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BRIEF COMMUNICATION

Toxic effects of high copper content on physiological processes in *Pinus sylvestris* L.

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Abstract

The aim of this study was to determine the impact of increased copper contents on selected physiological processes in one-year-old *Pinus sylvestris* L. needles from a former German timber storage area in Warcino Forest District, a subject to an environmental quality survey. Samples were collected from the area with the high copper content in the soil. The control area was a nearby pine tree stand showing unimpeded growth. The significant growth inhibition was found in dwarf shoots and whole needles, increased water content, and reduced dry mass were also observed. The chlorophyll content was lowered, while 20% higher electrolyte leakage was found. Chlorophyll *a* fluorescence indicated only higher values of the nonphotochemical quenching in *P. sylvestris* from the Cu-site. Significant differences were shown in the rate of gas exchange measured by changes in carbon dioxide or oxygen concentration. The intensity of photosynthesis in needles of *P. sylvestris* from the Cu-site measured by CO_2 uptake was considerably higher than that of oxygen production. The rate of respiration in the needles from the Cu-site measured by the amount of released CO_2 was higher only by 15%, while according to O_2 consumed, the rate increased by 30% in relation to the control. Our results suggest that the copper accumulation in *P. sylvestris* needles affected the morphology and physiology of the studied organs.

Additional key words: chlorophyll fluorescence; growth analysis; photosynthesis; respiration.

In their natural environment, plants are exposed to numerous substances that have a positive or negative impact on plant metabolic processes, depending on their concentration. The occurrence of heavy metals in the environment constitutes a serious problem at the present days. The main sources of pollution include industrial development, the application of mineral fertilisers, and plant protection products, as well as municipal waste. Heavy metals in soils pose a risk to the chemical balance and may lead to the degradation of the environment. The impact of high concentrations of heavy metals may entail creating stressful conditions for plants. Every stressor induces changes in plant cell metabolism, resulting in disruption of homeostasis. Stressors may activate modification mechanisms, which allow the plant to develop features and adaptations determining higher resistance (Tukendorf and Wójcik 1995, Clijsters *et al*. 1999, Baranowska-Morek 2003, Siwek 2008).

The factors that determine the accumulation of heavy metals in soils, particularly, their solubility and bioaccessibility, include soil pH, organic substances, hydroxides, clay minerals, and interactions with other elements. The harmful effect of heavy metals manifests itself when they occur in the environment at particular concentrations. The response of plants to heavy metals depends on the plant's individual vulnerability, the duration of exposure, the

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Abbreviations: Chl – chlorophyll; control – control areas of Warcino Forest District; Cu-site – areas with higher copper concentration in Warcino Forest District; DM – dry mass; F_0 – minimal fluorescence yield of the dark-adapted state; F_v/F_m – maximal quantum yield of PSII photochemistry; NPQ – nonphotochemical quenching; P_N – net photosynthetic rate; R – respiration; q_P – photochemical quenching coefficient.
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intensity of stress, and the form in which the metals are accessible. Moreover, not only a proper concentration of an element in the tissues is required for the normal development of a plant, but also the suitable proportion of this element to other substances present in the soil (Yruela 2005, Gruca-Królikowska and Wacławek 2006).

Macronutrients (N, P, K, S, Ca, Mg) and micronutrients (Fe, B, Mo, Zn, Cu, Mn) occurring as natural components of the environment, are essential for the normal functioning of a plant. Copper is acquired and transported in the form of copper ions or chelates most intensively by young plants. In the soil, it occurs in combinations with organic substances, clay minerals, and precipitates, such as sulphates, sulphides, and carbonates. It takes part in defensive mechanisms, biochemical reactions as a cofactor of enzymes and an electron carrier (Yruela 2005). It is also involved in the metabolism of nitrogen compounds and sugars, regulates DNA and RNA production processes, and plays a role in cell wall lignification (Ouzounidou *et al*. 1992, Wisniewski *et al*. 2003, Alaoui-Sossé *et al*. 2004, Valko *et al*. 2005). An excess of copper causes disruption in plant growth, plant pigment production, photosynthesis, and respiration, as well as in the rearrangement of protein and lipid structures (Maksymiec 1997, Vinit-Dunand *et al*. 2002, Burkhead 2009).

The copper content in plants is considerably varied depending on the plant organ and developmental stage, species, and variety, as well as climatic conditions (Ostrowska *et al*. 2006). According to Vries and Heij (1991) and Schachtman *et al*. (1998), the concentration of nutrients in the photosynthetic apparatus of plants indicates the content of their supply of mineral elements.

Scots pine (*Pinus sylvestris* L.) is an evergreen tree species from the Pinaceae family. It reaches an average height of approximately 30 m. It has stiff, hard, pointed, finely serrated, and spirally curved needles. Set in pairs on dwarf shoots, they are often used in bioindication research (Dmuchowski and Bytnerowicz 1995, Kurczyńska *et al*. 1997, Yilmaz and Zengin 2004, Parzych and Sobisz 2012).

In this study, we investigated the impact of the high copper concentration in soil on several physiological processes in Scots pine (*P. sylvestris*). We compared two forest stands in the Warcino Forest District, Poland, one stand with high copper contents and poorly growing pine trees (Cu-site) and a reference area with pine trees showing unimpeded growth (control).

The study area in the Warcino Forest District was characterized by highly acidic pH and acidic contents of mineral soils (pH in KCl = 4.03–4.98). Base saturation assumed low values, reaching a maximum –27% in the bedrock. The content of organic carbon in the soil under weakly growing pine trees was low and did not exceed 0.93%. The control soil carbon content in the well-developed organic level was 16.62%. The mineral fraction of soil was predominated by sand ($Ø$ = 0.05–2 mm), which content ranged from 92 to 98%. The clay ($Ø$ < 0.002 mm) fractions content ranged from 1 to 3%. Soil texture may limit the water supply to plants during periods of dry weather. The former German timber storage area is characterized by the higher copper content, the origin of which is associated with long-term use of wood preservatives. In the past, this area functioned as a repository of wood harvested in the surrounding stands. The average copper content in the content of mineral humus under weakly growing pine trees was 175 mg kg^{-1} (soil) and under the control stand it was 41 mg kg^{-1}(soil). In the area of the former German timber storage area, not covered by plants, the copper content in the humus reached 913 mg kg^{-1}(soil), whereas the organic carbon content was 0.73% (Wanic *et al*. 2013).

Plant material in the form of annual shoot increments of Scots pine (*P. sylvestris* L.) was sampled from the former wood storage area, where exceeded limits of copper content were reported (Cu-site), and from areas with pine tree showing unimpeded growth (control) in the Warcino Forest District.

Biometric analysis of dwarf shoots and needles was performed using a magnifying glass with a scale (*Hund Wetzlar FLQ150*, *Wetzlar*, Germany).

The fresh mass (FM) was determined for each of the studied needles on scales (*Ohaus Adventurer Pro Av 264c*, Melrose, USA). The needles were subsequently frozen in –80°C and lyophilised in a lyophiliser (*Scanvac CoolSafe™ 55-4 PRO*, Lynge, Denmark). The lyophilised leaves were weighed in order to establish the dry mass (DM) and calculate the water content.

In order to perform mineralisation, dried (*Wamed SUP-100*, Warsaw, Poland) and weighed (*Radwag WPA 60/C*, Warsaw, Poland) to an accuracy of ± 0.0001 g of needles were placed in glass flasks of a digestion apparatus (*Velp Scientifica DK20*, Usmate, Italy). They were subsequently poured over with 65% HNO_3 and left to dissolve at a temperature of 25°C. The samples were then gradually heated for 30 min at a temperature of 100°C and 120°C and for 120 min at temperature 140°C, until they reached full mineralisation. Excess acid was removed by heating the material without leading to its complete evaporation. Mineralised material was transferred to scaled test tubes, which were then filled up to 10 ml with deionised water (*Direct-Q 3 UV*, Millipore, USA). Copper content was determined with an atomic absorption spectrometer (*Analyst 200*, *PerkinElmer*, Waltham, USA), using HCL lamps.

Two morphologically similar *P. sylvestris* needles were placed in polypropylene falcon tubes in 30 ml of deionised water with specific conductance of 0.05 µS cm^{-1}. Tubes were then placed on a shaker (*Rocker Labnet International*, New York, USA) and Vortex (*Biomix BVX-10*, Blizne Jasinski, Poland) for 3 h. After that time, the electroconductivity of diffusates (L_Z) was measured using a multifunction device (*CX-701 Elmetron*, Zabrze, Poland). After the measurement, plant material was frozen at –80°C to cause the degradation of plasma membranes.

The material was successively defrosted and subjected to the same shaking procedure as alive *P. sylvestris* needles; the electroconductivity of the whole electrolyte content in the tissue was measured (L_M). The percentage of electrolyte leakage through plasma membranes was calculated according to the following formula: EL [%] = (L_Z/L_M) × 100, where EL is an electrolyte leakage, L_M is an electrolyte leakage from the dead cells, and L_Z is an electrolyte leakage from the living cells.

The net photosynthetic rate (P_N) and respiration (R) of one-year-old *P. sylvestris* needles were determined using an infrared gas analyser *ADC-225 MK-3* (*ADC Bio-Scientific Ltd.*, Herts, UK). The intensity of the light during the measurements was 100 µmol(photon) m^{-2} s^{-1}. The temperature during measurements was constant and kept at 25°C. The rates of these two processes were determined in air containing 21% of oxygen, with CO_2 concentration at 300–400 µmol mol^{-1}, in a closed system (Rut *et al*. 2010). The net photosynthetic rate and respiration were expressed in µmol(CO_2) g^{-1}(DM) h^{-1} taken up/released.

The rate of these processes was also measured using the Clark electrode (*Hansatech Instruments Ltd.*, Norfolk, UK). The electrode was placed in a 5-ml chamber *LD/2*, where a constant temperature of 25°C was maintained. The electrode was connected to a data-reading device *CB1D*. Computer software '*Acquire*' was employed for data reading and analysis. The measurement conditions were the same as in measurements with the infrared gas analyser. The rates of photosynthesis and respiration were expressed in µmol(O_2) g^{-1}(DM) h^{-1} released/taken up.

The content of chlorophyll (Chl *a* and *b*) was determined in dimethyl sulfoxide (*Sigma Aldrich*, England) using Barnes *et al*. (1992) method at a wavelength of 665 and 648 nm, with spectrophotometer *Aquarius 9500* (*Cecil Instruments*, Cambridge, UK). The amount of Chl was converted into a concentration in DM.

The functioning of PSII was examined with the use of fluorometer (*Fluorescence Monitoring System – 1*, *Hansatech Instruments Ltd.*, Norfolk, UK). Dark-acclimated needles after 30 min were exposed to excitation light 600 µmol(photon) m^{-2} s^{-1}. The values of the following parameters were analysed: (*1*) the fluorescence intensity indicators: minimal Chl fluorescence, when all dark-adapted PSII reaction centers are open (F_0), photochemical efficiency of PSII (F_v/F_m) (van Kooten and Snel 1990), and (*2*) fluorescence quenching parameters: photochemical quenching (q_P) and nonphotochemical quenching (NPQ) (Maxwell and Johnson 2000).

The significance of differences between mean values ± SD (n = 5) were assessed by analysis of variance (*ANOVA/MANOVA*) using *Tukey*'s test at p<0.05. Calculations were made in *STATISTICA 10.0* software.

Biometric analysis of *P. sylvestris* needles and dwarf shoots showed statistically significant differences in their length. Pine dwarf shoots and needles were markedly longer in the plants growing at control site compared with the needles of plants occurring at Cu-sites (Table 1).

Higher DM values were noted in the plants from the control compared to Cu-site. As regards water content, lower values were found in the plants from the control site, while higher amounts were found in the Cu-site plants (Table 1).

A statistically significant increase in the copper content was observed in *P. sylvestris* needles from the Cu-site relative to the control plants (Table 1).

Significant increases in cell membrane permeability were noted in needles from the Cu-site in relation to the plants from the control site. In the case of *P. sylvestris* from the Cu-site, the values of electrolyte leakage fluctuated from 90 to 100%, which is nearly 20% more of total electrolyte content in tissues in relation to the outflow of ions in samples from the control (Table 1).

Significant differences were noted in the gas-exchange rates measured by changes in carbon dioxide and oxygen concentration in *P. sylvestris* needles. The intensity of photosynthesis measured by the amount of carbon dioxide taken up was much higher in *P. sylvestris* needles from the control than that in the needles of plants growing at the Cu-site (Fig. 1).

Table 1. Comparison of morphological and physiological parameters in *Pinus sylvestris* needles from control area of the Warcino Forest District and Cu-site. Values are means (± SD), n = 5. Means followed by *the different letter* in each row are significantly different (p<0.05). DM – dry mass; Chl – chlorophyll; F_0 – minimal fluorescence yield of the dark-adapted state; F_v/F_m – maximal quantum yield of PSII photochemistry; NPQ – nonphotochemical quenching; q_P – photochemical quenching coefficient.

Parameter	Control	Cu-site
Dwarf shoot length [mm]	5.82^a	3.85^b
Needle length [mm]	54.32^a	41.75^b
DM [g]	0.018^a	0.013^b
Water content [%]	46.09^b	52.12^a
Cu [µg g^{-1}(DM)]	6.36^b	7.78^a
Electrolyte leakage [%]	72^b	93^a
Chl *a* [mg g^{-1}(DM)]	4.25^a	2.24^b
Chl (*a* + *b*) [mg g^{-1}(DM)]	5.23^a	2.80^b
Chl *a*/*b*	4.34^a	4.00^a
F_0	230.70^a	197.90^a
F_v/F_m	0.828^a	0.829^a
NPQ	0.068^b	0.091^a
q_P	0.938^a	0.940^a

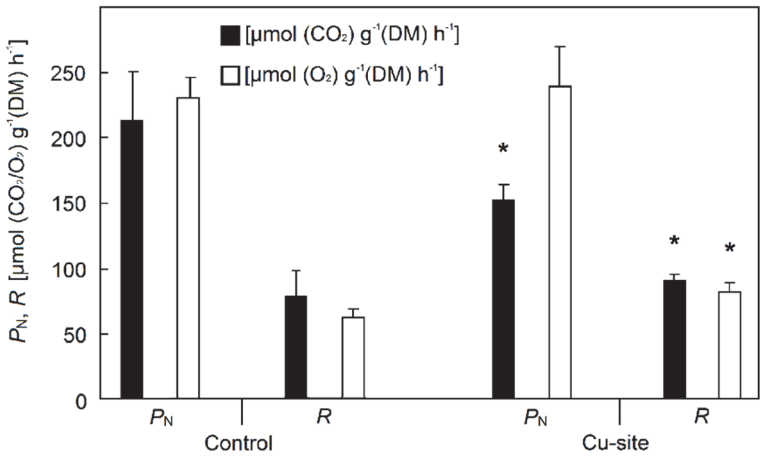

Fig. 1. Net photosynthetic (P_N) and respiration (R) rates of *Pinus sylvestris* needles from control area of the Warcino Forest District and Cu-site, expressed in CO_2 or O_2 uptake/output for plant dry mass. * – significant differences from control plants at $p<0.05$. Means ± SD, $n = 5$.

The net photosynthesis of needles measured by the amount of released oxygen obtained from the plants of the two sites with different Cu content showed no significant difference (Fig. 1). The rate of photosynthesis measured by carbon dioxide consumption in needles from the Cu-site was considerably lower than that measured by changes in oxygen concentration. Plants grown at the control site showed comparable CO_2 and O_2 rates (Fig. 1).

The *R* of the needles growing at the Cu-site was markedly higher compared with those from the plants of the control site (Fig. 1). The *R* of the Cu-site plants was higher merely by 15% when measuring the amount of released CO_2. However, the rate of the studied process which one *R* increased by 30% in the case of measured oxygen consumption (Fig. 1). No significant differences were noted in the rate of *R* measured by the oxygen consumption or CO_2 release between the control and the Cu-site. The *R* measured by CO_2 release was slightly higher than the rate of this process measured by oxygen uptake.

The content of Chl *a* and *b* in *P. sylvestris* needles was significantly lower at the Cu-site compared with that of the control area (Table 1). The analyses of selected fluorescence parameters showed no statistically significant differences in the studied *P. sylvestris* needles. The only exception was the parameter of NPQ, which showed higher values in *P. sylvestris* from the Cu-site compared with the control (Table 1).

The occurrence of heavy metals in the environment and their easy access through the root system and leaves cause a serious problem for plants, interfering with their metabolic and physiological processes. Prior to the manifestation of copper toxicity, the inhibition of plant growth and the reduction of yield occurs (Jiang *et al*. 2001, Gupta and Abdullah 2011). Ouzounidou *et al*. (1994), Monni *et al*. (2000), MacFarlane and Burchett (2002), and Elleuch *et al*. (2013) found that copper contamination disturbs physiological processes from the seed germination phase to the growth phase. The studies carried out in the Warcino Forest District showed that the length of pine dwarf shoots and whole needles and needle biomass of *P. sylvestris* were substantially higher in the control areas than that in the plants occurring on polluted soils (Cu-site), which suggested an inhibitory effect of copper (Table 1).

According to Kopittke and Menzies (2006), copper hinders mass production in roots to a larger extent than in shoots and leaves of *Vigna unguiculata*, and the restriction of shoot growth is higher due to the nutrient deficiency ensuing from the reduced nutrient uptake in damaged roots rather than the direct effect of toxic metal. Rouphael *et al*. (2008) believe that heavy metals disrupt the transport and absorption of nutrients, exerting phytotoxic effects on organisms. In the case of *P. sylvestris* from the Warcino Forest District, increased Cu accumulation was observed in the needles. The toxic effect of Cu on life processes results from its interaction with the functional groups of molecules that are found in cells, particularly, proteins and polynucleotides (Yruela *et al*. 2008, Moreira *et al*. 2015). The toxic effect mainly involves the damage of cells and inhibition of their growth (Liu *et al*. 2004). As a result, cell division and elongation of new cells in the growth zones of roots and aboveground plant parts are restrained and cell membrane permeability is altered (Kukkola *et al*. 2000, Schützendübel and Polle 2002, Liu *et al*. 2009, Bouazizi *et al*. 2010). High Cu concentrations seemed to have a detrimental effect on the functioning of plasma membranes in the cells of *P. sylvestris* needles. In the experiment, electrolyte leakage from the needle cells of plants growing on soils with high Cu content increased by 20% compared with the control needles obtained from the tree stand showing unimpeded growth (Table 1). According to Hall (2002), cell membranes are the first structures to be affected by heavy metals. Their disintegration allows the permeation of metals and augments the outflow of ions, which disturbs water and ion balance. Damages are induced, among others, by protein oxidation and changes in the composition and fluidity of plasma membrane lipid components.

The direct effects of heavy metals are observed in the functioning of chloroplasts, the destabilisation of cell membranes, the inhibition of Chl synthesis, and the disruption of the Calvin cycle (Clijsters *et al*. 1999). As

reported by Monni *et al*. (2001), *Empetrum nigrum* plants growing in the vicinity of a copper and nickel smelting Harjavalta plant in south-western Finland contained markedly lower Chl concentrations than plants growing away from the contaminated sites. Narrowed leaves, as well as progressive chlorosis and necrosis were observed at high Cu concentrations in *Trigonella foenum-graecum* (Elleuch *et al*. 2013). Caspi *et al*. (1999) showed an inhibitory effect of Cu on Chl synthesis in the leaves of barley. In the case of the studied needles from the Warcino Forest District, a reduced content of Chl *a* and *b* was demonstrated. The total Chl content at the Cu-site was less than half compared with those from control areas (Table 1). High accumulation of heavy metals causes the destruction of Chl-protein complexes, disrupts mineral nutrition, and decreases the energy balance of cells (Barón *et al*. 1995, Chen *et al*. 2000, Pilon *et al*. 2006, Rouphael *et al*. 2008). Another result of an excessive Cu concentration is the deactivation of ferredoxin *via* oxidation of thiol groups (SH) of this enzyme by Cu. Studies by Stolarska *et al*. (2006) have shown that the application of Cu salts at concentrations exceeding 0.05 mmol kg^{-1}(soil) causes a significant decrease in Chl concentration, CO_2 assimilation rate, and water-use efficiency in photosynthesis. The consequence was a reduced plant productivity, the suppressed growth of the root system and foliage development. The lowered photosynthesis measured by CO_2 uptake in *P. sylvestris* needles from the timber storage area might be caused by a higher Cu content in the needles compared with the plants from the control site. Increased Cu concentration and its accumulation in the aboveground parts of pines is most likely due to the higher content of this metal in the soil at the former timber storage site (Wanic *et al*. 2013). An excess of Cu in plants causes disruption in the Calvin cycle (lowered activity of Rubisco and other enzymes of this cycle) (Williams and Mills 2005, Yruela 2009, Pádua *et al*. 2010).

The lower rate of photosynthesis may be associated with the reduced Chl content in *P. sylvestris* needles from the poorly growing-tree stand and might occur due to difficulties with the uptake of other macronutrients (necessary for the Chl synthesis) from soils with higher Cu contents or due to the larger losses of absorbed photon energy dissipated as heat *via* the xanthophyll cycle (Osmond *et al*. 1997) (Table 1). The noted increase of the *R*, determined by the quantity of CO_2 released and oxygen taken up, may result from a higher demand for ATP in *P. sylvestris* needles from the contaminated tree stand which was aimed for reparation of the damage caused by the excess of copper (Fig. 1).

Study of the kinetics of Chl *a* fluorescence by fluorimeter is one of the best ways to explore the function of PSII and its reactions in response to changes in environmental conditions and plant growth (Kalaji and Nalborczyk 1991, Kalaji *et al*. 2012a,b, 2014; Peng *et al*. 2012). Copper ions in excess cause reduction of PSII activity (Pätsikkä *et al*. 1998). PSII efficiency is associated with the thylakoid membranes of chloroplasts (Pätsikkä *et al*. 2002) and the quantum yield of PSII electron transport (Vinit-Dunand *et al*. 2002). Copper has inhibitory effects on plastoquinone through reduction and interference with electron donation to photochemical reactions in PSII (Jegerschöld *et al*. 1995, Yruela *et al*. 1993, 1996). F_0 parameter indicates the excitation energy loss during transmission of the antenna energy to PSII (Baker and Rosenquist 2004). This parameter characterizes fluorescence emission of the excited Chl molecules in PSII antenna when all PSII reaction centers are open and ready to accept new electrons. The variable values of F_0 may be the result of an increased number of inactive reaction centers, where electrons cannot be transferred out by reduced plastoquinone, low energy transfer from the LHCII to PSII reaction center or caused by the dissociation of LHCII from PSII core, or D1 protein degradation and inactivation in PSII reaction centers (Havaux 1993, Rintamäki *et al*. 1995). F_v/F_m and q_P reflect the true efficiency of PSII by the amount of light energy absorbed by PSII to the energy consumed by an open reaction centers. They are responsible for reducing the amount of electrons in the stabilization of CO_2 (Maxwell and Johnson 2000, Lichtenthaler *et al*. 2005). The values of F_v/F_m in higher plants are close to 0.83 (Björkman and Demmig 1987), and the q_P values range between from 0 to 1 (Maxwell and Johnson 2000). NPQ associated with heat losses can range from 0 to infinity depending on the species and acting stressor (Lichtenthaler *et al*. 2004, Porcar-Castell 2011) and is responsible for excess light energy that is harmlessly dissipated as heat (Müller *et al*. 2001). Copper ions caused reduction of light-saturated PSII coincided with increased NPQ, which is normally associated with xanthophyll cycle-dependent energy dissipation in higher plants (Ruban and Horton 1999, Ebbert *et al*. 2001). In our study, the needles of *P. sylvestris* from the Cu-site compared with the control ones showed high photochemical efficiency of PSII (Table 1). Only NPQ significantly increased in the needles of *P. sylvestris* from the Cu-site relative to the value of the control (Table 1). No changes in the values of other parameters of fluorescence may indicate a physiological adaptation of plants to cope with oxidative stress (Shaw *et al*. 2014). Copper ions may target chloroplast membrane H^+-ATPases, thus lowering the demand for H^+ and electron transport, indirectly resulting in excitation energy entrapment in PSII. Inhibitory effects of Cu ions on ATPases and ion channels are known from several plant systems (Demidchik *et al*. 1997, 2001; Maksymiec 1997). Chloroplasts have developed flexible mechanisms to cope with changes in demand for energy. They are activated in stressful environmental conditions and metabolic disorders. Their purpose is sustainable production and consumption of ATP and NADPH by increasing the production of intermediates or preventing the accumulation of excess intermediates. The mismatch in the regulation restricts photosynthesis (Cruz *et al*. 2005,

Kramer and Evans 2011).

In the presence of excessive quantities of heavy metals, the symptoms of toxicity may be produced by a number of interactions at cellular and molecular levels. At cellular level, they may be involved not only in detoxification, but also in developing tolerance to stressors. They all seem to be active in preventing toxic concentrations from accumulation in vital sites of the cell, thus averting the related harmful effects (Barón *et al*. 1995, Hall 2002).

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