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Electrochemistry of copper(II) induced complexes in mycorrhizal maize plant tissues

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ABSTRACT

Aim of the present paper was to study the electrochemical behavior of copper(II) induced complexes in extracts obtained from mycorrhizal and non-mycorrhizal maize (*Zea mays* L.) plants grown at two concentrations of copper(II): physiological (31.7 ng/mL) and toxic (317 μg/mL). Protein content was determined in the plant extracts and, after dilution to proper concentration, various concentrations of copper(II) ions (0, 100, 200 and 400 μg/mL) were added and incubated for 1 h at 37 °C. Further, the extracts were analyzed using flow injection analysis with electrochemical detection. The hydrodynamic voltammogram (HDV), which was obtained for each sample, indicated the complex creation. Steepness of measured dependencies was as follows: control 317 μg/mL of copper < control 31.7 ng/mL of copper < mycorrhizal 31.7 ng/mL of copper < mycorrhizal 317 μg/mL of copper. Based on these results it can be concluded that mycorrhizal fungus actively blocks transport copper(II) ions to upper parts of a plant by means of adsorbing of copper(II) in roots. Rapid complex formation was determined under applied potentials 300, 500 and 600 mV during the measuring HDVs. It was also verified that mycorrhizal colonization reduced root to shoot translocation of Cu(II) ions.

1. Introduction

The majority (~80%) of terrestrial plant species form arbuscular mycorrhizas, a mutualistic symbiosis established with soil-borne fungi belonging to the Glomeromycota. This symbiosis play an important role in plant nutrition by providing access to soil-derived nutrients from sources not necessarily otherwise accessible to roots. The fungi receive a supply of carbohydrates in return. The formation of the arbuscular mycorrhizal symbiosis can improve plant uptake of (especially) phosphorus, and a range of other nutrients including Zn, Cu and K [1]. Although arbuscular mycorrhizas are most often considered important for uptake of immobile nutrients, they also play an important role in reducing uptake of heavy metals, including copper, where soil concentrations are high [2]. Thus, arbuscular mycorrhiza has various roles in terms of plant–copper interactions. Copper is essential for plant's photosynthesis and mitochondrial respiration, carbon and nitrogen metabolism, oxidative stress protection, and is required for cell wall synthesis, to name at least a few of its cellular functions [3]. Existence of copper in two oxidation states (Cu(I) and Cu(II)) allows this element to play a role as a reducing or oxidizing cofactor in various biochemical reactions [3]. But at the same time, this feature makes copper also potentially toxic since copper ions can catalyze the production of free radicals, in particular through Fenton chemistry, thus leading to the damage of proteins, DNA, and other biomolecules [4–7]. Therefore, immediately after its uptake the majority of copper ions are bound by scavenging proteins like metallothioneins to prevent copper from accumulating in a toxic form. However, part of the imported copper escapes this system and becomes captured by small binding proteins, so called copper chaperones [8,9] that spare copper from the detoxification systems and guide it to the target sites in the cell. The genome of *Arabidopsis thaliana* possesses over 30 sequence homologs that might encode for copper chaperones; however, only very few of these proteins are already characterized [10,11]. There are also discussed copper(II) ions complexes with other plant biologically important molecule as phytochelatin2,

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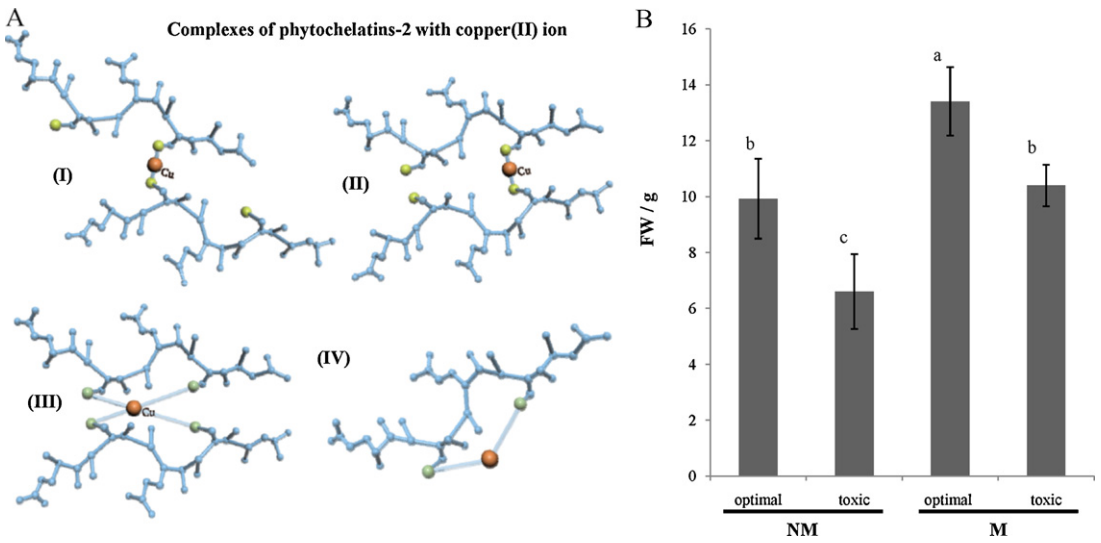

Fig. 1. (A) 3D models of complex between copper(II) ion and one **(IV)** or two **(I, II, III)** molecules of phytochelatin2. (B) Fresh weight (FW) of non-mycorrhizal (NM) and mycorrhizal (M) maize plants fertilized with Hoagland solution containing a physiological (31.7 ng/mL) or a toxic (317 μg/mL) dose of copper(II) ions. Data are means ± SD of six replicates. Data not sharing a letter in common differ significantly ($p < 0.05$).

which are shown in Fig. 1A. Interestingly, copper metabolism is intimately linked to iron metabolism [12]. In particular, the contribution of mugineic acid, nicotianamine, organic acids, histidine and phytate to metal homeostasis and mutual transporting is discussed by Haydon and Cobbett [13] and Curie et al. [14].

To study the fate of copper(II) ions in an organism, various bioanalytical tools with both advantages and disadvantages are used. Various types of spectrometric methods are often used for determination of copper content in real samples of various origins including plant tissues [15]. However, any real sample must be pre-treated before spectrometric analysis which may cause a loss of important information about copper and its complexes. On the other hand, electrochemistry represents a tool by which copper(II) ions content may be determined and also the complexes depending on treatment and type of a sample studied [16–18]. The aim of this paper was to study the effect of the arbuscular mycorrhizal symbiosis and two doses of biologically available copper(II) ions on the content of copper(II) ions and copper(II)-induced thiol-rich complex formation in maize by using flow injection analysis with electrochemical detection.

2. Materials and methods

2.1. Flow injection analysis coupled with electrochemical detection

The instrument for flow injection analysis with amperometric detection (FIA-ED) consisted of solvent delivery pump operating in range of 0.001–9.999 mL/min (Model 582 ESA Inc., Chelmsford, MA, USA), a reaction coil (1 m), and an electrochemical detector. The electrochemical detector includes one low volume (5 μL) flow-through analytical cell (Model 5040, ESA, USA) consisting of glassy carbon working electrode, hydrogen-palladium electrode as reference electrode and auxiliary electrode, and Coulochem III as a control module. The sample (5 μL) was injected manually using 6-way injection valve. The data obtained were processed by Clarity software (Version 3.0.04.444, Data Apex, Czech Republic). The experiments were carried out at room temperature. A glassy carbon electrode was polished mechanically by 0.1 μm of alumina (ESA Inc., USA) and sonicated at room temperature for 5 min using a Sonorex Digital 10 P Sonicator (Bandelin, Berlin, Germany) at 40 W [19,20]. Other experimental parameters were optimized; see in Section 3.

2.2. Chemicals and pH measurements

HPLC-grade methanol (>99.9%; v/v) was from Merck (Dortmund, Germany). Other chemicals were purchased from Sigma–Aldrich (St. Louis, USA) in ACS purity unless noted otherwise. Working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through 0.45 μm Nylon filter discs (Millipore, Billerica, MA, USA) prior to HPLC analysis. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany) and controlled by software MultiLab Pilot, Weilheim, Germany. The pH-electrode (SenTix H, pH 0.14/0.100 °C/3 M KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany).

2.3. Biological material

Maize (*Zea mays* L.) seeds were surface-sterilized and sown in wet vermiculite that had been autoclaved at 121 °C for 30 min. Plantlets were transplanted to 500 mL pots containing a sterile mixture of soil/sand (1/3, v/v). The soil was collected from Sierra Nevada (Granada, Spain), sieved through a 2 mm mesh, sterilized by tyndalization for three consecutive days and air dried. The sand was sterilized by autoclaving at 121 °C for 30 min. The soil had the following characteristics: pH 6.58, 10.84% organic matter, 0.47% total N, 0.03% total P, 0.38% Ca, 0.42% K and 65.85 ppm total Cu.

The set of experimental plants was divided into two main groups: (i) maize plants non-inoculated with the mycorrhizal fungus (non-mycorrhizal or control plants), and (ii) maize plants inoculated with the arbuscular mycorrhizal fungus *Glomus intraradices* (mycorrhizal plants). Each main experimental group was halved and treated with a physiological (31.7 ng/mL) or a toxic (31.7 ng/mL) dose of copper (II) ions. Each group consisted of six individual plants. Mycorrhizal plants were inoculated with the arbuscular mycorrhizal fungus *G. intraradices* (DAOM 197198, Smith & Schenck Biosystematic Research Center, Ottawa, Canada) by adding 50 g of a soil–sand-based inoculum to 450 g of growing substrate. The inoculum consisted of thoroughly mixed rhizosphere samples containing spores, hyphae and mycorrhizal root fragments. Control plants received a filtrate (>20 μm) of the inoculum

to provide the non-mycorrizal soil microbiota. Plants were grown in a greenhouse (16 h photoperiod, 25/18 °C day/night temperature, and 60% relative humidity) and watered three times per week with 30 mL Hoagland nutrient solution containing optimum or toxic doses of copper(II). Plants were harvested 9 weeks after inoculation. The roots and shoots of all plants were frozen in liquid nitrogen and stored at −80 °C until use. Mycorrhizal root colonization was estimated in root samples after trypan blue staining [21] using the gridline intersects method [22].

2.4. Preparation of the plant sample

Frozen roots and shoots (200 mg) were ground with liquid nitrogen in mortar with a pestle and then 1500 mL of 20 mM phosphate buffer (pH 7.5) was added to the obtained powder. The mixtures were pre-treated using ultrasonic waves (Bandelin Sonorex Digital 10P ultrasonic bath) for 5 min at room temperature. The homogenate was centrifuged (14,000 × *g*) for 20 min at 4 °C. The supernatant was diluted to final concentration of 7 mg of total proteins per 1 mL of the extract and then injected into the FIA-ED system with or without addition of various copper(II) concentrations.

2.5. Total protein content

For determination of the total protein content, the biuret solution (15 mM potassium sodium tartrate, 100 mM NaI, 15 mM KI, and 5 mM $CuSO_4$) was used. As a standard albumin (1 mg/mL in phosphate buffer, pH 7) was used. The measurement was done as follows: 180 μL of the biuret solution was mixed with 45 μL of a real sample or standard; after stirring and incubation (10 min at 37 °C) the absorbance at 546 nm was measured. Spectrometric measurements were carried out using an automated chemical analyser BS-200 (Mindray, China). Reagents and samples were placed in cooled sample holder (4 °C) and automatically pipetted directly into plastic cuvettes. Incubation was performed at 37 °C. Mixture was consequently stirred. The washing steps with distilled water (18 MΩ) were done in the midst of the pipetting. Apparatus was operated using manufacturers' software.

2.6. Descriptive statistics

Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (SD) unless noted otherwise (EXCEL®). Statistical significances of the differences between measured parameters were determined using STATISTICA.CZ. Differences with $p < 0.05$ were considered significant and were determined by using of one way ANOVA test (particularly Scheffe test), which was applied for means comparison.

3. Results and discussion

3.1. Biological experiment

In this study, the attention was primarily aimed on studying of copper(II) ions effects on maize plants inoculated or not with the arbuscular mycorrhizal fungus *G. intraradices*. The set of experimental plants was divided into two main groups: (i) maize plants non-inoculated with the mycorrhizal fungus (non-mycorrhizal or control plants), and (ii) maize plants inoculated with the arbuscular mycorrhizal fungus *G. intraradices* (mycorrhizal plants) as it is shown in Material and methods section. Percentage of root colonization by the mycorrhizal fungus was 65 ± 6 and 55 ± 10% for the plants grown under physiological and toxic Cu doses, respectively, being these values not statistically significant at $p < 0.05$. No colonization was observed in the non-inoculated plants. Biomass of plants inoculated with the arbuscular mycorrhizal fungus was higher than biomass of non-inoculated plants (Fig. 1B). Particularly, *G. intraradices* increased fresh weight of plants treated with the physiological dose of copper(II) ions by 35% and to a higher extent (about 60%) when treated with the toxic dose of it. Further, the effect of copper(II) treatment was studied by FIA-ED. The differences between the experimental groups were statistically treated. We found that differences between toxic and normal dose were significant in both non-mycorrhizal and mycorrhizal plants. However, differences between these groups, i.e. plants treated with the same dose of copper(II) ions but differed in the presence of mycorrhiza, was also significant at $p < 0.05$.

3.2. Electrochemical detection of copper(II) ions

In this study, glassy carbon electrode was used for determination of copper(II) ions in a flow system. Copper(II) ions were detected most sensitively at the surface of the working electrode at potential of 1000 mV (other analytical details will be published elsewhere). The measured copper(II) calibration dependence was strictly linear ($y = 15.865x - 56.639$; $R^2 = 0.9965$, $n = 5$) within tested concentration interval (10–400 μg/mL). Based on the good sensitivity of FIA-ED on the copper(II) ions, this method was employed in the following experiment: the question was whether it is possible to study the effect of mycorrhizal colonization and various doses of copper(II) ions on maize plants. Besides content of copper(II) ions the effect of external additions (0, 100, 200, and 400 μg Cu(II)/mL) of these ions to plant extracts was also studied. The extracts were analyzed at 1000 mV and results obtained are shown in Fig. 2A and B. In extracts from shoots without external additions of copper(II) ions, the content of copper(II) ions in mycorrhizal experimental groups were lower compared to both control groups where the increase of copper(II) ions content in control group treated with higher concentration of copper(II) ions for more than 70 μg/mg proteins was observed (Fig. 2A). The additions of copper(II) ions to extracts can be expressed by linearly increasing dependency ($y = 15.826x + 101.58$; $R^2 = 0.9603$, $n = 5$) in control treated with physiological dose of copper(II) ions. More gradual increase in the signal expressed as $y = 14.168x + 174.02$; $R^2 = 0.734$, $n = 5$ was determined in control treated with the toxic dose. Mycorrhizal experimental groups showed the following trends: (i) physiological dose ($y = 17.033x + 87.965$; $R^2 = 0.9068$, $n = 5$), and (ii) toxic dose ($y = 25.353x + 78.776$; $R^2 = 0.8954$, $n = 5$). Statistical treatment of data obtained revealed that the content of copper(II) ions increased significantly in all experimental groups after addition of 400 μg Cu(II)/mL compared to extracts with no copper(II) addition. Interesting finding was that control group of plants treated with toxic dose of copper(II) ions as 317 μg Cu(II)/mL had significantly higher content of Cu(II) compared with the other experimental groups.

The results obtained by analysis of root extracts shows the unambiguously positive effect of the arbuscular mycorrhizal fungus on immobilization of copper(II) ions in maize roots (Fig. 2B). In the extracts without external addition of copper(II) ions, the content of these ions was approximately 130 μg/mg proteins. Moreover, control group treated with toxic dose of copper(II) ions had slightly lower copper(II) ions content compared to physiological non-mycorrhizal groups which indicates the disruption of root regulation mechanisms and direct transport of the ions to upper parts of a plant. Experimental groups of plants cultivated in the presence of *G. intraradices* actively immobilized copper(II) ions in the root system and blocked other transport. This is clearly evident on the content of copper(II) ions in roots. Their concentration increased from 180 μg/mg proteins (physiological dose) to 340 μg/mg proteins (toxic dose). To test binding capacity of

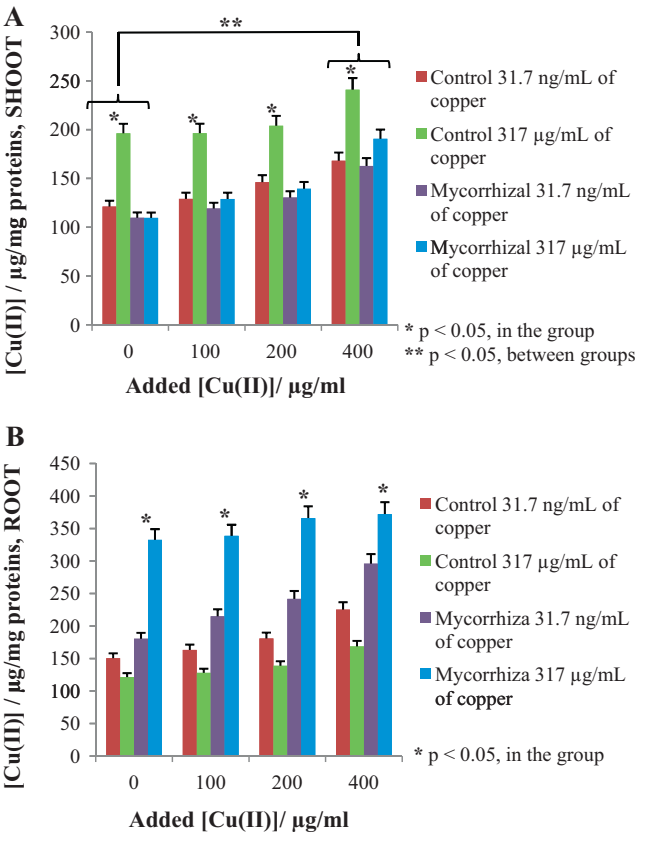

Fig. 2. The effect of various doses of copper(II) ions and mycorrhizal symbiosis on the content of Cu(II) in shoots and roots electrochemically revealed. Concentration of copper(II) ions in pure plant extracts and with addition of copper(II) ions (0, 100, 200, and 400 µg/mL). Current response was determined at 1000 mV in (A) SHOOT and (B) ROOT.

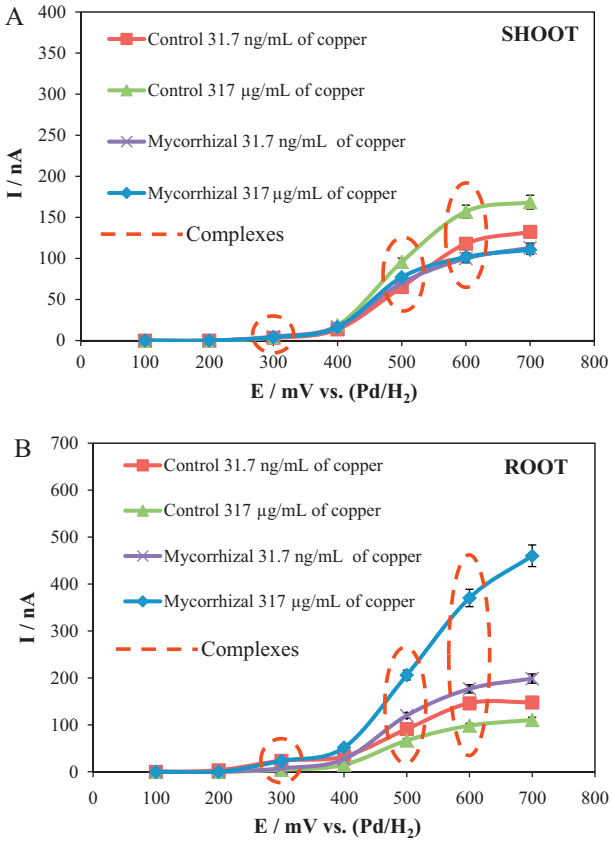

Fig. 3. Flow injection analysis with electrochemical detection used for analysis of tissue extracts. Hydrodynamic voltammograms of plant extracts obtained from maize mycorrhizal and non-mycorrhizal plants with physiological (31.7 ng/mL) and toxic (317 µg/mL) doses of copper(II) ions in (A) SHOOT and (B) ROOT. Main complex sensitive potentials are highlighted by red-dash. In both cases the concentration of added copper(II) ions was 0, 100, 200, and 400 µg/mL ($n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

plant extracts, another external additions of copper(II) ions were supplemented and plotting with linear regressions was done. All obtained dependencies had linear trend with the following equations: (a) non-mycorrhizal plants treated with physiological dose ($y = 24.202x + 119.49$; $R^2 = 0.9135$, $n = 5$); (b) non-mycorrhizal plants treated with toxic dose ($y = 15.249x + 101.16$; $R^2 = 0.8881$, $n = 5$); (c) mycorrhizal plants treated with physiological dose ($y = 37.282x + 140.1$; $R^2 = 0.9780$, $n = 5$); (d) mycorrhizal plants treated with toxic dose ($y = 14.552x + 315.9$; $R^2 = 0.9225$, $n = 5$). In the roots, statistical analysis revealed that mycorrhiza experimental group differed significantly in the case of any addition of copper(II) ions compared to other groups. In addition, there was not other difference between behavior of all extracts.

To abbreviate names of particular experimental groups the following titles were used: (i) maize plants without mycorrhizal fungus ("control") and (ii) maize plants with mycorrhizal fungus ("mycorrhizal"). Based on the above mentioned results, it was found that shoots of non-mycorrhizal plants treated with toxic dose had lower binding capacity for copper(II) ions (approximately 10%) compared with those plants treated with physiological dose of copper(II) ions. On the contrary, cultivation of maize plants in the presence of mycorrhizal fungus markedly enhanced binding capacity of their above ground parts for these ions without growth depression. This is clearly indicated by increasing binding capacity of plants treated with toxic dose for more than 40% compared to those treated with physiological dose. Moreover, it was found that occurrence of the symbiosis due to inoculation of the plants induced an increase in shoot copper binding capacity in plants grown in the presence of physiological dose of copper(II) ions. The binding capacity for copper(II) ions in roots of non-mycorrhizal plants decreased for more than 30% comparing physiological and toxic doses. The symbiosis had an effect on the copper binding capacity of the roots cultivated in physiological conditions (Fig. 2A and B). However, the similar trend is observed in the case of mycorrhizal experimental groups in which the binding capacity decreased for more than 250% as compared to physiological doses. The changes in the binding capacities can be associated with the synthesis of biologically active compounds, mainly with those rich in —SH moieties [23–25]. In addition, our results can be clearly associated with other findings showing that arbuscular mycorrhizal fungi engage a mutualistic symbiosis with the roots of most plant species and alleviate heavy metal stress in plants [26–28].

3.3. Complex formation

It is well known that —SH moiety is very reactive and is often conjugated with other moieties and reactive ions including metal ions. It is not surprising that peptides rich in —SH moieties called phytochelatins (PC; a basic formula $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2$–11) [29]) play an important role in detoxification of metal ions in a plant cell. Phytochelatins participate in the detoxification of heavy metals because they are able to bind heavy metal ions via —SH groups of cysteine units and consequently transport them to vacuole [30,31] where an immediate toxicity of metal ions does not pose any risk. The synthesis of phytochelatins proceeds from glutathione by transferring γ-Glu-Cys moiety from a donor to an

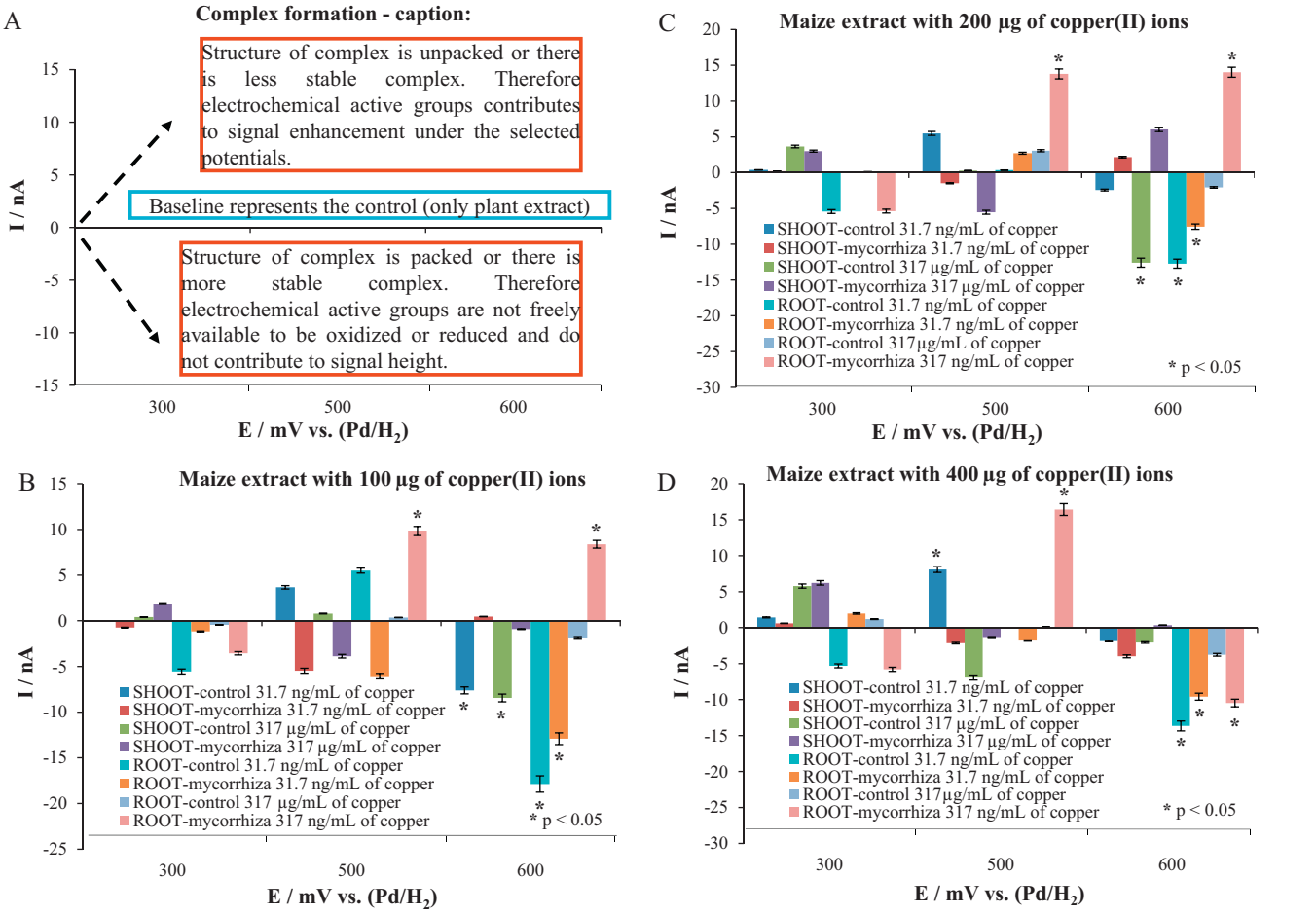

Fig. 4. The effect of external additions of copper(II) ions on complex formation in tissue extracts. (A) Explanation of approach, which has been used during data interpretation. As zero line, it was used HDV of extract of maize non-mycorrhizal and mycorrhizal plants both treated with physiological (31.7 ng/mL) and toxic (317 µg/mL) doses of copper(II) ions with no addition of copper(II) ions. Other values are shown as differences from this baseline. Differences of the signal obtained from pure extracts of the experimental plants and those with additions of (B) 100 µg/mL, (C) 200 µg/mL, and (D) 400 µg/mL of copper(II) ions.

acceptor molecule. Particularly, the reaction involves the transpeptidation of the γ-Glu-Cys moiety of reduced glutathione (GSH) onto initially a second GSH molecule to form PC_2 or – at later stages of the incubation – onto a PC molecule to produce an $n+1$ oligomer catalyzed by γ-Glu-Cys dipeptidyl transpeptidase (EC 2.3.2.15) called phytochelatin synthase [32–35]. Schemes of possible formation of PC complexes with copper(II) ion are shown in Fig. 1A. Besides thiols, other complexes of some plant biologically active compounds with metal ions such as isoflavones, naphthoquinones and terpenes can be formed [36].

3.3.1. *FIA-ED as a tool for studying metal ion-plant metabolite complexes*

Based on papers previously published by our group, it was found that formation of metal complexes with various types of biologically active compounds caused changes in current response depending on potential applied on a working electrode [37–39]. In this study, a new way to investigate the complex formation in a real sample extract for characterization metal-induced response in plants was suggested. Extracts prepared according to protocol described in Experimental section were analyzed using FIA-ED and hydrodynamic voltammograms (HDV, dependence of current response on applied potential) were obtained. Typical HDVs of plant extracts obtained from shoots and roots are shown in Fig. 3A and B, respectively. In the shoots of maize plants, changes in the single HDVs were determined. The lowest HDVs were measured in both experimental groups with inoculated mycorrhizal fungi. HDV of control plants treated with physiological dose of copper(II) ions were slightly higher compared to mycorrhizal plants. The steepest HDV was determined in control plants treated with the toxic dose of copper(II) ions. This marked increase is associated with higher content of copper(II) ions in the plants. On the other hand, mycorrhizal plants probably triggered some protective mechanisms against transporting of copper(II) within a plant. Therefore, courses of both HDVs were less steeper compared with control plants. In roots, more distinct changes were determined (Fig. 3B). Compared to shoots, the trends of HDVs were reversed. Steepness of measured dependencies was as follows: control 317 µg/mL of copper < control 31.7 ng/mL of copper < mycorrhizal 31.7 ng/mL of copper < mycorrhizal 317 µg/mL of copper. Based on these results it can be concluded that mycorrhizal fungus actively blocked transport copper(II) ions to upper parts of a plant by means of adsorbing of copper(II) in roots.

3.3.2. *Signals of complexes*

To follow changes in plants in greater details, three regions selected according to changes in current response at various potentials were chosen (Fig. 3A and B). Based on previously published results [39], we expect that different types of substances are reduced and/or oxidized under selected potentials. Low molecular mass compounds such as phenols or polyphenols are electroactive under 300 mV, thiols – mainly reduced glutathione – and

phytochelatins under 500 mV, and thiol–metal ion complexes under 600 mV. To highlight the effect of copper(II) ions on maize plants, all obtained HDVs were subtracted from HDV obtained by measuring of extract from maize plant cultivated without addition of copper(II) ions and without presence of mycorrhizal fungus. The basic explanations of possible observed changes expressed as increase or decrease of current response are shown in Fig. 4A (all changes marked with "*" are significant at $p < 0.05$). The enhancement of current response shows on the unpacking of electroactive substances; the formed complex may be less stable. The decrease in the current response indicates that the electroactive substances are packed and the formed complexes are stable. Besides pure extracts, the effect of three additions of copper(II) ions (100, 200, and 400 μg/mL) was investigated (Fig. 4B–D). Based on the results obtained by the addition of 100 μg Cu(II)/mL to plant extracts and plotting of linear regression it can be concluded that synthesis of compounds rich in —SH groups and complexes with copper(II) ions in shoots of control plants treated with physiological dose of copper(II) ions is enhanced. This statement is based on the slope of linear plotting of the measured dependence shown in Fig. 4B–D, because the obtained slope as −3.8 indicates that the dependence is decreasing. Compared to this, mycorrhizal plants treated with physiological dose of copper(II) ions synthesized compounds rich in cysteine only. The trend is opposite in the experimental groups treated with toxic dose of copper(II) ions. This means that complexes with copper(II) ions are synthesized in control group of plants (slope of the linear regression was 4.4) compared to mycorrhizal plants, where both thiol rich compounds and Cu(II)-complexes were determined (slope of the linear regression was −1.3).

In case of roots, packed complexes at 300 and 600 mV (slope of the linear regression was −6.1) in control plants treated with physiological dose were determined. The presence of packed complexes was also detected in mycorrhizal plants treated with physiological dose of copper(II) ions (slope of the linear regression was −5.8). The presence of mycorrhizal fungus had great effect on the complex formation in plants treated with toxic dose of copper(II) ions: marked enhancement of the complex formation was determined in mycorrhizal plants compared to plants cultivated without the presence of mycorrhizal fungus but treated with the toxic dose of copper(II) ions (slope of the linear regression was 5.9). The structure of newly formed biologically important Cu(II) complexes is different and is much more unpacked and therefore more electroactive. It must be associated with a high content of biologically important compounds and also with higher content of copper(II) ions. Applied externally increasing concentration of copper(II) ions in various extracts did not change dramatically profiles of studied complexes (Fig. 4C and D). Adapted inocula of ectomycorrhizal and endomycorrhizal strains can confer stress tolerance to higher plants by promoting nutrient and water interception, providing them macronutrients and/or plant growth hormones, and buffering contaminant accumulation by accumulation in the hyphae or increasing sorption through the production of a glycoprotein [40,41]. Such beneficial effects are described in this paper as well as by Bes et al. for ectomycorrhizal fungi – *Paxillus involutus*, *Hebeloma crustuliniforme*, and *Hebeloma salix* with *Populus nigra* and *Salix viminalis* in Cu-contaminated soils [42].

4. Conclusion

This manuscript deals with the topic of copper effects on plants. Copper is an essential micronutrient for plants, but at higher concentrations it is a toxic metal. Moreover, root symbiosis with mycorrhizal fungi is known to enhance the uptake of this essential element when present in low soil levels, and there are evidences that avoid excessive root uptake and/or shoot translocation in polluted soils. This study investigated the formation of complexes in plants that contribute to plant copper tolerance, and the effects of arbuscular mycorrhizal fungi on it, by the use of a methodological tool based on flow injection analysis with electrochemical detection. The advantages of both stationary and flow electrochemical techniques include high sensitivity, selectivity towards electroactive species, a wide linear range, portable and low-cost instrumentation, speciation capability, and a wide range of electrodes that allow assay of unusual environments. Extremely low (sub-nanomolar) detection limits can be achieved even with very small sample volumes (units of mL), thus allowing the determination of analyte amounts of 10^{-14}–10^{-16} mol on a routine basis [43,44]. Based on these advantages, it is not surprising that electrochemical methods are used not only for direct determination of target molecules but also for studying of their interactions. In this study, we show easy-to-use and low cost method for assessment of changes in plant tissues after their treatment with copper(II) ions. The observed behavior is very interesting and may become a basis for other types of studies aimed at particular complexes or at testing the effect of other both essential and toxic metals.

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