ORIGINAL ARTICLE

EFFECTS OF EXCESS Cu ON GROWTH AND PHOTOSYNTHESIS OF BARLEY PLANTS. IMPLICATION WITH A SCREENING TEST FOR Cu TOLERANCE

ЕФЕКТИ НА ИЗЛИШЪКА НА Cu ВЪРХУ РАСТЕЖА И ФОТОСИНТЕЗАТА НА ЕЧЕМИЧНИ РАСТЕНИЯ. ВРЪЗКА СЪС СКРИНИНГ ТЕСТ ЗА ТОЛЕРАНТНОСТ КЪМ Cu

A. VASSILEV*, LIDON F., RAMALHO J. C., DO CÉU MATOS M., DA GRACA M.

ABSTRACT

Twenty-day-old barley (*Hordeum vulgare* L. cv. Ribeka) plants grown as sand culture were exposed to Cu treatment (0, 10, 15 and 20 mg Cu kg^{-1} sand) for ten days. The effects of excess Cu on both growth and photosynthetic performance were studied in order to identify the most sensitive probes implicating a further development of screening test for Cu tolerance within barley genotypes. The results obtained indicated that stomata conductance and photosynthetic electron transport linked to PSII+OEC exhibited the highest sensitivity to excess Cu, followed by plant dry weigh accumulation, leaf area formation, net photosynthetic rate, photosynthetic electron transport linked to PSI and PSII-OEC. It was concluded that leaf gas exchange parameters, plant dry weight accumulation and leaf area formation present an effective plant test system for screening for barley genotypes with higher Cu tolerance.

KEYWORDS: Cu, barley (Hordeum vulgare L.), growth, photosynthesis, Cu tolerance, screening test

РЕЗЮМЕ

Двадесетдневни ечемични (*Hordeum vulgare* L. сорт Ribeka) растения, отглеждани като пясъчна култура, са подложени на третиране с Cu (0, 10, 15 and 20 mg Cu kg^{-1} пясък) в продължение на 10 дни. Проучени са ефектите на излишъка на Cu върху растежа и фотосинтетичната активност на растенията с цел да се определят най-чувствителните индикатори във връзка с понататъшното разработване на тест за скрининг на толерантни към Cu ечемични генотипове. Получените резултати показват, че с най-висока чувствителност към Cu са устичната проводимост, фотосинтетичния електронен транспорт свързан с PSII+OEC, следвани от натрупването на суха маса в растенията, формирането на листната площ, нето скоростта на фотосинтезата, фотосинтетичния електронен транспорт свързан с PSI и PSII-OEC. Заключава се, че листният газообмен, натрупването на суха маса и формирането на листна площ представляват ефективна растителна система за скрининг за ечемични генотипове с по-висока толерантност към Cu.

КЛЮЧОВИ ДУМИ: Cu, ечемик (*Hordeum vulgare* L.), растеж, фотосинтеза, Cu толерантност, скрининг тест

Manuscript received: May 15, 2003
Review: May 15, 2003
Accepted for publication: June 15, 2003

JOURNAL
Central European Agriculture
ISSN 1332-9049

DETAILED ABSTRACT

The almost one century long use of Bordeaux mixture against vine downy mildew has lead to copper (Cu) contamination of topsoil layers in many wine-producing regions. There are some expectations for land use changes in wine-producing Mediterranean regions, but the Cu contamination may create a problem for shallow rooting plant species, *e.g*. cereals. The information concerning barley responses to excess Cu is insufficient and much work should be done concerning the evaluation and mechanisms responsible for cultivar differences in Cu tolerance. Thus, there is a need to develop and conduct screening tests aimed to identify genotypes with higher Cu tolerance.

Generally, the screening tests need sensitive indicators, providing fast and reliable information. The wide spectrum of Cu-induced effects on plant physiology, and especially on photosynthesis, may be used as suitable indicators. For example, leaf gas exchange and chlorophyll fluorescence are easily measured and, on the other hand, may be determined *in situ*, thus reflecting not only Cu phytotoxicity itself, but also metal interactions with different environmental factors. On the other hand, due to well known self-regulation of the photosynthesis, down-regulation processes may appear in Cu-exposed plants leading to reduced demand for photosynthates. In this situation, integral estimates, such as CO_2 fixation might be less sensitive than the destructively measured ones - photosynthetic pigments content, electron transport activities, enzyme activities, etc.

The phytotoxic effects of Cu (as well as other metals) are strongly dependant on many factors with different origin. Thus, the sensitivity of the proposed bio-indicators should be tested for the actual species in an experimental design well suited for screening studies. The aim of this work was to study the effects of excess Cu on barley growth and photosynthesis indicators in order to develop screening test for cultivar difference in Cu tolerance, based on simple mathematical approaches.

The results obtained in this study indicated that a number of different estimates of barley plants are significantly influenced by excess Cu and might be used as bio-indicators in plant test system. The calculated "effective concentrations" leading to 10% decrease in the value of the used estimates (EC_{10}) varied around 12 - 16 mg Cu kg^{-1} sand for plant dry weight (DW) accumulation, leaf area (LA) formation as well as for net photosynthetic rate (A), photosynthetic electron transport linked to photosystem I (PSI) and photosystem II without water evolving complex (PSII-OEC). Stomata conductance (gs) and PSII+OEC exhibited the highest sensitivity to excess Cu, their EC_{10} values were about 4 - 5 mg Cu kg^{-1} sand. However, this study showed that in the experimental design used photosynthetic pigments content and chlorophyll fluorescence are less sensitive parameters of Cu toxicity.

In conclusion, excess Cu produces a variety of toxic effects on barley growth and photosynthesis. Based on both high sensitivity of leaf gas exchange parameters (g_s and A) to excess Cu and fastness of their measurement, we propose these parameters together with plant dry weight accumulation and leaf area formation as suitable parameters for plant test systems, which can be utilized for successful screening for higher Cu tolerance among barley cultivars.

INTRODUCTION

Agricultural soils in many parts of the world are contaminated by heavy metals. The use of Bordeaux mixture for almost one century against vine downy mildew lead to copper (Cu) contamination of topsoil layers in many wine-producing regions. While arable land usually presents Cu contents between 5 and 30 mg kg^{-1}, many wine-growing areas have been found to exhibit values between 200 and 500 mg kg^{-1} [2]. There are some expectations for land use changes in wine-producing Mediterranean regions due to the European Community policy concerning agricultural subsidy [3], but the Cu contamination may create a problem for the use of shallow rooting plant species, *e.g.*, cereals. In fact, there is some evidence that some cereals, as rice [22], wheat [16], oat [26] and barley [31] are able to tolerate (to some extend) excess Cu, but much work should be done concerning the evaluation and mechanisms for cultivar tolerance differences in Cu. Thus, there is a need to develop and conduct screening tests aimed to identify genotypes with higher Cu tolerance.

The unspecific visual symptoms of chronic Cu phytotoxicity includes growth reduction, interveinal foliar chlorosis, wilted leaves, necrotic leaf tips, etc. However, with a low degree of metal contamination, the visible symptoms are less pronounced, or even be absent, although reduction of plant yield quality and inhibition of biomass production may persist [30].

Commonly, metal tolerance is evaluated by the toxic threshold value in the tissue leading to a definitive inhibition of dry matter yield and/or root elongation [23]. Some metabolic biomarkers, such as peroxidase (POD) activity and (iso)peroxidase pattern have also been proposed to assess Cu phytotoxicity [25]. Furthermore, other Cu-induced effects on plant physiology may be additionally used as suitable indicators. For example, Cu toxicity impact on photosynthesis is well documented (for reviews see 15, 24, 18). Briefly, excess Cu triggers an oxidative stress in plant cell [4], disturbs chloroplast and thylakoid ultrastructure [1], diminishes content of photosynthetic pigments, electron carriers and subsequently photosynthetic electron transport [19, 20], decreases enzymes activities and leaf gas exchanges [28, 22], changes sink-source interactions [5, 27], etc.

Generally, the screening tests need sensitive indicators, providing fast and reliable information. In this aspect some *in vivo* measured photosynthetic parameters have advantages over some *in vitro* determinations. For example, leaf gas exchange and chlorophyll fluorescence are easily measured and, on the other hand, may be determined *in situ*, thus reflecting not only Cu phytotoxicity itself, but also metal interactions with different environmental factors. On the contrary, due to well known self-regulation of the photosynthesis, down-regulation processes may appear in Cu-exposed plants leading to reduced demand for photosynthates [5]. In this situation, integral estimates, such as CO_2 fixation might be less sensitive than the destructively measured ones - photosynthetic pigments content, electron transport activities, enzyme activities, etc. The same might be suggested for chlorophyll fluorescence, which in heavy metal-stressed plants seems to be indirectly affected [14] unlike in plants exposed to light and heat stresses.

The phytotoxic effects of Cu (as well as other metals) are strongly dependant on many factors with different origin. Thus, the sensitivity of the proposed bio-indicators should be tested for the actual species in an experimental design well suited for screening studies. The present work was conducted with barley plants exposed to Cu-enriched sand. The aim of this work was to study the effects of excess Cu on barley growth and photosynthesis indicators in order to develop screening tests for cultivar difference in Cu tolerance, based on simple mathematical approaches.

PLANT MATERIAL AND GROWTH CONDITIONS

Barley (*Hordeum vulgare* L. cv. Ribeka) plants were grown in pots lined with polyethylene bags and filled in with 2.5 kg of sand in a glasshouse under natural conditions of light, temperature and humidity as described elsewhere [31]. Twenty days after emergence, the plants were given 125 ml of nutrient solution containing 0, 1.6, 3.2, 4.8 and 6.4 mM Cu, which corresponds to 0, 5, 10, 15 and 20 mg total Cu kg^{-1} sand, respectively. Cu ion was applied as $CuSO_4.5H_2O$, plants were exposed to Cu treatment for 10 days. At the end of the treatment period different photosynthetic parameters were measured. After harvest, the plants were cut to leaves and roots. The roots were thoroughly washed with deionized water. Dry mass of leaves and roots was determined after drying the sample at 70 °C to constant weight.

Leaf gas exchange measurements

Leaf gas-exchange measurements included net photosynthesis (A), stomatal conductance (g_s), transpiration rate (E) and intercellular CO_2 (c_i) were performed on the first fully expanded leaf from the top, using a infrared gas analyzer (Li-6200, Li-Cor Inc., USA). The measurements were carried out in a growth chamber, under controlled conditions of temperature (20±1ºC), air humidity (75±1%), photon flux density (*ca*. 800 μmol m^{-2} s^{-1}), and external CO_2 concentration (*ca*. 380 μmol mol^{-1}).
Measurements of oxygen evolution, expressing photosynthetic capacity (A_{max}), were performed using a leaf-disc oxygen electrode (LD2/2, Hansatech, UK), in 2 cm^2 leaf pieces (taken from undamaged zones) placed under saturating conditions of light (900 μmol m^{-2} s^{-1}, provided by a Björkman lamp (LS2, Hansatech) and CO_2 (*ca*. 7%, supplied by 400 μL $KHCO_3$, 2 M), at a stabilized temperature of 25ºC.

Chlorophyll *a* fluorescence measurements

Chlorophyll fluorescence parameters were measured using a PAM 2000 system (H. Walz, Effeltrich, Germany) on leaf discs (from undamaged areas) placed inside the LD2/2 O_2 electrode, under CO_2 saturating conditions, at 25ºC. Measurements of the minimal fluorescence from the antennae, F_o, and photochemical efficiency of PSII, F_v/F_m, were taken from overnight dark-adapted leaves. The photochemical quenching, q_P, [29], the estimation of quantum yield of photosynthetic non-cyclic electron transport, ϕ_e [9] and the PSII efficiency of energy conversion, F_v'/F_m', [14] were determined under photosynthetic steady-state conditions, using a photon flux density of 550 μmol m^{-2} s^{-1} as actinic light and 4200 μmol m^{-2} s^{-1} for the saturating flashes (with a duration of 0.8 s).

Photosynthetic electron transport rates

Determination of photosynthetic activities coupled to PSII and PSI were measured in a Clark-type oxygen electrode (LW2, Hansatech), using subchloroplast fractions obtained as described by Droppa *et al.* [7], with minor modifications [20]. The electron transport rates were determined according to Droppa *et al.* [7] in 1 ml of reaction mixture containing 100-150 μg chl, at 25°C and with photon flux density of 3000 μmol m^{-2} s^{-1}, given by a Björkman lamp.

Ethylene production associated with thylakoid membranes

Ethylene production was measured in 500 μl of thylakoid extracts incubated in 2 ml flasks with a light intensity of 500-600 μmol m^{-2} s^{-1}, provided by a Björkman lamp. After two hours of incubation a 1 ml gas sample was withdrawn from the headspace gas of the incubating flask using a gas-tight syringe.
This gas sample was analysed in a Pye Unicam gas chromatograph (Series 204, UK) equipped with a Porapak Q column and a flame ionization detector (FID). Ethylene was identified and quantified through an external standard with a known concentration (29 ppm).

Photosynthetic pigments content

Chlorophylls *a* and *b* and total carotenoids were extracted in 100% acetone, measured spectrophotometrically and calculated according to the formulae of Lichtenthaler [17].

Mineral analysis

The concentrations of Cu in roots and Cu, Fe and Mg in shoots were determined after dry mineralisation of samples by atomic absorption spectrophotometry (Perkin-Elmer 5000, country). Three replicates of plant tissue, with 1 g dry mass, were put in oven at 500 ºC for 24 hours. Each sample was dissolved in 5 ml of a 20 % HCl solution before analysis.

Statistical analysis

Statistical analysis was performed using a one way ANOVA (for $P<0.05$). Based on the ANOVA results, a regression analysis was performed, for a 95% confidence level.

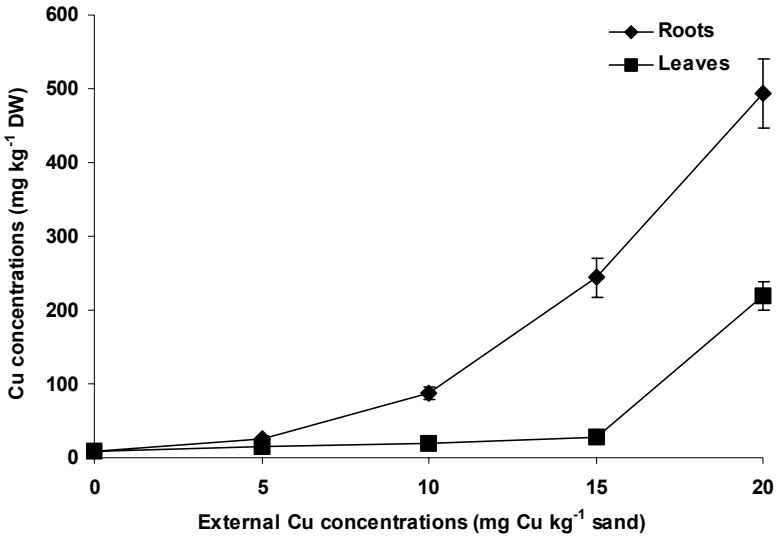
RESULTS

Cu content of plant parts and growth

No visual toxicity symptoms on barley leaves appeared when 20-day-old plants were exposed for 10 days in Cu-enriched sand having contamination level of 5, 10 and 15 mg total Cu kg^{-1} sand. However, well expressed leaf chlorosis and necrosis have been observed at the highest treatment 20 mg Cd kg^{-1} sand 5 days after the treatment beginning, resulted in dying of the lower leaves and root browning at the end of treatment period.
Mean Cu concentrations in both roots and leaves increased with Cu-exposure. In roots Cu

concentrations rised more gradually from 8 (0 Cu treatment, control) to 489 mg kg DM^{-1} (20 mg Cu kg^{-1}); the respective values in the leaves were 8 and 220. However, leaf Cu concentrations barely increased untill 15 mg Cu kg^{-1} showing a sharp rise only for the 20 mg Cu kg^{-1} treatment. The regression equations describing best the dependence of Cu concentrations (Y) in barley organs on sand Cu concentration (X) are given in Figure 1.

Figure 1: Cu concentrations in roots and shoots of Cu-exposed barley plants.



Mean values are the average of three replicates ± SE. The regression equations describing the dependence of Cu concentrations (Y) of plant organs on external Cu concentrations (X) are following:

$Y_{(leaves)} = {}^{*}1.07X^2 - {}^{*}12.7X + {}^{*}23.91$ $\quad r^2 = 0.87$

$Y_{(roots)} = {}^{*}1.63X^2 - {}^{*}8.68X + {}^{*}14.06$ $\quad r^2 = 0.94$

*Significant at the 0.05 probability level. X - external Cu concentration.

Dry mass and leaf area of barley plants exposed for 10 days to 5, 10 and 15 mg Cu kg^{-1} sand were not significantly differed from control plants values (0.58 g and 96.7 cm^2 per plant, respectively). However, these plant parameters at the highest Cu treatment (20 mg Cu kg^{-1}) were significantly lowered, averaging only 69% of dry weight and 56% of leaf area of the control plants. The regression equation best describing DW accumulation on leaf Cu concentration (X) was following: $Y_{(DW)} = -0.00081X + 0.5769$ ($r^2 = 0.92$; P=95%)(data not shown).

PHOTOSYNTHETIC PERFORMANCE

The photosynthetic performance of Cu-exposed barley plants was evaluated *in vivo* by leaf gas exchange and chlorophyll fluorescence measurements. The increase of Cu dose decreases all leaf gas exchange values, having the strongest negative effect on A and g_s (Table 1). The values of A and g_s at the highest dose (20 mg Cu kg^{-1}) averaged 56-57% of those of control plants. The effect of Cu on E and A_{max} was smaller, with a decrease below 20% for the 20 mg Cu kg^{-1} treatment. c_i values were slightly lowered by Cu treatments. According to the regression analysis a quadratic function described best A and g_s responses to external Cu concentration (P=95%). Chlorophyll fluorescence response showed only minor changes in Cu-exposed barley plants (Table 2).

Table 1: Net photosynthesis rate (A, μmol m^{-2} s^{-1}), transpiration rate (E, mmol m^{-2} s^{-1}), stomatal conductance (mol m^{-2} s^{-1}), c_i (ppm) and photosynthetic capacity (A_{max}, μmol m^{-2} s^{-1}) of Cu-exposed barley plants 10 days after treatment

Parameters	Cu treatments (mg kg^{-1} sand)									
	0		5		10		15		20	
A	13.97	(100)	11.93	(85)	11.34	(81)	9.17	(66)	7.85	(56)
E	5.88	(100)	5.27	(90)	5.38	(91)	4.65	(79)	4.80	(82)
g_s	0.292	(100)	0.227	(78)	0.232	(79)	0.170	(58)	0.165	(57)
c_I	289.7	(100)	275.5	(95)	271.8	(94)	272.5	(94)	285.0	(98)
A_{max}	23.05	(100)	-		21.58	(94)	20.73	(90)	19.63	(85)

Parameters	Regression Equation	r^2
A	$Y = -0.0004X^2 - {}^{*}0.292X + {}^{*}13.83$	0.66
g_s	$Y = {}^{*}0.00015X^2 - {}^{*}0.0091X + {}^{*}0.2867$	0.57

*Significant at the 0.05 probability level. X - external Cu concentration.

Table 2: Selected chlorophyll fluorescence parameters and quenching analysis coefficients of leaves (undamaged zones) of Cu-exposed barley plants 10 days after treatment

Parameters	Cu treatments (mg kg^{-1} sand)			
	0	10	15	20
F_0	42.8	41.4	40.5	41.3
F_v/F_m	0.812	0.815	0.817	0.805
F_v'/F_m'	0.530	0.511	0.525	0.527
q_P	0.536	0.507	0.538	0.530
q_{NP}	0.625	0.794	0.788	0.787
q_E	0.489	0.512	0.510	0.529
ϕ_e	0.285	0.260	0.272	0.280

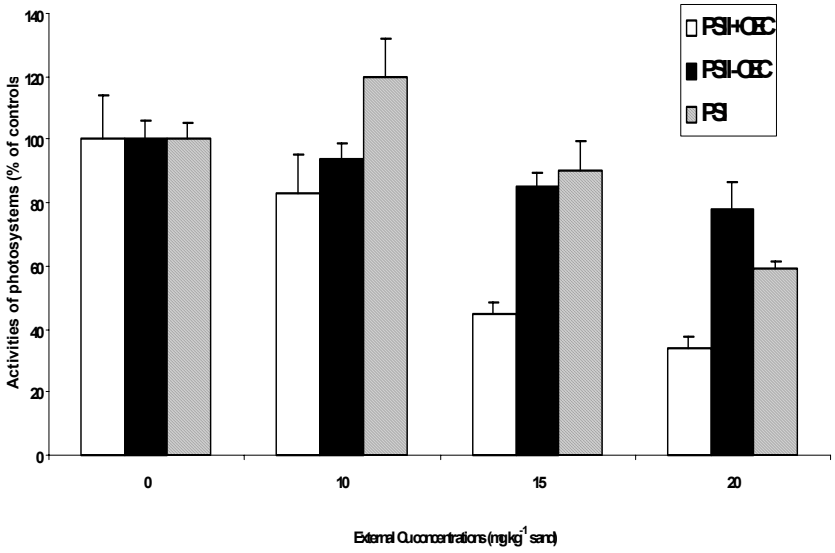
Only q_{NP} and q_E values showed a consistent tendency to increase, thus denoting a rise in the non-photochemical processes, but no significant differences were observed even in the highest Cu treatment. However, it is necessary to note that the measurements have been done on the undamaged leaf zones of Cu-exposed plants and under CO_2 saturating conditions (without CO_2 limitation due to g_s decrease).

The contents of chlorophyll *a* and *b* were severely reduced by Cu treatments relative to untreated plants (Table 3), specially with the 20 mg Cu kg^{-1} treatment, when the contents of Chl *a* and Chl *b* fall 53% and 41%, respectively.

At the same treatment, excess of Cu induced also a decrease in the carotenoids content by 35%. The Chl (*a*/*b*) and (Total Chl/Total Carotenoids) ratios remained quite stable except for the highest Cu dose, when the values decreased 19 and 23%, respectively.

The activity of photosynthetic electron transport was retarded in Cu-exposed plants (Figure 2).

Figure 2: Rates of photosynthetic electron transport in thylakoid membranes isolated from leaves of barley plants grown in Cd-enriched sand.



Electron transport rates were measured between: (1) H_2O and 2,6-dichlorophenolindo-phenol (DCPIP) (PSII+OEC, oxygen evolving complex), (2) 1,5-diphenyl-carbohydrazide (DPC) and DCPIP (PSII-OEC) and (3) reduced $DCPIPH_2$ and methyl viologen (MV) (PSI). Control values (representing 100%) were 92, 86 and 496.1 µmol O_2 mg chl^{-1} h^{-1} for PSII+OEC, PSII-OEC and PSI, respectively. Mean values are the average of three replicates ± SE. The regression equations describing photosynthetic electron transport rates on external Cu concentrations are following:

$Y_{(PSII+OEC)} = {}^{*}\text{-}0.088X^2 + {}^{*}1.537X + {}^{*}93.14 \quad (r^2 = 0.75)$

$Y_{(PSII-OEC)} = {}^{*}\text{-}0.038X^2 + {}^{*}0.221X + {}^{*}86.15 \; (r^2 = 0.46)$

$Y_{(PSI)} = {}^{*}\text{-}1.87X^2 + {}^{*}26.52X + {}^{*}499.6 \; (r^2 = 0.76).$

*Significant at the 0.05 probability level. X - external Cu concentration

Significant decreases were obtained for electron transport involving both PSII and PSI. Nevertheless, the PSII seems to be affected at lower Cu concentrations than PSI, when the OEC is involved. In fact, after 10 days exposure to 20 mg Cd kg^{-1}, the decrease in PSII activity without OEC represented about 67% (a lower effect than that of PSI), whereas with OEC only 22% of the control values. In this treatment the PSI activity decreased to ca. 41% of its initial value.

The excess Cu provoked a significant impact in Mg and Fe leaf contents, which decreased to 80 and 45%, respectively, of their control values at the highest treatment - 20 mg kg^{-1} sand.

Since ethylene production associated thylakoid membrane degradation can be final product of acyl lipid peroxidation [21], it can be used as a suitable indicator of the thylakoid peroxidation status. The results obtained in this study showed that excess Cu caused an increase of ethylene production of barley plants at 10 and 15 mg Cu kg^{-1} treatments (Table 3).

Table 3: Photosynthetic pigments content and their ratios, ethylene production associated with thylakoids as well as some minerals content in the leaves of Cu-exposed barley plants 10 days after treatment.

Parameters	Cu treatments (mg kg^{-1} sand)			
	0	10	15	20
Chl *a* (mg g^{-1}FW)	1.35 (100)	1.39 (103)	1.15 (85)	0.63 (47)
Chl *b* (mg g^{-1}FW)	0.49 (100)	0.55 (112)	0.41 (84)	0.29 (59)
Total Carotenoids (mg g^{-1}FW)	0.54 (100)	0.50 (93)	0.46 (85)	0.35 (65)
Chl (*a*/*b*)	2.74 (100)	2.54 (93)	2.79 (102)	2.21 (81)
Total Chl/ Total Car.	3.41 (100)	3.89 (114)	3.39 (100)	2.62 (77)
(µl C_2H_4 mg^{-1} Chl h^{-1})	58.3 (100)	75.4 (129)	84.2 (144)	-
Mg (g kg^{-1})	1.5 (100)	1.4 (93)	1.3 (87)	1.2 (80)
Fe (mg kg^{-1})	150 (100)	91 (61)	70 (47)	68 (45)

Parameters	Regression Equation	r^2
Chl. *a*	Y = *-0.031X + *1.52	0.63
Chl. *b*	Y = *-0.01X + *0.54	0.55
Total Carotenoids	Y = *-0.009X+ *0.57	0.81
Mg (g kg^{-1})	Y = *-0.009X + *1.468	0.66
Fe (mg kg^{-1})	Y = *-4.35X + *143.2	0.87

*Significant at the 0.05 probability level. X - external Cu concentration.

DISCUSSION

The inhibition of dry weight (DW) accumulation in plants suffering heavy metal stress is widely observed effect in phytotoxicity studies. The idea of a critical or threshold toxicity is often used to establish the point at which metals in the growth substrate cause significant growth and/or yield decrease [23]. Several methods have been used to find that threshold, one of them is the determination of the tissue metal concentration resulting in a 10% reduction in growth [8]. According to the established equation describing the dry weight accumulation of barley plants on leaf Cu concentration, the tissue Cu concentration at 90% of DW was about 60 mg kg^{-1} DW. The established Cu threshold in barley leaves is very similar to the values reported for bean (62.4 mg kg^{-1}, [6]) and blackgram (*Vigna mungo*) plants (67 mg kg^{-1}, [13]).

Physiological range of Cu concentrations in mature leaf tissue varies from 5 to 30 mg kg^{-1} [12]. Cu accumulation over this range usually results in a number of toxic effects, mostly on photosynthesis, as the majority of leaf Cu content is associated with the photosynthetic apparatus [10]. The observed decline in A of Cu-exposed barley plants could be due both to stomatal and non-stomatal factors (Table 1). For the plants submited to 5, 10, and 15 mg Cu kg^{-1} doses the significant decrease in A could result from stomatal limitation, since g_s and c_i decreased but A_{max} suffered a 10% reduction. With the exposure to the highest Cu level (20 mg Cu kg^{-1}), mesophyll impairments seems to have increased, since the decrease in A was accompanied by a strong drop in photosynthetic pigments content as well as in A_{max}. Similar effects were observed earlier in other barley cultivar (cv. CE 9704) grown with the same experimental design [31].

The mesophyll impairments may result from Cu-induced oxidative stress having great impact on cell physiology. The strong pigment reduction and the enhanced ethylene production associated with chloroplast membranes of plants submited up to 15 mg Cu kg^{-1}, indicated an increased lipid peroxidation at chloroplast level (Table 3). On the other hand, the lower photosynthetic pigment content could result from mineral deficiency. Excess Cu decreased significantly Fe and Mg concentrations in barley leaves, but Fe value was above the deficiency threshold value of 25 mg Fe kg^{-1} reported for barley [11]. Thus, it seems the significant decrease in the

photosynthetic pigments of Cu-exposed plants was mainly due to their enhanced degradation. Those Cu effects, related with the thylakoid functioning was also observed at the PS performance, mostly at the OEC level. In fact, lipid peroxidation at the chloroplast level may cause ultrastructure changes in the thylakoids as it was shown by Lidon *et al*. [22] in Cu-exposed rice plants. Additionally, the electron transport might be limited by changes in the concentrations of the electron carriers [20]. Furthermore, excess Cu may indirectly decrease barley photosynthesis by changing the sink-source relationship, with a consequent diminished requirement for photosynthetic products [5]. The Cu-induced growth inhibition may cause phloem overloading leading to decrease in enzymes activity and energy consumption by Calvin's cycle reactions, and finally reflecting in down regulation in PSII. In this situation a slight tendency to increase in q_{NP} and q_E values (Table 2) may be interpreted as a way to avoid over-production of primary electron acceptor Q_A and to reduce the load on the electron transport chain, as was earlier suggested by Cd-exposed plants [14].

The results discussed here indicated that a number of different estimates are significantly influenced by excess Cu. Thus, they might be used as bio-indicators in plant test system. Both morphological and metabolic bio-indicators should be included in these systems to fully integrate potential metal phytotoxicity [30]. Data presented in Table 4 showed that dry weight (DW) accumulation and leaf area (LA) formation of barley plants have similar sensitivity to excess Cu, as A and photosynthetic electron transport at PSI. In fact, the calculated "effect concentrations" leading to 10% decrease in the value of the used estimates (EC_{10}) varied around 12 - 16 mg Cu kg^{-1} sand for DW, LA as well as for A, PSI and PSII-OEC. The highest sensitivity to Cu was exhibited by g_s and PSII+OEC, since their EC_{10} values were obtained at the low Cu content of 4 - 5 mg Cu kg^{-1} sand. Our study showed that at the experimental design used photosynthetic pigments content and chlorophyll fluorescence are less sensitive parameters of Cu toxicity.

Table 4: External Cu concentrations (mg Cu kg^{-1} sand) decreasing by 10% (EC_{10}) the values of different estimates of Cu-exposed barley plants

Parameters	Regression Equation	EC_{10}
DW	$Y = {}^{*}-0.0007X^2 + {}^{*}0.0068X + {}^{*}0.5728$	15.1
LA	$Y = {}^{*}-0.23X^2 + {}^{*}2.9X + {}^{*}93.4$	15.4
A	$Y = {}^{*}-0.0004X^2 - {}^{*}0.0292X + {}^{*}13.83$	15.6
g_s	$Y = {}^{*}0.00015X^2 - {}^{*}0.0091X + {}^{*}0.2867$	4.3
Chl *a*	$Y = {}^{*}-0.031X + {}^{*}1.52$	44.2
Chl *b*	$Y = {}^{*}-0.01X + {}^{*}0.54$	49.0
PSII+OEC	$Y = {}^{*}-0.088X^2 + {}^{*}1.537X + {}^{*}93.14$	5.1
PSII-OEC	$Y = {}^{*}-0.038X^2 + {}^{*}0.221X + {}^{*}86.15$	11.8
PSI	$Y = {}^{*}-1.87X^2 + {}^{*}26.52X + {}^{*}499.6$	16.1

*Significant at the 0.05 probability level. X - external Cu concentration

CONCLUSION

In conclusion, excess Cu produces a variety of toxic effects on barley growth and photosynthesis. Based on both high sensitivity of leaf gas exchange parameters (g_s and A) to excess Cu and fastness of their measurement, we propose these parameters together with plant dry weight accumulation and leaf area formation as suitable parameters for plant test systems, which can be utilized for successful screening for higher Cu tolerance among barley cultivars.

ACKNOWLEDGMENTS

The authors thank Tech. Eng. Carlos S. Carvalho and Ana P. Ramos for technical assistance, Engs. José M. Semedo and Nuno C. Marques (all from EAN, Oeiras, Portugal) for help with gas-exchange measurements as well as the Dept. Plant Physiology in EAN for the working facilities provided.

REFERENCES

[1.] ANGELOV, M., T. TSONEV, A. UZUNOVA, K. GAIDARDJIEVA, 1993. Cu^{2+} effect upon photosynthesis, chloroplast structure, RNA and protein synthesis of pea plants. *Photosynthetica*, 28, 3, 341-350.

[2.] BRUN, L. A., J. MAILLET, P. HINSINGER, M. PEPIN, 2001. Evaluation of copper availability to plants in copper-contaminated vineyard soils. *Environmental Pollut*., 111, 293-302.

[3.] CHERREY, A., V. CHAIGNON, P. HINSINGER, 1999. Copper bioavailability in Mediterranean vineyard soils as assessed by chemical and plant extractions. In: Extended Abstracts of the 6th. Int. Meeting on Soils with Mediterranean Type and Climate. Ed. J. Bech. pp 848-850. University of Barcelona Publ. Barcelona, Spain.

[4.] CLIJSTERS, H., A. CUYPERS, J. VANGRONSVELD, 1999. Physiological responses to heavy metals in higher plants; defence against oxidative stress. *Z. Naturforsch*., 54c, 730-734.

[5.] CISCATO, M., R. VALCKE, K. VAN LOVEN, H. CLIJSTERS, F. NAVARI-IZZO, 1997. Effects of *in vivo* copper treatment on the photosynthetic apparatus of two *Triticum durum* cultivars with different stress sensitivity. *Physiol. Plant*., 100, 901-908.

[6.] COOK, C. M., A. KOSTIDOU, E. VARDAKA, T. LANARAS, 1997. Effects of copper on the growth, photosynthesis and nutrient concentrations of *Phaseolus* plants. *Photosynthetica*, 34, 2, 179-193.

[7.] DROPPA, M., J. MASOJIDEK, Z. ROSZA, A. WOLAK, L. HORVATH, I. FARKAS, E. HORVATH, 1987. Characteristics of Cu deficiency–induced inhibition of photosynthetic electron transport in spinach chloroplasts. *Biochim. Biophys. Acta*, 891, 75-84.

[8.] EDWARDS, D. G., C. J. ASHER, 1982. Tolerance of crop and pasture species to manganese toxicity. In: Plant Nutrition, 1982. Proceedings of the Ninth Inter. Plant Nutr. Colloquium, Warwick University, England (ed. Scaife, A,), pp. 145-151. Commonwealth Agricultural Bureau.

[9.] GENTY, B., J. BRIANTAIS, N. BAKER, 1989. The relation between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta,* 990, 87-92.

[10.] HEWITT, E. J., 1983. A perspective of mineral nutrition: essential and functional metals in plants. In: Robb, D. A., Pierpoint, W. S. (editors): Metals and Micronutrients: Uptake and Utilization by Plants, 227-323. Academic Press, London

[11.] JONES, B., JR., J. WOLF, H. MILLS, 1991. Plant Analysis Handbook; Micro-Macro Publishing, Inc., Athens, GA.

[12.] KABATA-PENDIAS, A., H. PENDIAS, 1984. Trace elements in soil and plants. CRC Press, Boca Raton.

[13.] KALYANARAMAN, S. B., P. SIVAGURUNATHAM, 1993. Effect of cadmium, copper, and zinc on the growth of blackgram. *J. Plant Nutr*., 19, 2029-2042.

[14.] KRUPA, Z., G. ÖQUIST, N. HUNER, 1993. The effects of cadmium on photosynthesis of Phaseolus vulgaris –—a fluorescence analysis. *Phisiol. Plant*., 88, 626-630.

[15.] KRUPA, Z., T. BASZYNSKI, 1995. Some aspects of heavy metals toxicity towards photosynthetic apparatus – direct and indirect effects on light and dark reactions. *Acta Physiol. Plantarum*, 17, 2, 177-190.

[16.] LANARAS, T., M. MOUSTAKAS, L. SYMEONIDIS, S. DIAMANTOGLOU, S. KARATAGLIS, 1993. Plant metal content, growth responses and some photosynthetic measurements on field-cultivated wheat

growing on ore bodies enriched in Cu. *Physiol. Plant*., 88, 307-314.

[17.] LICHTENTHALER, H., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods of Enzymology*, 148, 350-382.

[18.] LIDON, F. C., 1999. An overview of the effects of excess copper on the photosynthesis of rice plants. *Agronomia Lusitana*, 47, 69-88.

[19.] LIDON, F., F. S. HENRIQUES, 1991. Limiting step on photosynthesis of rice plants treated with varying copper levels. *J. Plant Physiol*., 138, 115-118.

[20.] LIDON, F., F. S. HENRIQUES, 1992. Changes in the contents of photosynthetic electron carriers, RNAse activity and membrane permeability. *Photosynthetica*, 26, 371-380.

[21.] LIDON, F., F. HENRIQUES, 1993. Oxygen metabolism in higher plant chloroplasts. *Photosynthetica*, 29, 249-279.

[22.] LIDON, F. C., J. C. RAMALHO, F. HENRIQUES, 1993. Copper inhibition of rice photosynthesis. *J. Plant Physiol*., 142, 12-17.

[23.] MACNICOL, R. D., P. H. T. BECKETT, 1985. Critical tissue concentrations of potentially toxic elements. *Plant Soil* 85, 107-129.

[24.] MAKSYMIEC, W., 1997. Effects of copper on cellular processes in higher plants. *Photosynthetica*, 34, 3, 321-342.

[25.] MOCQUOT, B., J. VANGRONSVELD, H. CLIJSTERS, M. MENCH, 1996. Copper toxicity in young maize (*Zea mays* L.) plants: effects on growth, mineral and chlorophyll contents, and enzyme activities. *Plant Soil*, 182, 287-300.

[26.] MOUSTAKAS, M., T. LANARAS, L. SYMEONIDIS, S. KARATAGLIS, 1994. Growth and some photosynthetic characteristics of field grown Avena sativa under copper and lead stress. *Photosynthetica*, 30, 3, 389-396.

[27.] OUZOUNIDOU, G., I. ILIAS, H. TRANOPOULOU, S. KARATAGLIS, 1998. Amelioration of copper toxicity by iron on spinach physiology. *J. Plant Nutr*., 21, 10, 2089-2101.

[28.] STIBOROVA, M., M. DOUBRAVOVA, S. LEBLOVA, 1986. A comparative study of the effect of heavy metal ions on ribulose-1,5-biphosphate carboxylase and phosphoenolpyruvate carboxylase. *Biochem. Physiol. Pflanz*., 181, 373-379.

[29.] VAN KOOTEN, O., J. F. H. SNELL, 1990. The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynth. Res*., 25, 147-150.

[30.] VANGRONSVELD, J., H. CLIJSTERS, 1992. A biological test system for the evaluation of metal phytotoxicity and immobilisation by additives in metal contaminated soils. In: Metal compounds in environment and life, 4. Special supplement to Chemical Speciation and Bioavailability. (Eds. E. Merian, W. Haedi), Wilmington: Science Reviews Inc., 1992, 117-125.

[31.] Vassilev, A., F. C. Lidon, M. do Céu Matos, J. C. Ramalho, I. Yordanov, 2002. Photosynthetic performance and some nutrients content in cadmium- and copper-treated barley plants. *J. Plant Nutr*., 25, 11, 2343-2360.

ADDRESSES OF AUTHORS

A. Vassilev*
Agricultural University of Plovdiv, Dept. Plant Physiology and Biochemistry,
12 Mendeleev St., 4000 Plovdiv, Bulgaria

F. Lidon
Plant Biology Unit, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa,
2825-114 Caparica, Portugal

J. C. Ramalho
Instituto de Investigação Científica Tropical - CEPTA,
Tapada da Ajuda, Ap. 3014, P-1301-901 Lisboa, Portugal

M. do Céu Matos, M. da Graca
Plant Physiology Department, Estação Agronómica Nacional-INIA, Quinta do Marquês,
Av. da Republic, 2784-505 Oeiras, Portugal

*Author for correspondence