Changes of some photosynthetic parameters were studied in younger and older leaves of cabbage plants (*Brassica oleracea* L., cv. Gloria di Enkhouizen 2) grown in water solution (pH 5.7) containing Cu(II)-EDTA chelate, in which the molar ratio of the metal (M) to the ligand (L) was 1:0.5 and 1:1. Such parameters as the total leaf area of the plant, younger and older leaf lamina surface and thickness, chloroplast number in mesophyll cells, the concentration of total chlorophyll and photosystem 2 activity were studied at the same concentration of chelate M:L 1:0.5 and M:L 1:1 in the medium ($\text{Cu}^{2+}$ $\text{L}^{-1}$ $\text{dm}^{-3}$) and at a very similar concentration of Cu in leaves, absorbed from these forms by the plants. The Cu(II)-EDTA chelate in which the molar ratio M:L was 1:0.5 was more toxic than the chelate in which the molar ratio M:L was 1:1. The Cu(II)-EDTA chelate, regardless of the molar ratio M:L, reduced the assimilative surface of plants, decreased the content of the total chlorophyll in the leaves and reduced their phytochemical efficiency. It also reduced the number of chloroplasts in mesophyll cells and induced changes in their ultrastructure similar to the changes taking place during the ageing of leaves. The chelate also caused the reduction of the area of young leaves, accompanied by a higher accumulation of chlorophyll than in the control. The leaves were photochemically efficient and their chloroplasts showed no damage.

**Keywords:** Cabbage, copper chelate, cooper toxicity, EDTA, photosynthesis

Copper in trace amounts is necessary for the development of plants; however, it becomes toxic to plants when it is absorbed by them in excessive amounts [1–4]. Copper in excess concentration causes such phenomena as inhibition of plant growth, malformation of the root, leaf chlorosis and necrosis; it also disturbs many metabolic and physiological processes, especially photosynthesis [1, 2, 5–9]. Copper is a $d$-electron element (a metal of the transition group) easily forming complexes, including chelate complexes with organic compounds [1, 10, 11]. Consequently, it occurs mostly in the form of organic chelates both in abiotic environment (soil, water) and in biological
systems [1]. Also in soil solution, besides a free ion Cu\(^{2+}\), this element occurs mostly in the form of complex ions and mainly in combination with organic ligands; thus, it is available to plants in these forms [1–3, 12–15].

Recently, copper has been introduced into the environment in the form of organic chelates in increasingly larger amounts, including the form of Cu(II)-EDTA chelate, which due to the chemical nature of a ligand, its chemical structure and a high stability constant \((K = 18.78 [16])\), is relatively resistant to chemical and biological degradation [1, 17]. Since this chelate is used in many different branches of industry, it is introduced into the environment together with municipal and industrial waste and with the sludge used in soil fertilization [1, 2]. It is also introduced together with microelement fertilizers, fungicides and pesticides [1, 15]. Chelation of copper by EDTA is one of the most popular methods of remediating the soil polluted with this metal [17–19]. It is also one of the methods used in medicine in the case of copper poisoning, i.e. in the so-called chelatotherapy [1].

Unlike the phytotoxicity of inorganic compounds of copper, the phytotoxicity of Cu(II)-EDTA chelate has not been studied so extensively and relatively little is known about it. Consequently, the study presents the results of experiments concerning the toxic effect of Cu(II)-EDTA chelate, in which the molar ratio of the metal to the ligand was 1:0.5 and 1:1, on some parameters of the photosynthetic apparatus of cabbage plants cv. Gloria di Enkhouizen 2.

**Materials and methods**

**Plant culture and experimental design.** Fifteen-day seedlings of cabbage (*Brassica oleracea* L. cv. Gloria di Enkhouizen 2) were cultivated hydroponically in aerated Hoagland’s nutrient solution [20] at pH 5.7. The nutrient solutions were supplemented with Cu(II)-EDTA chelate. The Cu(II)-EDTA was prepared by mixing Cu (as CuSO\(_4\) · 5 H\(_2\)O) and Na\(_2\)EDTA in molar ratio of metal (M) to ligand (L) 1:0.5 and 1:1. The blue colour that appeared after mixing indicated a very rapid formation of the complex. The copper chelates (M:L 1:0.5 and 1:1) were added to the Hoagland’s solution in concentration of 24 \(\mu\)mol Cu · dm\(^{-3}\), the Cu(II)-EDTA (M:L 1:1) was added also in concentration of 72 \(\mu\)mol Cu · dm\(^{-3}\). Plant growth conditions were day and night, 16 and 8 h, respectively, under actinic red light illumination 375 \(\mu\)E · m\(^{-2}\) · s\(^{-1}\), at a temperature of 25 ± 1 °C and a relatively humidity of 75 ± 5%.

After 14 days of Cu(II)-EDTA treatment, the plants were harvested, next the root length was measured and crop of plant dry mass was determined. Such parameters of the photosynthetic apparatus as the area and thickness of leaf lamina, the content of chlorophyll, the number and ultrastructure of chloroplasts and the activity of photosystem 2 (PS2) *in vitro* were studied in the youngest leaves (i.e inner ones) and in older leaves (but not in the oldest ones, which lost turgor and withered relatively fast). Young leaves were differentiated and grew in the presence of copper chelate, whereas older leaves were formed at the time when the seedlings were transferred to a medium with copper chelate addition and their further growth took place in its presence. During
harvest and measurement of the parameters under study these leaves were in the state of turgescence.

**Cu(II)-EDTA toxicity indexes.** On the basis of the root length the root index toxicity (RIT) was determined according to Willkins [21] and on the basis of the crop of plant dry mass the toxicity index (TI) was determined according to Berry and Wallace [22].

**Cu concentration.** Dried leaf material was ground and wet-digested in a nitric(V)-chloric(VII) acid (4:1 v/v) solution. Total concentration of Cu was determined by atomic absorption spectrophotometer (Perkin Elmer 1100) according to the method described by Ostrowska et al. [23].

**Morphometry.** Leaf blade area (total per plant, younger and older) was measured using MK2 area meter. The leaf lamina thickness was measured by light microscope (LM) with micrometer in ocular. The number of chloroplasts per cell section was determined by TEM. For light and transmission electron (TEM) microscopes study, the sections were prepared according to the methods described by Gerlach [24], Toth [25] and Wróbel et al. [26].

**Leaf chlorophyll assay.** To determine the total leaf chlorophyll \((a + b)\), 1 g of fresh inner and outer leaves, they were homogenized in 80 % acetone and centrifuged at 10 000 g for 15 min [27]. The transparent supernatant was filtered and made up to 10 cm³ with 80 % acetone. Absorbance was measured at \(\lambda = 663\) and 645 nm in a LKB Ultraspec spectrometer.

**DCPIP photoreduction measurement.** The electron transport through PS2 was determined by photoreduction of DCPIP (2,6-dichlorophenol-indophenol) as an electron acceptor, measured by the spectrophotometer used in the split beam mode [28]. The DCPIP photoreduction was assayed by recording the absorbance changes at \(\lambda = 600\) nm after illuminating chloroplast equivalent to 15 µg of chlorophyll in 3 cm³ of reaction buffer containing 20 µmol DCPIP. The intensity of the illuminating red actinic light was 1500 \(\mu E \cdot m^{-2} \cdot s^{-1}\). The chloroplasts were isolated according to Sabat et al. [29].

**Statistical analysis.** Variance analysis of experimental plant parameters (ANOVA) was carried out, followed by a test checking the significant difference (LSD) with \(p = 0.05\).

**Results and discussion**

The toxicity of copper in the form of Cu(II)-EDTA chelate to the photosynthetic apparatus of cabbage plants cv. Gloria di Enkhouizen 2 depended on its concentration in the leaves and on the degree of Cu chelated by EDTA. The concentration of Cu in the leaves of plants treated with Cu(II)-EDTA in which the molar ratio M:L was 1:0.5 was over two times higher than in the leaves of plants treated with Cu(II)-EDTA in which the molar ratio M:L was 1:1, when the amount of chelate in the medium was the same, \(ie\) 24 \(\mumol\) Cu \(\cdot dm^{-3}\) (Table 1). Similar content of Cu in leaves of plants treated with Cu(II)-EDTA (M:L 1:1) was observed when its amount in the medium was three times higher than that of the chelate in which the molar ratio M:L was 1:0.5 (Table 1). The
content of Cu in cabbage leaves corresponded to the calculated Cu toxicity indexes (ie RTI and TI). The degree of toxicity of the Cu(II)-EDTA chelate in which the molar ratio M:L was 1:1 was smaller than that of the chelate in which the molar ratio was 1:0.5, even when the content of this metal in the leaves of cabbage cultivar under study was very similar (Table 1).

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Growth medium Cu concentration [μmol · dm⁻³]</th>
<th>LSD(p ≤ 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cu(II)-EDTA (M:L 1:0.5)</td>
<td>Cu(II)-EDTA (M:L 1:1)</td>
</tr>
<tr>
<td>Cu concentration in leaves [mg · kg⁻¹ d.m.]</td>
<td>12.25</td>
<td>110.13</td>
<td>50.24</td>
</tr>
<tr>
<td>Cu toxicity index:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RTI* [%]</td>
<td>0.0</td>
<td>75.2</td>
<td>31.5</td>
</tr>
<tr>
<td>TI** [%]</td>
<td>0.0</td>
<td>37.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* RTI – root toxicity index of Cu, **TI – toxicity index of Cu.

These results confirm the studies in which it was shown that chelating Cu by EDTA reduces the phytosorption of this metal in acid and slightly acid environment [1, 3, 12–15, 18, 19]; at the same time, as the authors’ experiments show, when the chelating of Cu by EDTA increased, the assimilability of this metal by plants decreased. It can be suggested that these differences resulted from ionic speciation of the forms of copper used. In water solution whose pH was 5.7 (temp. 25 °C, μ = 0.1) only 48.76 % of Cu chelated by EDTA in the molar ratio 1:0.5 occurs in the form of complex anions [Cu(II)-EDTA]²⁻, whereas the remaining amount of this element, ie 51.24 % forms ions of greater phytoassimilation, such as Cu²⁺, CuOH⁺, Cu(OH)₂ and Cu₂(OH₂)²⁺ and CuO [30]. In the case of Cu(II)-EDTA chelate in which the molar ratio M:L was 1:1 in water solution (pH 5.7, temp. 25 °C, μ = 0.1) as much as 98.58 % of Cu formed complex anions [Cu(II)-EDTA]²⁻ [30].

Toxic effect of inorganic forms of Cu on photosynthetic apparatus is varied. Excess of Cu reduces the assimilative surface of plants, disintegrates the anatomic structure of leaves and the ultrastructure of chloroplasts and reduces the content of plastid pigments, including chlorophyll [6–8]. Photosynthetic electron transport process is extremely sensitive to the excess of Cu, particularly on the PS2-driven electron transfer chain [5, 31–33].

As the results of experiments conducted by the authors show, also Cu in the form of Cu(II)-EDTA chelate has a negative effect on the phytosynthetic apparatus of higher plants; it should be noted that the toxicity of this chelate depends on the molar ratio M:L, its concentration in the medium, the concentration of Cu in leaf rosette and the physiological age of the leaf (Tables 2, 3).
In the plants treated with Cu(II)-EDTA in which the molar ratio M:L was 1:0.5 polymorphism of leaves differing with respect to age was observed, a phenomenon typical of inorganic forms of this metal [1, 3, 8, 31]. The area of the youngest leaves was reduced by over 55 % and the thickness of leaf lamina was bigger by over 14.5 % than the corresponding leaves of plants in control (Table 2). These leaves had turgor and stiffness and their colour was intense green and blue. They contained about 22 % more chlorophyll than the corresponding leaves of plants in control and were photochemically efficient (Table 3).

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Growth medium Cu concentration [μmol · dm⁻³]</th>
<th>LSD((p \leq 0.05))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf area [dm² per plant]</td>
<td>6.05</td>
<td>24.0 Cu(II)-EDTA (M:L 1:0.5) 24.0 Cu(II)-EDTA (M:L 1:1)</td>
<td>0.87</td>
</tr>
<tr>
<td>Lamina surface of older leaves [dm²]</td>
<td>0.86</td>
<td>Cu(II)-EDTA (M:L 1:0.5) 0.61 Cu(II)-EDTA (M:L 1:1)</td>
<td>0.12</td>
</tr>
<tr>
<td>Lamina surface of younger leaves [dm²]</td>
<td>0.38</td>
<td>0.17 Cu(II)-EDTA (M:L 1:0.5) 0.39 Cu(II)-EDTA (M:L 1:1)</td>
<td>0.28</td>
</tr>
<tr>
<td>Lamina thickness of older leaves [μm]</td>
<td>618.9</td>
<td>684.9 Cu(II)-EDTA (M:L 1:0.5) 622.7 Cu(II)-EDTA (M:L 1:1)</td>
<td>21.3</td>
</tr>
<tr>
<td>Lamina thickness of younger leaves [μm]</td>
<td>665.40</td>
<td>762.5 Cu(II)-EDTA (M:L 1:0.5) 670.3 Cu(II)-EDTA (M:L 1:1)</td>
<td>17.4</td>
</tr>
<tr>
<td>Number of chloroplasts per older leaf mesophyll cell section</td>
<td>8.7</td>
<td>6.2 Cu(II)-EDTA (M:L 1:0.5) 8.5 Cu(II)-EDTA (M:L 1:1)</td>
<td>1.7</td>
</tr>
<tr>
<td>Number of plastoglobuli per plastid section</td>
<td>1.4</td>
<td>5.7 Cu(II)-EDTA (M:L 1:0.5) 1.3 Cu(II)-EDTA (M:L 1:1)</td>
<td>1.12</td>
</tr>
</tbody>
</table>

Also the area of leaf lamina of next (older) leaves in the rosette as compared with the corresponding leaves of plants in control was reduced but to a lesser degree than in the case of young leaves, ie by 29.1 % as compared with the control (Table 2). These leaves were green; the intensity of the colour increased at the initial stage of the treatment of the leaves with copper chelate and then it decreased. After 7–8 days and nights of Cu-treatment these leaves showed slight, intervascular chlorosis. Reduction of leaf area was accompanied by an increase in the thickness of leaf lamina (by 10.7 % as compared with control) and a reduction of the number of chloroplasts in mesophyll cells (Table 2). Most of the plastids were shrunken and contained more plastoglobuli than the chloroplasts in control plants. As a rule, no damage to the membranes of grana and stroma thylakoids was observed, although the number of thylakoids in grana decreased.
Changes in the ultrastructure of chloroplasts were similar to the changes which take place during leaf ageing or during water deficit which is also observed during the process of ageing of this organ [34]. The leaves whose biometric parameters were changed in this way contained about 17 % less chlorophyll than the leaves of plants in the control and were far less photochemically efficient (Table 3).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Growth medium Cu concentration [μmol · dm⁻³]</th>
<th>LSD_{p ≤ 0.05}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24.0</td>
<td>24.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cu(II)-EDTA (M:L 1:0.5)</td>
<td>Cu(II)-EDTA (M:L 1:1)</td>
</tr>
<tr>
<td>Chl ((α + β)) concentration in older leaves [mg · g⁻¹ f.m.]</td>
<td>2.02</td>
<td>1.68</td>
<td>2.08</td>
</tr>
<tr>
<td>Chl ((α + β)) concentration in younger leaves [mg · g⁻¹ f.m.]</td>
<td>2.07</td>
<td>2.52</td>
<td>2.11</td>
</tr>
<tr>
<td>PS II activity in older leaves [μmol DCPIP · (mg Chl)⁻¹ × h⁻¹]</td>
<td>117.0</td>
<td>65.0</td>
<td>115.0</td>
</tr>
<tr>
<td>PS II activity in younger leaves [μmol DCPIP × (mg Chl)⁻¹ · h⁻¹]</td>
<td>124.0</td>
<td>129.0</td>
<td>125.0</td>
</tr>
</tbody>
</table>

The older leaves (ie outer ones) lost turgor relatively quickly (within 3–5 days) and then dried out.

The leaves of plants treated with Cu(II)-EDTA in which the molar ratio M:L was 1:1, in concentration 24 μmol Cu · dm⁻³ were similar to the leaves of plants in the control with respect to morphology, marked biometric parameters, colour, chlorophyll content and photochemical efficiency (Table 2).

When the amount of the chelate was three times bigger, ie 72 μmol Cu · dm⁻³, the leaves of plants treated with this chelate (M:L 1:1) did not show such polymorphism as the leaves treated with the chelate in which the molar ratio was 1:0.5; however, their photosynthetic parameters changed. The area of the youngest leaves was reduced by 26.3 % and the thickness of leaf lamina was a bit bigger than in the control, ie only by 3.05 % (Table 2). Reduction of these parameters was accompanied by a slight (about 4 %) increase in the content of total chlorophyll in photochemically-efficient leaves (Table 3). The surface of older leaves was reduced by 16.3 % and the thickness of leaf lamina increased by about 5 % as compared with the control (Table 2). Mesophyll cells contained fewer chloroplasts than control cells (Table 2). These plastids were not damaged; however, they contained more plastoglobuli than the chloroplasts of control plants (Table 2). Older leaves contained significantly less chlorophyll and were less
photochemically efficient than the corresponding leaves of control plants (Table 3). These leaves were not as green as the leaves of control plants and showed no chlorosis at all. The oldest leaves were pale green as slightly withered.

According to Baszyński [31] and Maksymiec and Baszyński [32, 33] young leaves are less sensitive to Cu toxicity than older leaves, which was confirmed by the study conducted by the authors. At the initial stage of leaf growth the leaves are photochemically efficient, their chloroplasts do not disintegrate and excessive accumulation of chlorophyll in these leaves does not result from its intensified biosynthesis; it results rather from a slower reduction of its synthesis than from the reduction of leaf area [31–33].

The studies conducted so far have shown that older leaves which are at the stationary stage of growth are the most sensitive to the toxicity of Cu [31, 32]. At this stage a drop in the content of chlorophyll, damage of chloroplasts and reduction of photochemical activity of the leaf were observed [31–33]. Heavy metals, including Cu, decrease the level of chlorophyll by inhibiting its biosynthesis [7, 8, 31] or by its degradation with the help of chlorophyllase [31, 35]. Inhibition of biosynthesis of chlorophyll in the presence of Cu in excessive amount results from Fe deficiency, among other things [6, 8]. In the case of many plant species exposed to excess of Cu a drop in PS2 activity was also observed at the stationary stage of leaf growth [5, 32, 33]. It is suggested that the main cause of reduction of activity of this photosystem is water stress in leaves [36, 37] and destruction of PS2 protein centre [37, 38].

**Conclusion**

The toxicity of the chelate form of copper, i.e. Cu(II)-EDTA depended on the molar ratio Cu to EDTA. The chelate in which the molar ratio M:L was 1:1 was much less toxic to the photosynthetic apparatus of cabbage cv. Gloria di Enkhouizen 2 than the chelate in which the molar ratio M:L was 1:0.5. The toxicity of the chelate (M:L 1:1) was smaller even when the concentration of Cu (absorbed from this form by plants) in leaves was very similar. These results suggest that depending on the degree of its chelation by EDTA, Cu can be absorbed in the form of different ions and/or it occurs in vivo in different chemical forms. These differences may also result from a different readiness for initiating defence mechanisms during assimilation and transfer of Cu in the plant or, perhaps, different defence mechanisms are initiated in the plant in response to these forms of Cu. The problem remains open and needs to be explored in further studies.

The Cu(II)-EDTA chelate used in the experiments reduced the assimilation area of cabbage plants of cv. Gloria di Enkhouizen 2, the level of chlorophyll in older leaves and their photochemical activity to a greater or lesser extent (depending on the molar ratio Cu to EDTA). It also reduced the number of chloroplasts in mesophyll cells and induced changes in their ultrastructure similar to the changes taking place during the process of leaf ageing. Young leaves were less sensitive to the toxicity of this chelate but even their area was reduced and the thickness of leaf lamina increased. However, these biometric changes were not accompanied by a drop in the photochemical
efficiency of the leaf and damage to the photosynthetic apparatus at the ultrastructural level of its organisation. What these changes were accompanied by was a bigger accumulation of chlorophyll than in the control.

References

ZMIANY NIEKTÓRYCH PARAMETRÓW FOTOSYNTETYCZNYCH
LIŚCI KAPUSTY (Brassica oleracea L.)
POD Wpływem Nadmieru Chelatu Cu(II)-EDTA

Streszczenie

W pracy przedstawiono zmiany niektórych parametrów fotosyntetycznych liści roślin kapusty (Brassica oleracea L., cv. Gloria di Enkhouizen 2) wywolane nadmiarem chelatu Cu(II)-EDTA w środowisku ożywczym roślin. Hodowlę roślin przeprowadzono w kulturach wodnych na pożywce Hoaglanda o pH 5,7 uzupełnionej chelatem Cu(II)-EDTA, w którym stosunek molowy metalu (M) do liganda (L) wynosił 1:0,5 oraz 1:1. Badano takie parametry fotosyntetyczne, jak: powierzchnia i grubość blaszki liściowej, ogólna powierzchnia asymilacyjna rośliny, liczba chloroplastów w komórce mezofilu, liczba plastoglobul w chloroplastach, koncentracja chlorofilu \( a + b \) oraz aktywność fotochemiczną fotosystemu II. Parametry te oznaczano przy tej samej koncentracji chelatu, w której M:L wynosił 1:0,5 oraz M:L 1:1, w pożywce (tj. 24 \( \mu \)mol Cu/\( \text{dm}^{-3} \)) oraz przy bardzo zbliżonej koncentracji Cu w liściach, którą rośliny pobrały z tych form. Chelat Cu(II)-EDTA (niezależnie od stosunku molowego M:L) redukował powierzchnię asymilacyjną liści starszych, obniżył w nich zawartość chlorofilu oraz redukował ich sprawność fotochemiczną. Redukował także liczbę chloroplastów w komórkach mezofilu oraz indukował zmiany w ich ultrastrukturze podobne do zmian zachodzących podczas starzenia się liści. Chelat ten powodował także redukcję powierzchni liści młodych,
której towarzyszyła większa niż w kontroli akumulacja chlorofilu. Liście te były sprawne fotochemicznie, a chloroplasty nie wykazywały żadnych uszkodzeń. Chelat Cu(II)-EDTA, w którym stosunek molowy M:L wynosił 1:0.5, był bardziej toksyczny w stosunku do wszystkich oznaczanych parametrów fotosyntetycznych liścia niż chelat, w którym stosunek molowy M:L wynosił 1:1.

Słowa kluczowe: chelat miedzi, EDTA, fotosynteza, kapusta, toksyczność miedzi