F-20-P

HYDROXYL RADICAL GENERATION AND CARBON CENTRE DEPLETION IN THE ROOT CELL WALL ISOLATE ENRICHED WITH COPPER

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Abstract
Copper is often accumulated in contaminated soils such as open cast mines, and is toxic to plants. Effect of excess Cu (20 μM) on free radicals generation in the cell wall was investigated in the root cell wall isolated from *Verbascum thapsus* L. plants. Electron paramagnetic resonance (EPR) spectroscopy of cell wall isolates containing the spin-trapping reagent, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO), was used for detection and differentiation between free radicals. EPR analysis showed an increase of DEPMPO/OH adduct concentration induced by Cu accompanied by the decrease of concentration of carbon center (DEPMPO/CH_3) adduct originally determined in the cell wall of control plants. This reaction was suppressed by boiling the cell wall with SDS to denaturate proteins. We show that Cu induced •OH accumulation in the cell wall is enzyme dependent.

Introduction
Excess copper in the soil originating from anthropogenic activities is taken up by the roots leading to perturbation of redox processes at first in the apoplastic space and cell wall matrix, and then to deleterious effects for cell metabolism [1]. As a redox active metal, Cu^{2+} can catalyze formation of reactive oxygen species (ROS) *via* Fenton-type reactions. Apoplastic antioxidants such as ascorbate, phenolic compounds, and enzymes superoxide dismutase and peroxidase, play an important role in maintenance of redox homeostasis [2]. The involvement of ROS in the processes of lignification, suberinization, redox signalling and elongation has been well known. This implies a great potential of Cu to interfere with plant development and biomass production, but also a signalling pathway upon pathogen attack. It has been shown that addition of Cu to *Nicotiana tabacum* cell cultures resulted in extracellular accumulation of H_2O_2 (so called, oxidative burst) qualifying Cu as an elicitor of hypersensitive response [3]. Despite the numerous amounts of descriptive data, no explanation has been put forward on the mechanism of copper induced oxidative stress so far.

Material and Methods
Eight weeks old *Verbascum thapsus* L. plants were exposed to 20 μM Cu in hydroponic solution for three weeks. Roots were washed in 10 mM Na_2EDTA

(pH 7.0) and rinsed with distilled water. Cu was quantified with ICP-OES. Accumulation of free radicals in the root cell wall isolates was determined by EPR, according to [4]. Cell wall proteins were denaturated after addition of 10% SDS and heat treatment of cell wall isolates for 10 min at 95ºC, and pellet containing cell wall constituents was used. Simulation parameters used for EPR spectra analysis were: DEPMPO/OH (aP = 46.70; aN = 13.64; aH= 12.78), DEPMPO/CH_3 (aP = 46.95; aN = 14.56; aH = 21.80).

Results and Discussion

Cu is accumulated in a concentration dependant way in the roots but not in the leaves (Table 1). It is known that plants cope with Cu toxicity by retention of excess Cu in the roots and immobilization in the cell wall [1].

Cu (μM)	Cu (mg/kgDW)	
	root	leaf
Control	9.9±7	7±1
6	239±12	12±3
10	497±11	11±5
20	1546±13	12±3

Table 1. Copper concentration in the roots and leaves of *V. thapsus* after 3 weeks of treatment with different Cu concentrations in the hydroponic solution. Values are means ± STDEV from 8 plants.

We have previously shown that the quantity of the redox active metals (Fe and Cu) did not change significantly by the treatment of cell wall isolates with SDS/heat [4], which confirmed that Cu, taken up by the roots, was tightly bound to cell wall components. EPR measurements of root cell wall isolates were performed using spin trap DEPMPO, which can distinguish among various radicals [4]. EPR measurements showed an increased accumulation of both, DEPMPO/OH and DEPMPO/CH_3 adducts, in Cu treated roots compared to the controls (Fig. 1A,B). To test the involvement of enzymes in these reactions, we performed EPR measurements with cell wall fractions after protein denaturation (Fig. 1 C,D). Signals of DEPMPO adducts in the cell wall fractions were completely suppressed in both control and Cu treated plants, indicating the importance of cell wall associated proteins in free radicals generation. Incubation of such denaturated cell wall fraction with 20 μM Cu did not cause any increase of the EPR signal intensity upon addition of DEPMPO (Fig. 1E). Only after addition of 1 mM H_2O_2, the significant increase of DEPMPO/OH was measured which could be explained by the influence of the Fenton reaction (Fig. 1F). Contribution of CH_3 radical increased during incubation of the cell wall with DEPMPO in the roots of control plants (data not shown). However, concentration of DEPMPO/CH_3 adduct decreased in the Cu treated roots, as shown by the spectral simulation, while the concentration of DEPMPO/OH has increased (Fig. 2). The results indicated that consumption of either phenolics or polygalacturonic acid occurred during the production of •OH.

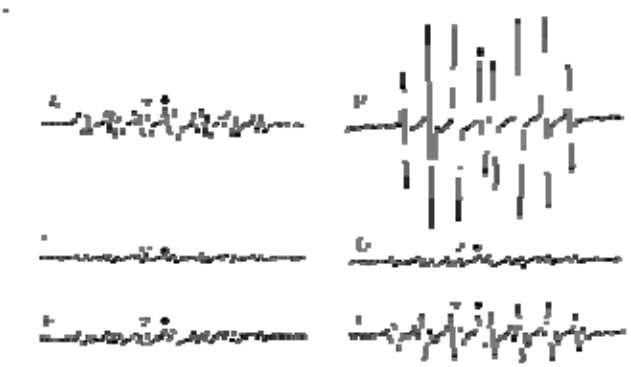

Figure 1. EPR spectra of root cell wall isolates of A) control and B) Cu treated plants; after protein denaturation with SDS/heat treatment; C) control and D) Cu treatment 20 min after addition of DEPMPO; E) cell wall with denaturated proteins incubated with 20 μM Cu and F) 10 min after addition of 1 mM H_2O_2. ● and ∇ mark characteristic si-gnal of DEPMPO/OH and DEPMPO/ CH_3 respectively.

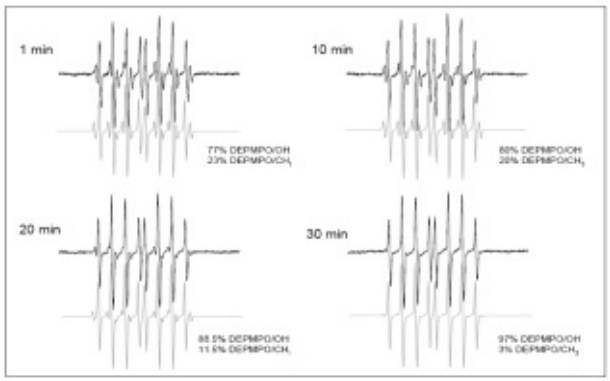

Figure 2. EPR spectra of the root cell wall of Cu treated plants 1, 10, 20 and 30 min upon addition of DEPMPO. Computer simulations of the contribution of DEPMPO/OH and DEPMPO/CH_3 spin adducts were performed using parameters described in Materials and Methods.

Conclusion

We have shown that the excess of Cu tightly bound to the cell wall in Cu treated plants induced •OH generation through the enzyme dependent consumption of the CH_3 radical.

Acknowledgments

This research was supported by the III43010 project funded by the Ministry of Education and Science of the Republic of Serbia.

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