

REVIEW

Effect of copper on cellular processes in higher plants

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Copper (Cu) is a heavy metal which in recent studies has been attributed an increasing role in metabolic processes of plant cells. It is an indispensable component of oxidative enzymes or of particular structural components of cells. At elevated concentrations, Cu can act strongly on chromatin, the photosynthetic apparatus, growth, and senescence processes. The mechanisms of the metal toxicity depending largely on the growth stage of treated plants are presented in this review.

Additional key words: genetic apparatus; growth; oxidative enzymes; photosynthetic apparatus; senescence; toxicity.

Indispensability of copper in biochemical processes

Copper is taken up by higher plants largely in the form of Cu^{2+} due to the action of still not well-known mechanisms. For its absorption from the rhizosphere, in which it is almost totally bound to various ligands, probably the functioning of Fe-dependent reductase (Welch *et al.* 1993) is indispensable. In its further transport phases, a significant role may be played by nicotinamide (Pich *et al.* 1995). One of the Cu accumulation sites in higher plants are chloroplasts. This metal is directly involved as a component of plastocyanin in the photosynthetic electron transport chain (see Kato 1977). However, Cu content in chloroplasts, usually several times higher than that found in plastocyanin (Plesničar and Bendall 1970, Baszyński *et al.* 1978) indicates a high significance of the metal. Cu has been found also in several other

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Abbreviations: Chl - chlorophyll; cyt - cytochrome; D1, D2 - polypeptides of PS2 reaction centre; DCMU - (3,4-dichlorophenyl)-1,1-dimethylurea; DPC - 1,5-diphenyl-carbohydrazide; EPR - electron paramagnetic resonance; ESR - electron spin resonance; P680 - primary electron donor of PS2; PA - polyamines; Phe - pheophytin; PS1, PS2 - photosystems 1 and 2; Q_A , Q_B - primary and secondary quinone acceptors of PS2.

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proteins or their complexes (Table 1). Some amount of Cu can also bind with chromosomes, maintaining probably their normal structure (see Li and Trush 1993).

On the basis of photosynthetic activity measurements of maize plants, Barr and Crane (1974) proposed functional involvement of Cu in electron transport within photosystem 2 (PS2). They found that Cu deficiency decreased the PS2 activity without simultaneous modification of the polypeptide composition of thylakoid membranes. Cu may affect photosynthetic activity as the prosthetic group of a polypeptide involved in electron transport. This was indirectly confirmed in other studies with Cu chelates (Barr and Crane 1976) and ESR technique (Gol'dfel'd and Khalilov 1979) indicating the occurrence of strong binding sites of the metal in PS2. The inhibition of PS2 activity under Cu deficiency was also confirmed in sugar beet (Horváth *et al.* 1983), the alga *Dunaliella* (Sandman 1985), and pea plants (Barón Ayala *et al.* 1992).

Table 1. Copper content in the functional components of plants.

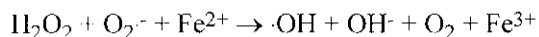
Protein	Cu	References
	atom/protein	
Polyphenol oxidase	1	Kączkowski 1980, Łyszcz 1980
Zn/Cu superoxide dismutase	2	Elstner 1979
Ascorbate oxidase	8	Marchesini and Kroneck 1970
Cu amine oxidase	2	Yamada <i>et al.</i> 1969
Galactose oxidase	1	Bereman and Kosman 1977
Plantacyanin	1	Aikazyán <i>et al.</i> 1974, Markossian <i>et al.</i> 1974
Plastocyanin	1	Danny <i>et al.</i> 1979
	atom/Chl	
PS2 particles	2-4/300	Droppa <i>et al.</i> 1984, Sibbald and Green 1987, Barón <i>et al.</i> 1990
LHC2	1.4-4.3/300	Sibbald and Green 1987, Barón <i>et al.</i> 1990

A relevant problem still to be solved is precise localization of copper in PS2 (see Barón *et al.* 1995). A certain Cu level is required to ensure the appropriate lipid microenvironment, among other things, for binding Q_B (Droppa *et al.* 1987), polypeptides and pigments of the photosystems (Barón Ayala and Sandmann 1988, Barón *et al.* 1990, Barón Ayala *et al.* 1992). However, although Cu indispensability for PS2 has been sufficiently demonstrated, the precise mechanism of action of Cu involved at this distance of electron transport chain is still a mystery.

Cu has a distinct regulatory role in electron transport between PS2 and PS1 as the constituent of plastocyanin. Cu-impairment in the plastoquinone region was also observed (Droppa *et al.* 1987, Horváth *et al.* 1987) in a fairly narrow range of metal deficiency [9-7 $\mu\text{mol}(\text{Cu}) \text{ m}^{-2}(\text{leaf area})$; Droppa *et al.* 1984]. If the Cu content is less than 7.5 $\mu\text{mol}(\text{Cu}) \text{ m}^{-2}(\text{leaf area})$, the strong inhibition of PS1 and PS2 electron transport becomes predominant (Droppa *et al.* 1984).

Copper ions as the constituents of Cu/Zn superoxide dismutase (Cu/Zn-SOD) are essential for photosynthetic electron transport to oxygen in the Mehler reaction.

In chloroplasts, superoxide radicals ($O_2^{\cdot -}$) are formed even under normal cell metabolism. CuZn-SOD, localized near the PS1 complex (Ogawa *et al.* 1995), considerably accelerates $O_2^{\cdot -}$ decomposition which results in the formation of hydrogen peroxide (H_2O_2) and O_2 . Weakened function of antioxidative systems (*i.e.*, SOD, catalase, peroxidases) that remove H_2O_2 and O_2 can lead to the formation of highly reactive hydroxyl radical $\cdot OH$ (Fig. 1). The hydroxyl radicals are believed to be formed *via* the metal-catalysed Haber-Weiss reaction:



Other transition metal ions (especially Cu^+) can similarly react with H_2O_2 (Wardman and Cadeias 1996). CuZn-SOD localized also in other cell compartments (see Longa *et al.* 1994) can play the role both of a "scavenger" of $O_2^{\cdot -}$ and of an H_2O_2 producer, often indispensable in making interpolymer cross-links (Fry 1986).

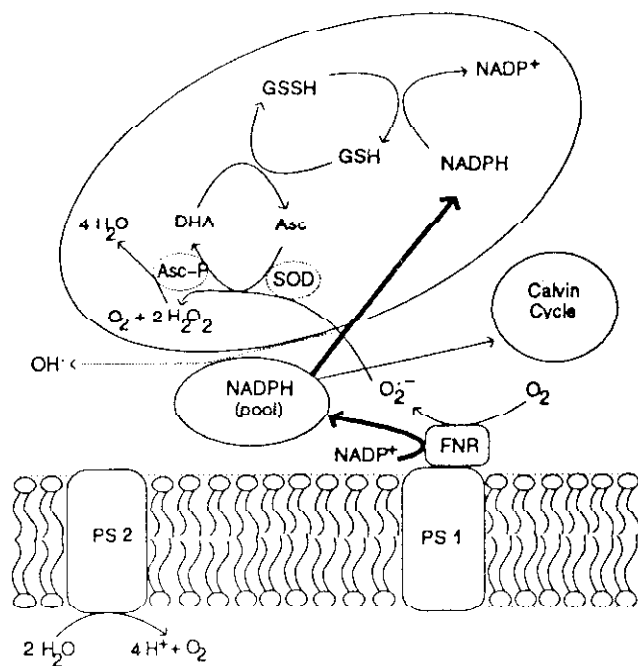
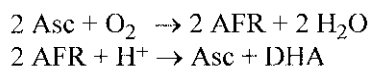


Fig. 1. Scheme of the formation of transient active forms of oxygen due to overproduction of NADPH. SOD - superoxide dismutase; Asc and DHA - reduced and oxidized ascorbate form; GSH and GSSH - reduced and oxidized glutathione form; FNR - ferredoxin/NADP reductase; Asc-P - ascorbate peroxidase.

Cu deficiency affects the photosynthetic apparatus also indirectly through metabolic processes. Baszyński *et al.* (1978), Droppa *et al.* (1984), and Henriques (1989) showed decreased rate of synthesis of chlorophyll (Chl) or plastoquinone in Cu deficient leaves. In such conditions the saturation degree of the particular acyl lipids of thylakoid membranes can be changed (Droppa *et al.* 1987, Horváth *et al.* 1987), as well as ultrastructural alterations in chloroplasts can occur (Baszyński *et al.* 1978, Henriques 1989). In *Anacystis nidulans* Cu caused bleaching of phycocyanin, but not of allophycocyanin (Gupta and Singhal 1996).

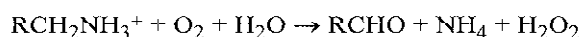
Cu affects the activity of enzymes involved in other life processes of plants which are usually connected with the action of chloroplasts. Savini *et al.* (1990) pointed to

indispensability of this metal in preserving the activity of ascorbate oxidase (OAsc). The biological function of OAsc still has to be elucidated. This enzyme is a dimer containing 8 Cu^{2+} ions which participate in reduction of O_2 molecules to water in the presence of a reducing substrate, *i.e.*, ascorbate (Asc), according to the scheme:



Transient products of this reaction are Asc radicals (AFR) and the final product, dehydroascorbate (DHA). Oxidation of Asc can also take place without the enzyme in the presence of transient metals (largely Cu^{2+}), or in the presence of equivalent amounts of Asc and DHA. The OAsc lacking a part of the metal is more sensitive to the action of stress agents such as, *e.g.*, low temperature or guanidine (Savini *et al.* 1990). Its synthesis or activation processes may be controlled by photochrome (Drumm *et al.* 1972, Leaper and Newbury 1989). Asc, the substrate of OAsc, has significant functions in animal and plant organisms (see Navas *et al.* 1994). Synthesized in cytosol, it is transported to apoplasts and chloroplasts (see Córdoba and González-Reyes 1994). In chloroplasts, where about 10-50 mM Asc is found (Foyer 1993), it can be involved in O_2^- dismutation cycle and in H_2O_2 removal from overreduced PS1 (Fig. 1). It can also participate in regeneration of zeaxanthin (Bratt *et al.* 1995) and in reduction of oxygen reactive forms that appear during peroxidation of lipids induced by stresses. Asc present in cytosol and in apoplast can modulate the hydroxyproline-rich synthesis of glycoproteins (Arrigoni *et al.* 1977), controlling cell divisions or elongation processes, respectively. Some forms of these proteins, *e.g.*, extensin, toughen cell walls (Cassab and Varner 1988). Asc inhibits cross-linking action of extensin, stimulating thereby cell elongation (Cooper and Varner 1984). A similar effect can be exerted by AFR, causing both increased vacuolization of cells and transport of sugars and nitrates into floem. This effect may result from the AFR action on membrane redox system including NADH-AFR reductase or cytochrome (cyt) *b* (Córdoba and González-Reyes 1994, González-Reyes *et al.* 1994). After strong oxidation of hydroxyproline-rich glycoproteins - often occurring after infection of plant cells - growth inhibition takes place in the presence of H_2O_2 due to the toughening of cell walls (Brisson *et al.* 1994).

Cu is also required for the function of amine oxidase (Dooley *et al.* 1991a,b), probably through its direct involvement in attaching O_2 molecules to the enzyme in the reducing medium. This enzyme, found in plants and animals, catalyzes the oxidative deamination of polyamines (PA) with the formation of corresponding aldehydes, ammonia, and H_2O_2 :



PA function has not been explained yet. These substances probably affect the division and differentiation of cells (Finazzi Agró and Rossi 1992, Huang *et al.* 1994, Linares *et al.* 1994). They occur in chloroplasts, conjugated to apoproteins of the Chl *a/b* antenna complex II (Kotzabasis *et al.* 1993, Del Duca *et al.* 1994), as well as the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBPCO (Del

Duca *et al.* 1994). Del Duca *et al.* (1995) suggest that PA stabilize the mentioned oligomers.

Cu-containing polyphenoloxidase (PPO) seems to function in the defence mechanisms of cells. After fungal or bacterial infections of the cells they produce hydroxyphenols and quinones having fungicidal or bacteriocidal properties (see Vaughn *et al.* 1988). However, because of PPO localization in chloroplasts the mentioned mechanism is questioned. Vaughn *et al.* (1988) propose that this enzyme might mediate photoreduction of O_2 (the Mehler reaction) by PS1.

The above mentioned results allow to make a new scheme (Fig. 2) illustrating Cu involvement in photosynthetic processes.

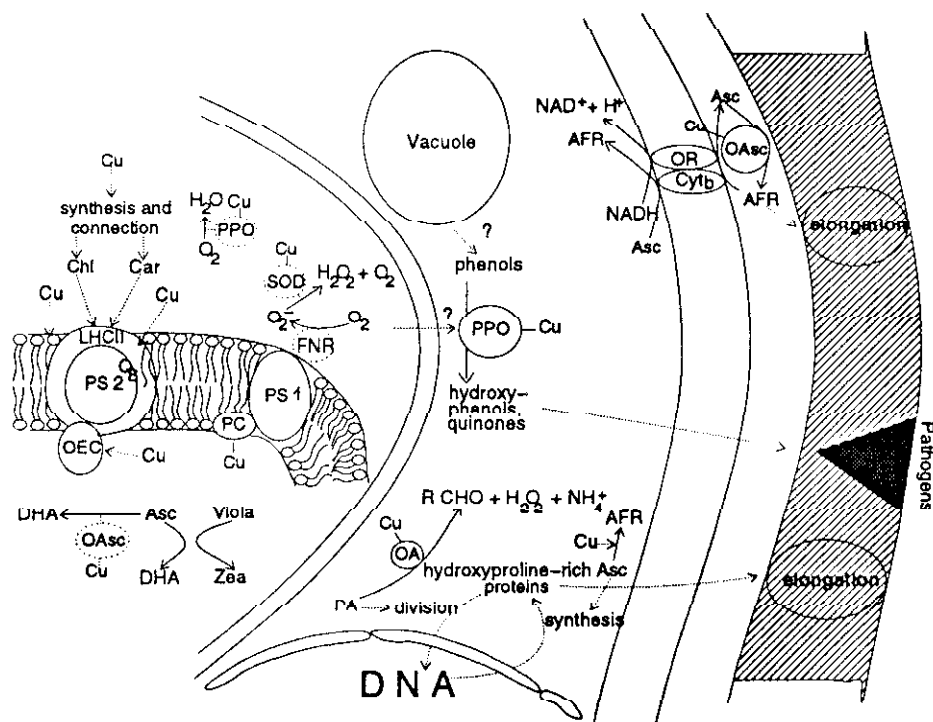


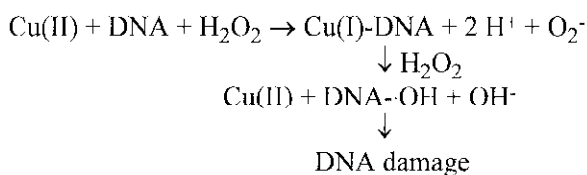
Fig. 2. Cu involvement in biochemical processes of plant cells. PPO - polyphenoloxidase; OAsc - ascorbate peroxidase; OA - ascorbate oxidase; SOD - superoxide dismutase Cu/Zn; OR - oxidoreductase NADH/AFR; Asc and DHA - reduced and oxidized ascorbate form; AFR - ascorbate free radical; PA - polyamines; Viola - violaxanthin; Zea - zeaxanthin; FNR - reductase ferredoxine/NADP.

Toxic effect of excess copper on plants

Despite its indispensability, overdosed Cu has a strong toxic effect both on plants and animals. The necessity to elucidate Cu toxicity results not only from its increasing use in industry but also from its use as a fungicide, algicide or bactericide in agriculture, and as a drug in medicine.

Visual symptoms of toxicity: In sensitive forms of higher plants, excess Cu causes various effects depending strongly, among other things, on the plant growth stage at which the metal was applied, and on the time of its action. Excess Cu applied at the initial growth stage strongly inhibits leaf expansion and increases content of pigments calculated per leaf area unit (Maksymiec *et al.* 1994, 1995, Maksymiec and Baszyński 1996b). Growth inhibition occurs already after one-day metal treatment (Weckx and Clijsters 1996). After a longer exposure to Cu (throughout the vegetation period), Chl concentration decreases which is associated with simultaneous destruction of the inner structure of chloroplasts (Eleftheriou and Karataglis 1989). A visual toxicity symptom in plants exposed to the metal at an advanced growth stage is reduction of Chl content in leaves (Rousos *et al.* 1986, 1989, Baszyński *et al.* 1988) connected with partial destruction of grana (Baszyński *et al.* 1988, Maksymiec *et al.* 1995), and a considerable modification of lipid-protein composition of thylakoid membranes (Maksymiec *et al.* 1992, Lidon and Henriques 1993, Maksymiec *et al.* 1994) depending on the duration of Cu action (Maksymiec *et al.* 1992). A common feature of action of excess Cu in most plants is the decrease in root mass (Rousos and Harrison 1987, Casella *et al.* 1988, Rhoads *et al.* 1989, Lidon and Henriques 1992).

The genetic apparatus: Decrease in plant yields and external symptoms of toxic Cu action are the consequence of periodically or permanently changed cellular metabolism. Can the genetic apparatus controlling the whole complicated organization of life be damaged by excess Cu? Some experimental results give a positive answer. At micromolar concentrations in the presence of H_2O_2 , Cu causes disturbances of DNA conformation. Free $\cdot\text{OH}$ radicals formed according to the following scheme probably damage chromatin (Sagripanti and Kraemer 1989, Prütz *et al.* 1990, Ueda *et al.* 1994):



The presence of some substances, *e.g.*, excess NaCl (Prütz 1990) or Mn(III) with hydrazine derivatives (Kawanishi and Yamamoto 1991) intensifies the reaction rate. Because the average diffusion distance of $\cdot\text{OH}$ in cells is very short, the deleterious effects may be site-specific, in close proximity to the metal-binding site (Samuni *et al.* 1983). Such localized Cu action probably occurs in the case of oxidative modification of proteins (see Stadtman 1990). However, a controversial problem is still the kind of free radicals involved in DNA-strand cleavage. Some authors suggest that they can be O_2^- but not $\cdot\text{OH}$ radicals (Li and Trush 1993), or both forms (Yamamoto and Kawanishi 1989). Unfortunately, the above results were obtained only in *in vitro* experiments on isolated DNA fragments. However, if Mn is present in the nucleus (Sakurai *et al.* 1985), and both photosystems produce H_2O_2 (Schröder and Åkerlund 1987, Salin 1991), the reaction can probably take place also in plants *in vivo*. Since natural "scavengers" of free radicals, such as SOD, catalase and

tocopherols, can inhibit this process, the involvement of genetic material in the development of Cu toxic action in biological systems is controversial. Recent studies have shown lack of a significant role of SOD in Cu detoxification. This function can be performed effectively by catalase (Sagripanti and Kraemer 1989, Prütz 1994, Makrigiorgos *et al.* 1995). *In vivo* the metal does not occur in free form, and thus the toxic action of Cu probably depends on the bound ligand. Ueda *et al.* (1994) observed that Cu complexes with several oligopeptides containing histidyl residue at the N-terminal caused DNA damage, whereas Cu complexes with peptides containing a histidyl residue in another position were without effect. Azenha *et al.* (1995) showed that toxicity of Cu to bacterial cells can be markedly reduced by the presence of weak and moderate organic ligands. Some metallic ions, usually present in plant cells such as, *e.g.*, $Mg^{2+} \geq Ca^{2+} \geq Mn^{2+}$, may antagonize Cu^{2+} binding to DNA (Sagripanti *et al.* 1991). Prütz (1994) suggests that Cu^{2+} is the main agent permitting DNA binding to proteins, *i.e.*, the metal in the form of Cu^{2+} is not harmful *per se*. Chromatin is damaged only due to Cu^{2+} replacement by Cu^+ in the presence of additional factors stimulating O_2^- formation.

The photosynthetic apparatus: Information about the toxic effects of Cu on the photosynthetic apparatus is in the reviews by Droppa and Horváth (1990) and Barón *et al.* (1995). However, some aspects of this problem require further consideration.

In the initial experiments on the influence of excess Cu on plants, a number of controversial results were obtained. The Cu effect on PS2 especially aroused much controversy. In 1969, Habermann discovered the ability of manganese to restore photosynthetic activity previously decreased by Cu ions, drawing thereby attention to the donor side of PS2 as a site susceptible to the metal action. This view was supported by restoration of electron transport activity after DPC application (Vierke and Struckmeier 1977). However, Shioi *et al.* (1978a,b) did not obtain such an effect. Similarly, Cedeno-Maldonado *et al.* (1972) and Samuelsson and Öquist (1980) did not observe restoration of photosynthetic activity after adding Mn(II). These discrepancies can be explained in part by the high Cu reactivity with reagents used both for isolation of chloroplasts and for activity measurements (Renganathan and Bose 1989, 1990, Prütz 1990, Azenha *et al.* 1995). From the information given by these authors, the results of experiments with applied DPC (see also Purcell and Carpentier 1993), ascorbate, hydroxylamine, DBMTB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone), dithionate, and some buffers - compounds reacting with Cu - must therefore be regarded as controversial. Taking into account the results of studies using non-invasive techniques or reagents nonreactive with Cu, some mechanisms of inhibitory Cu action can be proposed.

Excess metal can effectively bind to thylakoid membranes (Vierke and Struckmeier 1977), particularly in the light (Cedeno-Maldonado *et al.* 1972), causing inhibition of photosynthetic efficiency that depends, to some extent, on irradiance (MacDowall 1949, Steemann Nielsen *et al.* 1969). So far, several sites of Cu bonding have been proposed. The use of EPR technique allowed demonstration of Cu bonding with proteins of thylakoid membranes largely through oxygen atoms (Vierke and Struckmeier 1977). Such modes of metal binding allow direct effects on the

constituent components of membranes or effects mediated by free radical processes. This can result in disturbances of association of lipid-protein-pigment complexes connected with the photosystems (Samuelsson and Öquist 1980, Baszyński *et al.* 1988). A mechanism of peroxidative degradation of lipids around the photosynthetic complexes has been proposed by Sandmann and Böger (1980a,b) from results obtained both in *in vitro* and *in vivo* experiments. Cu ions can also associate with histidine (His) groups of polypeptides D1 and D2 (Renganathan and Bose 1989). Proteins separated by affinity chromatography form coordination bonds with Cu^{2+} through oxidized His and tryptophan exposed on the surface (Sulkowski 1985). A high Cu reactivity with His has been confirmed by several experiments *in vitro* or carried out on animals or bacteria (Brumas *et al.* 1995, see Stohs and Bagchi 1995).

On the basis of homologies with the bacterial reaction centre, His is a ligand to Q_A , and the nonheme iron is also associated with PS2 in plants (see Trebst and Heinze 1991). The presence of such amino acid sites near Q_A and Q_B has also been found in spinach PS2 (Diner *et al.* 1991). Increased Cu at these sites can destabilize the quinone acceptor, Q_B (Mohanty *et al.* 1989), or the Phe- Q_A -Fe domain of the PS2 reaction centre (Yruela *et al.* 1992, 1993). Renganathan and Bose (1989), however, excluded Cu inhibitory action on the reducing side of PS2. The order of addition and the time taken to make the measurements seem to have significantly affected the results obtained. Renganathan and Bose made measurements directly after adding the metal (the time is too short to saturate with Cu a sufficient number of binding sites), but Mohanty *et al.* (1989) made them after 10 min of incubation. Recent studies *in vitro* using preincubation with Cu^{2+} (Renger *et al.* 1993, Jegerschöld *et al.* 1995) as well as studies *in vivo* (Maksymiec *et al.* 1994, Maksymiec and Baszyński 1996a) confirmed inhibition of the PS2 acceptor side. Cu action on the PS2 acceptor side may also result from its high reactivity with some types of quinone compounds (Bates 1990, Dooley *et al.* 1990, 1991a,b). Indeed, increased metal concentrations intensify reactivity, possibly by dislodging other elements (*e.g.*, Fe) which may have formed complexes with the quinones. Singh and Singh (1987) showed the restoration of Cu-induced inhibition of PS2 activity after Fe addition.

Other potential sites of Cu-bonding are the tyrosine Y_Z sites (Hoganson *et al.* 1991). Electrostatic metal attachment to these sites can occur after the dissociation of oxygen evolving complex and apart of Mn^{2+} from thylakoid membranes enriched with PS2, finally resulting in weakened P680^+ reduction. On the basis of these results, Cu^{2+} inhibited photosynthetic oxygen evolution may be due to replacement of manganese ions, confirming earlier experiments of Shioi *et al.* (1978a,b). Assuming that His is the terminal ligand of Mn (Yachandra *et al.* 1993), the oxidation of which is connected to electron transfer (Ono and Inoue 1991), a block of PS2 donor side activity by Cu on this amino acid is likely. Recent studies (Renger *et al.* 1993, Schröder *et al.* 1994, Arellano *et al.* 1995, Jegerschöld *et al.* 1995) show that Cu specifically inhibits electron donation from Tyr_Z to P680^+ . The inhibition rate depends on the conditions of exposure of PS2 particles to Cu^{2+} . After exposing PS2 particles to Cu^{2+} after preirradiation periods longer than 30 s, the damage is irreversible (Arellano *et al.* 1995). Irradiation considerably intensifies Cu bonds because even a longer dark incubation (over 10 min) does not cause irreversible

inhibition (Jegerschöld *et al.* 1995). For Y_Z inhibition a higher Cu^{2+} concentration is required than that which leads to modification of the atrazine binding site (Renger *et al.* 1993).

Samson *et al.* (1988) pointed to the occurrence of a third inhibition site in the reaction centre (RC) of PS2. Hsu and Lee (1988) confirmed a possible disturbance of the primary charge separation at P680. Supposing no simultaneous transfer of energy to PS1 (as this photosystem is also inhibited), they interpreted the observed fluorescence efficiency decrease by PS2 RC damage followed by loss of excitation energy due to heat dissipation. So far, the way of excess Cu binding to P680 has not been elucidated. At least two interaction sites are likely to exist: Mg in Mg-porphyrins, and ligands binding P680 with D1/D2 protein.

Heavy metal-substituted chlorophylls and related porphyrins have been known *in vitro* for a long time (Rebeiz and Castelfranco 1973, Bolfo *et al.* 1975, Kowalewska *et al.* 1987). The *in vivo* experiments showed that the substitution of Mg in Chl observed *in vitro* also takes place in living plants (Agarwal *et al.* 1987, Kowalewska and Hoffmann 1989, Küpper *et al.* 1996). Cu-porphyrins dissipate energy in the form of heat, resulting in a strong decrease of fluorescence efficiency (Küpper *et al.* 1996). It was also observed in the total pool of pigments of thylakoid membranes after their exposure to the excess metal (Hsu and Lee 1988, Samson *et al.* 1988). Due to strong oxidizing properties, Cu^{2+} ions can affect cyt b_{559} associated with PS2 RC, intensifying non-productive cyclic electron transport around this photosystem. As a result, a part of the energy may be lost also on this way. *In vivo*, this can be a defence mechanism against excess Cu in young, developing plants (Maksymiec and Baszyński 1996a). In older plants, Cu^{2+} ions involved in free radical processes are considerably transformed to Cu^+ . In the reduced form, Cu can transform cyt to an inactive form increasing the sensitivity of stressed plants to irradiation. Moreover, cyt b_{559} has two components sensitive to excess Cu-Fe and His as a binding agent (see Whitmarsh *et al.* 1994).

The His residues coordinate many bacteriochlorophyll molecules to their apoproteins. By analogy, His residues play a major role in binding Chl molecules to proteins in the PS2 RC (see Wettstein *et al.* 1995). Replacing of His results in efficiency decrease of antenna probably through the formation of pheophytin in it (Shen *et al.* 1993). After exposure to excess Cu, heavy metal ions may interfere also with histidine residue in the heterodimers D1/D2 and/or in the Chl-binding protein of inner antenna RC.

Much less is known on the effect of excess Cu on other steps of the electron transport chain. Singh and Singh (1987) indicated inhibitory action of the metal outside the sites sensitive to DCMU, and on the concentration of cyt f . Shioi *et al.* (1978a) presented a direct PS1 inhibition due to interaction with ferredoxin. This photosystem, in agreement with the model of Sandmann and Böger (1980a), participates also in indirect inactivation of the photosynthetic apparatus consisting Cu-mediated lipid peroxidation processes in thylakoid membranes. Few experiments *in vivo* confirm the sensitivity of both PS1 and PS2 to the metal (Ouzounidou 1996, Daszyński *et al.* 1988), but the changes in PS1 after exposition to Cu are smaller than those occurring in PS2 (Ouzounidou 1996). In specific conditions, Cu is a potential

inhibitor of phosphorylation processes, acting with the coupling factor 1 (Uribe and Stark 1982).

A unique metabolic process in the plant kingdom is the Calvin cycle. At a concentration of 50 M, Cu ions cause both *in vitro* and *in vivo* an inhibition of the carboxylase function of the main enzyme of the pathway, RuBPCO (Stiborová *et al.* 1987, 1988). Because RuBPCO oxygenase activity is also inhibited (Lidon and Henriques 1991), the inhibition mechanism probably consists in oxidizing-SH groups of the enzyme by Cu or in replacing Mg ions indispensable for the formation of the enzyme-CO₂ complex (Stiborová *et al.* 1988). Heavy metals have a high affinity for sulphhydryl groups. Spectroscopic ESR studies at low temperatures (Ainscough *et al.* 1987) gave direct evidence of the formation of Cu²⁺ complexes with thiol. De Vos *et al.* (1989) point out that interactions between Cu and SH groups result in increased cell permeability and loss of internal K⁺. The loss of membrane integrity was also observed in conditions of increased ethylene production (Sylvestre and Paulin 1987). Sandmann and Böger (1980a,c) propose that production of ethylene can occur from Cu⁺ and Cu²⁺ ions-induced peroxidative decomposition of lipids.

In vivo, there is not such a direct contact of tissue structures with Cu ions, and the inhibition mechanism of the photosynthetic apparatus differs a little from that *in vitro*. In addition to the possible direct Cu action on the metal-binding sites, indirect effects are also suggested. The PS2 acceptor side, and in consequence the whole photosystem, can be inhibited on the basis of feedback control caused by accumulation of terminal metabolites of the light phase (Maksymiec *et al.* 1994, Moustakas *et al.* 1994, Maksymiec and Baszyński 1996a,b). A similar mechanism was also proposed in regard to the action of Ni²⁺ ions (Krupa *et al.* 1993). Excessive amounts of ATP and NADPH may occur due to direct or indirect inhibition of the Calvin cycle. In the latter case, CO₂ assimilation can be inhibited by poor triose phosphate utilization caused by slow sugar export to the sink tissue, which is observed when *Phaseolus vulgaris* seedlings are exposed to excess Co, Ni, and Zn (Rausser and Samarakoon 1980). Probably a competitive Cu-Fe interaction (Lidon and Henriques 1992, Ouzounidou 1994, Reboredo 1994, Alva and Chen 1995) induces not only chlorotic changes of leaves but also disturbs Fe incorporation into Q_A-Fe sites in constantly restored Chl particles, causing inhibition of the PS2 acceptor side. The PS2 donor side and its centre can be blocked through modification of the structure of thylakoid membranes observed in plants at an advanced growth stage (Ouzounidou *et al.* 1992, Lidon and Henriques 1993, Maksymiec *et al.* 1995). The structural changes are connected with rebuilding their lipid components and replacement of functional parts of proteins (Lidon and Henriques 1993, Maksymiec *et al.* 1994) or due to release of Ca²⁺, Mg²⁺, and H⁺ ions by excess Cu. Removal of Ca²⁺ from PS2 may induce peroxidative processes (Hillier and Wydrzyński 1993) leading to Cu-induced increase of futile electron transfer pathways (Maksymiec and Baszyński 1996a). These results allow to propose a possible mechanism of Cu action on the photosynthetic apparatus occurring when the plants are exposed to Cu at different growth stages (Fig. 3).

Summing up, *in vivo* the excess of Cu can act on various steps of the photosynthetic chain. Particularly sensitive are different PS2 sites showing a big

affinity for Cu, as indicated earlier in *in vitro* experiments. The degree of Cu action on the particular domains of the photosynthetic complexes depends, among other things, on the growth stage and plant condition during Cu application as well as on the duration of the stress or action. Taking into consideration the above dependences, the localization of the Cu action side seems to be settled to a considerable degree. The detailed mechanism of Cu action in individual cell compartments or their membranes remains an open problem. Its explanation will be the next step in learning some mysteries of life not only in plants but also in animals.

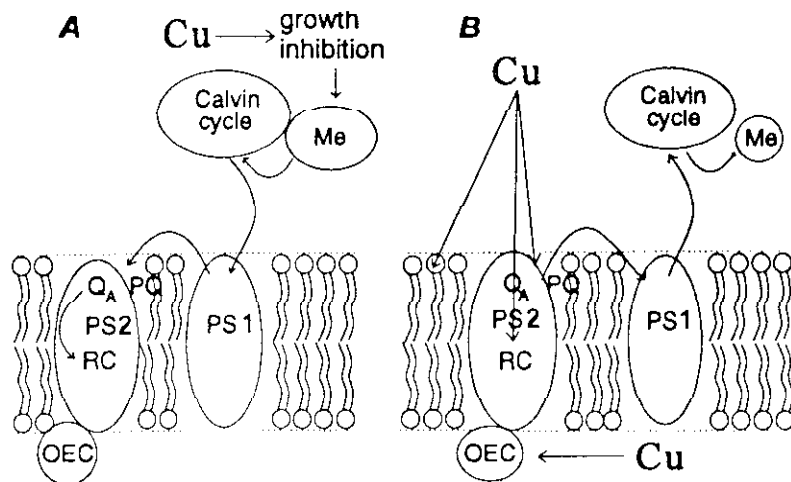


Fig. 3. Effect of excess Cu on the photosynthetic apparatus of higher plants in relation to their exposure to the metal. *A* - Cu-treated young plants at the initial growth stage; *B* - Cu-treated older plants at the stationary growth stage. Me - metabolites.

Enzymatic reactions, leaf senescence: Cu ions used in excess may also act indirectly on some enzymatic pathways of plant cells. Increased ethylene production, probably resulting from blockade of photosystems after application of excess Cu (Sandmann and Böger 1980a,c), may be caused also due to lipoxygenase activity increased by the metal (Kubacka *et al.* 1993). As lipoxygenase stimulating peroxidative decomposition of lipids can give a number of active indirect or terminal metabolites, the modifications of its activity (as well as stimulation of lipid peroxidation in another way) may have far-reaching consequences (Fig. 4).

Excess Cu can induce a number of free radical processes in protein (Stadtman 1990) and lipid cell membrane components (De Vos *et al.* 1989, 1991, Strange and Macnair 1991), causing destabilization of membranes and increase of their permeability (Wainwright and Woolhouse 1977, see Meharg 1993). Polypeptide components of membranes can also be modified through Cu action on genetic material. These initial changes, the nature of which has been speculative as yet, allow Ca^{2+} to move into cytoplasm from Ca^{2+} containing organelles such as chloroplasts, mitochondria, endoplasmic reticulum, and vacuoles, or from apoplast. The plant cell has various systems on different membranes which are important for cytosolic Ca^{2+} regulation. They include Ca^{2+} ATPases at the plasma membrane and

endoplasmatic reticulum (see Marmé 1988). If Cu^{2+} is capable to inhibit ATPase (Uribe and Stark 1982), the concentration of cytoplasmatic Ca^{2+} may increase due to

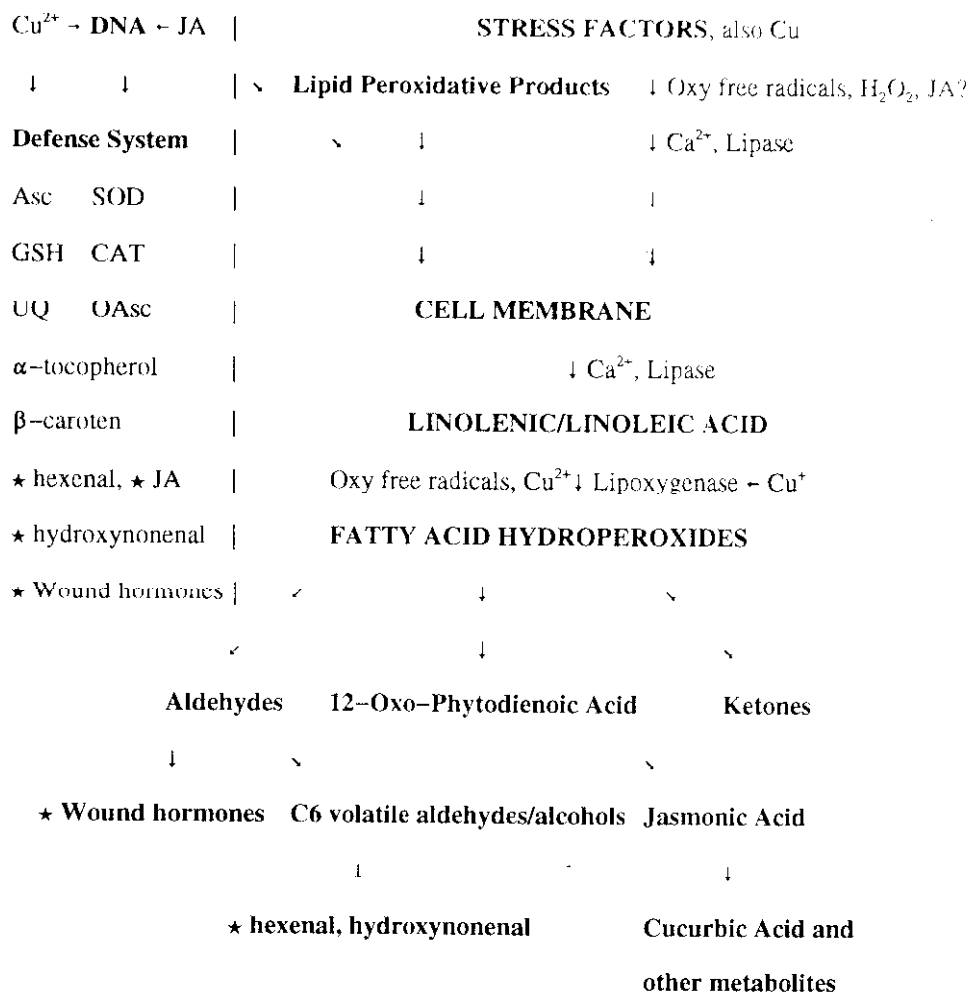


Fig. 4. Induction of "linolenyl cascade" in the presence of excess Cu. JA -jasmonic acid; CAT - catalase; SOD - superoxide dismutase; OAsc - ascorbate oxidase; UQ - ubiquinone; GSH - glutathione.

blockade ATP-dependent pumps removing its excess. Entry of Ca^{2+} into cytosol triggers catabolic processes by activation of phospholipases. This leads to liberation of linolenic or linoleic acids supplying substrates for lipoxygenase or free radical processes directly induced by Cu ions. From the built-in various forms of free radicals, a number of active metabolites are formed. A part of them can act as wound hormones (traumatin, traumatic acid, phaseolic acid) or actively combating pathogenes (hexenal, hexenol) (see Farmer 1994), and a part (ketones, jasmonic acid - JA, ethane, ethylene, free oxygen radicals) can accelerate the catabolic cycle

presented in Fig. 4, intensifying disintegration of membranes. JA formed in the cycle may additionally act, specifically on the particular biochemical processes, directly or as signal particle (Maslenkova *et al.* 1990, see Hamberg and Gardner 1992). The efficiency of the defence system (also modified by Cu) determines whether the processes assume the character of "cascade" leading to irreversible senescence changes comprising the whole organism, or it is only a signal mobilizing its defensive functions. The above presented changes concern rather the induction of senescence processes usually occurring under the influence of Cu in plants at the final growth stage (Maksymiec *et al.* 1994, 1995, Maksymiec and Baszyński 1996a,b) or after a longer exposure to the metal (Maksymiec *et al.* 1992, Eleftheriou and Karataglis 1989).

Cu action on synthesis of lipids is still unknown. Only Smith *et al.* (1984, 1985) indicated a possible effect of excess Cu on the elongation of 16-C acids and the desaturation at $\Delta 9$ binding. This opinion was based on finding in algae of a reversed relation in the contents of fatty acids 16:0 and 18:3; it was confirmed after Cu treatment of spinach (Maksymiec *et al.* 1992) and bean plants (Maksymiec *et al.* 1994). Similarly, Cu effect on Chl synthesis has been poorly learned. Inhibitory influence of Cu is assumed at the stage of δ -aminolevulinic acid formation (Stiborová *et al.* 1986). Also a direct Cu effect is possible, through inhibition of Fe uptake (Lidon and Henriques 1992, Alva and Chen 1995, Pich *et al.* 1995) which is indispensable for Chl formation. Chl concentration decrease by its catalytic degradation was found only to a limited extent, as a result of a small activity increase of chlorophyllase (Nag *et al.* 1981) in the presence of excess Cu.

Growth inhibition: Adding excess Cu as well as other heavy metals largely inhibits elongation growth (Wainwright and Woolhouse 1977, Maksymiec *et al.* 1994, 1995, Arduini *et al.* 1995, Maksymiec and Baszyński 1996b). Although some authors consider that inhibition of the cell cycle is the basis for growth inhibition (Eleftheriou and Karataglis 1989, Punz and Sieghardt 1993), the precise role of Cu in cell proliferation is as yet unknown and controversial. Several hypotheses interpreting the causes of growth inhibition involve free radicals, systemic responses, and competitive phenomena.

Elongation growth is a complex process including turgor changes, synthesis of cell membrane components, and the content of growth regulators. The free radical hypothesis considers the possibility of Cu action on cell wall building. At the initial growth stages, intensive fresh mass increase is accompanied by intensified peroxidation of lipids (Weekx and Clijsters 1996) and some proteins, and by Asc synthesis (Córdoba and Gonzáles-Reyes 1994). These are phenomena associated with attachment and stabilization of many new membrane components, and also of the cell wall. Too strong intensification of these processes, observed after the so-called oxidative "burst" induced by elicitors (Apostol *et al.* 1989, Brisson *et al.* 1994), leads to rapid inhibition of growth elongation probably due to intensified crosslinking of walls. As mentioned, Cu is an efficient catalyst in the formation of several reactive oxygen species and other free radicals. Progressing oxidation of tissue components can cause the appearance of the above effect. In such conditions, Asc elimination

from the medium occurs due to its direct oxidation (Navas *et al.* 1994) or by increased OAsc activity (Rodrigues-Aguilera and Navas 1994). At decreased Asc content, adequate inhibition of extensin crosslinking (Córdoba and Gónzales-Reyes 1994) and AFR production do not take place (Gónzalez-Reyes *et al.* 1994), which results in the inhibition of cell elongation. Moreover, simultaneous inhibition of division processes occurs in phases G1 or G2 (Liso *et al.* 1984). Because of a high Cu reactivity with most amino acids (Kossakowska *et al.* 1988), the metal may directly affect crosslinking of hydroxyproline-rich protein cell wall components. The generalization of this hypothesis of growth inhibition is hindered by a distinct localization of the processes of oxidative "burst". For this reason the possibility of induction, influenced by Cu, of systemic response should be additionally taken into account. Good candidates for signal factors, indispensable for excess Cu-induced growth inhibition seem to be: membrane potentials, JA, H₂O₂, Ca, and some aldehyde derivatives of fatty acids. They all depend on the functioning of redox system, and largely on terminal oxidase-NADH oxidase/peroxidase. The effects of this enzyme action in isolated soybean plasma membranes are strongly modified after addition of excess Cu (Zancani *et al.* 1995). However, a similar effect has not been obtained in the presence of other heavy metals which also cause growth inhibition.

Some divalent ions (especially Mg²⁺ and Ca²⁺), in relation to their concentration, inhibit or enhance growth (Thimann and Biradivolu 1994, Baluška *et al.* 1996). Mg and Ca have a similar chemical ionic form as Cu. For this reason, the competitive action of these elements may be possible in the phase of uptake and transport (Agarwal *et al.* 1977, 1987, Jensen and Adalsteinsson 1989), modifying in consequence plant growth. The recent initial studies *in vivo* have confirmed the possibility of Cu-Ca interaction (Ouzounidou 1994, Ouzounidou *et al.* 1995, Maksymiec and Baszyński 1995) and that of Cu-Mg (Ouzounidou *et al.* 1992). Ca addition can partially diminish the effect of growth in metal-cell interaction. Inhibition of growth processes after Cu application, through competition with other elements in the particular biochemical processes, has not been determined yet.

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