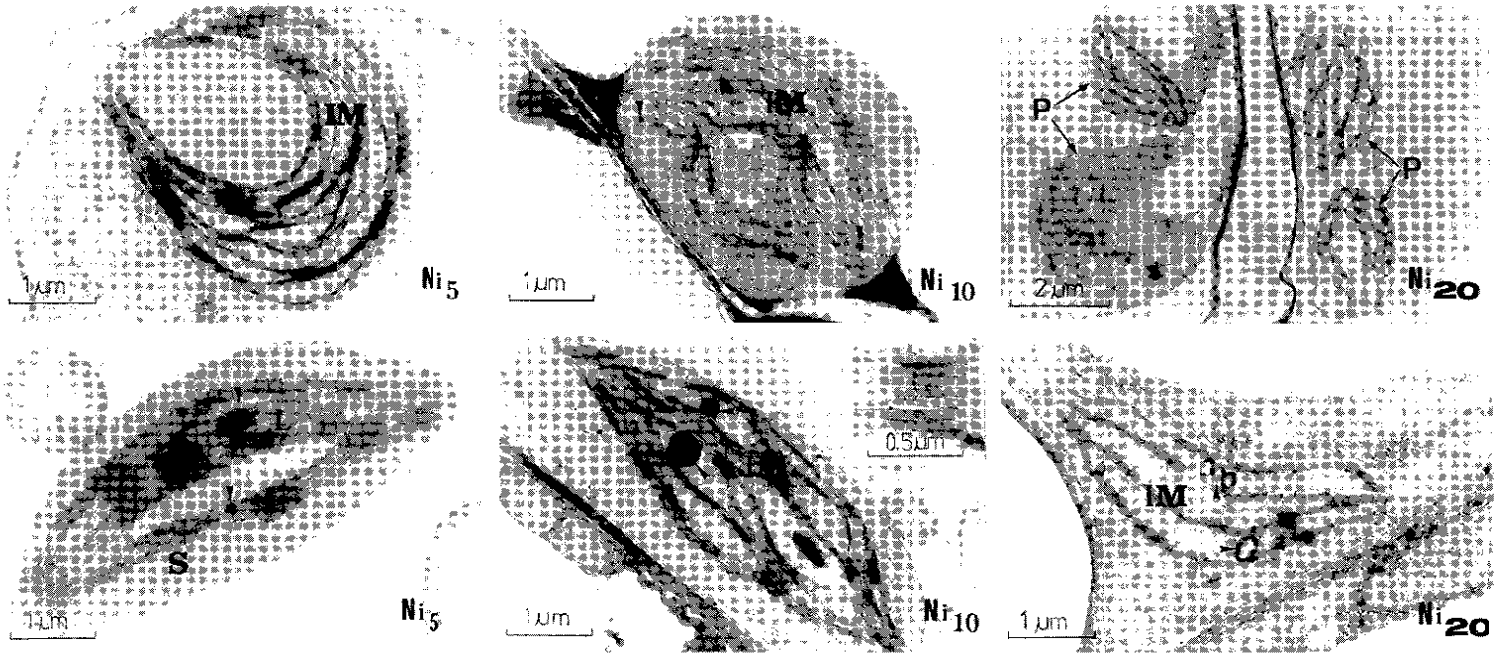

Fig. 2. Comparison of chloroplast morphology and their internal structure in outer leaves of cabbage Ni_5, Ni_{10}, and Ni_{20} plants. G - granum; IM - intercellular membranes; L - thylakoic; P - plastid; S - stroma.

staining. The chloroplasts of Ni_{20} plants were small, and often morphologically deformed (Fig. 2, *right*). Their internal membranes did not form grana or sporadically formed small grana, and the few non-appressed lamellae were usually swollen. They often contained plastoglobuli that were electron-empty inside (see *arrows*).

Morphological and anatomical changes of leaves and chloroplasts were accompanied by a change in the content of Chl. In comparison to Ni_0 plants, the content of Chl increased in Ni_5 plants, but in leaves of Ni_{10} and Ni_{20} plants it was reduced by 24.4 and 39.8 %, respectively (Table 3).

Discussion

Response of plants to heavy metals, including Ni, depends, among other things, on the metal concentration, on duration of its effect, and on the growth phase of a given plant or organ. The sensitivity of young plants and their organs (including leaves) to toxic influence of metals is smaller than the sensitivity of older plants and their organs (Barceló *et al.* 1988, Sheoran *et al.* 1990b, Maksymiec *et al.* 1995). My results confirm that the toxic effects on the examined cabbage leaf dependent on the concentration of Ni are observed in the early phase of leaf growth, *i.e.*, at the beginning of stage II. Of course, leaf growth in cabbage is longer than in other plant species.

In all applied concentrations, Ni affected morphological, anatomical, and ultrastructural features of a leaf that determine photosynthesis both on molecular and gas-exchange levels. The reduction of the surface of leaf blade, of the volume of intercellular spaces of mesophyll tissue (except for the Ni_5 sample), and of the number of total stomata and open stomata certainly affect gas exchange rate. Nickel reduces net photosynthetic rate, as shown already by Sheoran *et al.* (1990a) for *Cajanus cajan*. The damage or/and disturbance of stomata differentiation and function I observed is certainly reflected in a reduction of stomatal conductance.

The observed decrease of succulence and leaf density and the increase of specific leaf area point to water deficiency in leaves, that is one of the main factors which inhibit photosynthesis (Govindjee *et al.* 1981, He *et al.* 1995).

The reduction of Chl concentration in Ni_{10} and Ni_{20} leaves was accompanied by interbundle chlorosis, which is characteristic for the excess of nickel. Nickel reduces the Chl content in plant leaves of different plant species (Veeranjaneyulu and Das 1982, Clijsters and Van Assche 1985, Sheoran *et al.* 1990b, Krupa *et al.* 1993). Heavy metals decrease the concentration of Chl in leaves either by inhibition of its biosynthesis or by induction of its degradation. In algae, Ni stimulates the activity of chlorophyllase (Abdel-Basset *et al.* 1995), and a similar activity may be expected in higher plants. My results point to the inhibition of Chl biosynthesis, as shown by leaf chlorosis at the very early stage of leaf growth. As Ni is an element antagonistic to iron, and restricts its absorption by plants (Bergmann 1988), toxic effects may be induced by the Ni-Fe interaction. In Ni_5 plants, the decrease of mesophyll cell volume was accompanied by larger concentration of chloroplasts than in the control. In internal structure of these plastids the reduction of grana was accompanied by the

increase of amount of non-appressed lamellae and by their central arrangement. These changes may be an adaptation to the moderate stress. In Ni_{10} and Ni_{20} plants the number and size of chloroplasts were reduced. These plastids were usually deformed and shrunk, most probably because of loss of water. In their internal structure a reduction of internal membranes was observed (particularly of grana), and they were swollen. This is probably connected with disturbances in transport of electrons in the photochemical phase of photosynthesis, observed in the presence of Ni both *in vitro* and *in vivo* (Tripathy *et al.* 1981, Mohanty *et al.* 1989, Krupa *et al.* 1993, Csatorday *et al.* 1987). The mechanism of damage of thylakoid membranes in the presence of metals may be either a result of (*1*) lipid peroxidation of membranes (Sandmann and Böger 1980), or (2) their hydrolysis and liberation of fatty acids (Skórzyńska *et al.* 1991, Maksymiec *et al.* 1992). Fatty acids reduce the activity of photosystem 2 (Goldbeck and Warden 1984). The first suggestion may be confirmed by the fact that Ni induces free-radical reactions in plant cells (Pandolfini *et al.* 1992), including lipid peroxidation of cell membranes. Nickel also associates with chloroplast membranes (Veeranjaneyulu and Das 1982), and my results confirm it indirectly. Only on the membranes of chloroplasts of Ni-treated plants there were electron dense precipitates, probably precipitates of Ni or its compounds. Nickel associated with membranes can, therefore, directly induce free-radical reactions.

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