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ARTICLE

Generation of reactive oxygen and nitrogen species in pea cultivars under copper exposure

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ABSTRACT Copper is an essential microelement in plants, but its exposure can induce toxicity symptoms such as growth inhibition, chlorosis or necrosis. The aim of this study was to investigate the physiological responses of two pea cultivars (*Pisum sativum* L. cv. Rajnai törpe and cv. Lincoln) to long term copper exposure. Seven-day-old pea plants were treated with 25 or 50 μM $CuSO_4$, in nutrient solution for 14 days. We studied the growth parameters, the metal uptake, the levels of different reactive oxygen species (hydrogen peroxide, H_2O_2 and superoxide radical, $O_2^{\cdot-}$) and reactive nitrogen species (nitric oxide, $NO^{\cdot}$ and peroxynitrite, $ONOO^-$) together with lipid peroxidation and cell death in the meristem cells of pea roots using *in vivo* and *in situ* microscopic methods. Long-term copper exposure resulted in a serious decrease in shoot and root growth of both pea cultivars and the root system proved to be more sensitive to the stressful condition. The reason of higher sensitivity of the root system is that the largest proportion of copper accumulated in it, namely, pea plants exclude the toxic metals from their shoot. Copper treatment induced the elimination of $O_2^{\cdot-}$ and the concurrent H_2O_2 generation in root tips of both cultivars. The level of NO significantly decreased as the effect of Cu^{2+} exposure, while the level of $ONOO^-$ ($+OH^{\cdot}$) enhanced, suggesting the occurrence of the reaction between $O_2^{\cdot-}$ and NO yielding peroxynitrite. As the effect of copper, lipid peroxidation and cell death were detected in the root tips which led to growth inhibition and biomass decrease of pea plants.
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KEY WORDS

long-term copper exposure
reactive nitrogen species
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pea cultivar

Copper (Cu^{2+}), an essential microelement is considered to be a major heavy metal for plants which is toxic at high concentrations. It can accumulate in various plant organs, directly causing a decrease in photosynthetic activity, carbohydrate content enhancement, damages of lipids, proteins, DNA or cell death (Shao et al. 2010). In the presence of toxic copper concentrations (3-100 μM) plants show reduced biomass (decrease of the root and shoot volume, stem and leaf size), chlorotic and/or necrotic symptoms and inhibition of shoot and root growth. Chlorophyll content decrease and alterations of chloroplast structure have been found in leaves of spinach, rice, wheat, bean and oregano under copper exposure (refs. in Yruela 2009). In general, legume crops (including pea) are less tolerant to copper compared to cereals and grasses. According to the results published by Palma et al. (1987) antioxidant enzyme activities in cv. Lincoln were higher in response to serious copper exposure than in cv. Granada, which suggests the more intense resistance of cv. Lincoln to copper stress.

Moreover, Cu^{2+} and other transition metals induce the formation of reactive oxygen species (ROS) leading to oxidative stress condition. Hydroxyl radical ($OH^{\cdot}$), having the highest reactivity is able to react directly with biological membranes causing lipid peroxidation. Superoxide radical ($O_2^{\cdot-}$) has a short half-life, it does not penetrate membranes, but it reduces transition metal complexes of Fe^{3+} and Cu^{2+}, thus affecting the activity of metal containing enzymes. Hydrogen peroxide acts as a signal molecule and it may inactivate enzymes by oxidizing their thiol groups (Vranová et al. 2002).

Reactive nitrogen species (RNS) such as nitric oxide radical ($NO^{\cdot}$) and peroxynitrite ($ONOO^-$), can be also produced under different stress conditions. Excessive amount of RNS triggers nitrosative stress, which can damage DNA, lipids, proteins and carbohydrates leading to impaired cellular functions (Corpas et al. 2011). Similarly to ROS, reactive nitrogen species can act as signal molecules inducing defence gene expression. Nitric oxide production was detected in *Brassica juncea* L. Czern. and *Pisum sativum* L. roots under Cu or Cd stress or in Cu-treated *Panax ginseng* roots (Bartha et al. 2005; Tewari et al. 2008; Lehotai et al. 2011). However, NO levels were significantly reduced by Cd in pea leaves and roots after long periods of metal treatments (Barroso et al. 2006; Rodríguez-Serrano et al. 2006, 2009). Exogenous application of NO donor induces enzymatic and non-enzymatic antioxidants, regulates root cell wall decomposition, reduces heavy metal uptake and regulates tolerance-related gene expression under heavy metal stress (Xiong et al. 2010). Nitric

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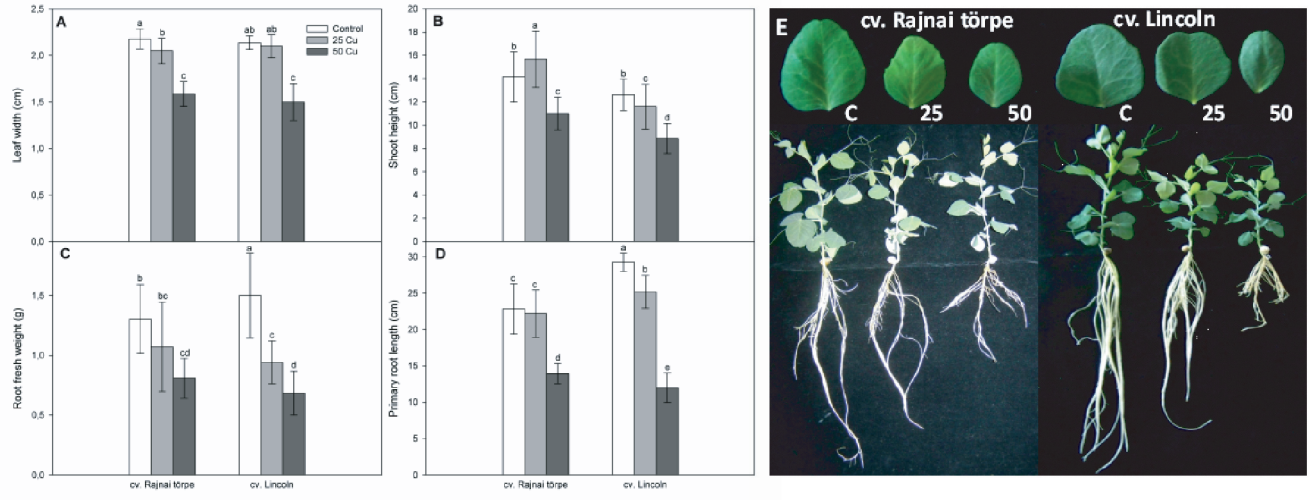

Figure 1. Leaf width (A), shoot height (B), root fresh weight (C) and primary root length (D) of *Pisum sativum* L. cv. Rajnai törpe and *Pisum sativum* L. cv. Lincoln treated with 0, 25 or 50 µM $CuSO_4$ for 14 days. Values are means of 10 plants ±SE. Different letters indicate significant differences (P<0.05) according to Duncan's test. Representative images illustating detached pea leaves and intact plants (E).

oxide may act as an antioxidant during heavy metal exposure through reacting with $O_2^{\cdot-}$ and producing peroxynitrite, a less toxic reactive nitrogen species (Hasanuzzaman et al. 2010).

The effect of long term copper exposure on ROS and RNS metabolism of plants is less known, therefore the aim of this study was to investigate the effects of 14-day-long copper treatment on growth, metal accumulation, ROS (H_2O_2, $O_2^{\cdot-}$) and RNS (NO, $ONOO^-$) levels and cell damages in root apices of two pea cultivars (cv. Rajnai törpe and cv. Lincoln).

Materials and Methods

Plant material and growth conditions

Two pea cultivars (*Pisum sativum* L. cv. Rajnai törpe = Petit Provençal and *Pisum sativum* L. cv. Lincoln) were used for our experiments. The seeds were surface sterilized with 5 % (v/v) sodium hypochlorite for 10 min, rinsed and imbibed for 2 h in running water. Seeds were germinated between moisture filter papers at 26°C for 2-3 days. Germs with radicles (about 2 cm) were placed into Hoagland solution (30 germinated seeds per 10 L growth basin). Plants were grown under controlled conditions in greenhouse at photo flux density of 150 µmol m^{-2} s^{-1} (12/12 day/night period) at a relative humidity of 55-60%, and 25 ± 2°C temperature for 7 days. The Hoagland solution contained the following chemicals: 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4. The micronutrient concentrations were: 10 µM H_3BO_3, 1 µM $MnSO_4$, 5 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0,1 µM $(NH_4)_6Mo_7O_{24}$, 10 µM $AlCl_3$, 10 µM Fe-EDTA. Seven-day-old pea plants were treated with 25 or 50 µM $CuSO_4$ for 14 days. As control, untreated plants were used. All the measurements were done 14 days after treatments. All chemicals were purchased from Sigma-Aldrich (St. Louis MO), unless specified otherwise.

Determination of growth parameters

Shoot height (cm), leaf width (cm) and primary root (PR) length (cm) were determined manually using a scale. Root fresh weight (g) was measured with the help of a compact scale.

Measurement of element contents by atomic absorption spectrophotometry (AAS)

The Cu content in root, stem and leaf samples of pea plants were determined using atomic absorption spectrophotometer (Hitachi Z-8200, Tokyo, Japan). After drying the plant material (90°C, 24 h) 100 milligrams of the samples were measured into destruction tubes. Nitric acid (HNO_3, 65% (w/v), Carlo Erba Reagents, Italy) and hydrogen peroxide (H_2O_2, 30% (w/v, Reanal, Hungary) were added to the dry material and the samples were destructed in microwave destructor (MarsXpress CEM, Matthews, USA) at 200°C on 1600 W for 20 min. Cooled samples were diluted with distilled water and were transferred to 20 ml Packard glasses. After further sufficient dilution of the samples the element contents were determined by AAS. Values of copper concentrations are given as µmol g^{-1} dry mass (DM).

In vivo and *in situ* light microscopy

For light microscopic investigations Zeiss Axioskope 2000-C (Carl Zeiss, Jena, Germany) stereomicroscope was used. Hydrogen peroxide was detected by 3,3'-diaminobenzidine (DAB) staining method (Guan et al. 2009). Pea root tip segments were incubated for 1.5 h in DAB solution (2 mg L^{-1}) then samples were washed once with 2-N-morpholine-ethansulphonic acid/potassium chloride (MES/KCl) buffer (10^{-3} M, pH 6.15) and were prepared on microscopic slides.

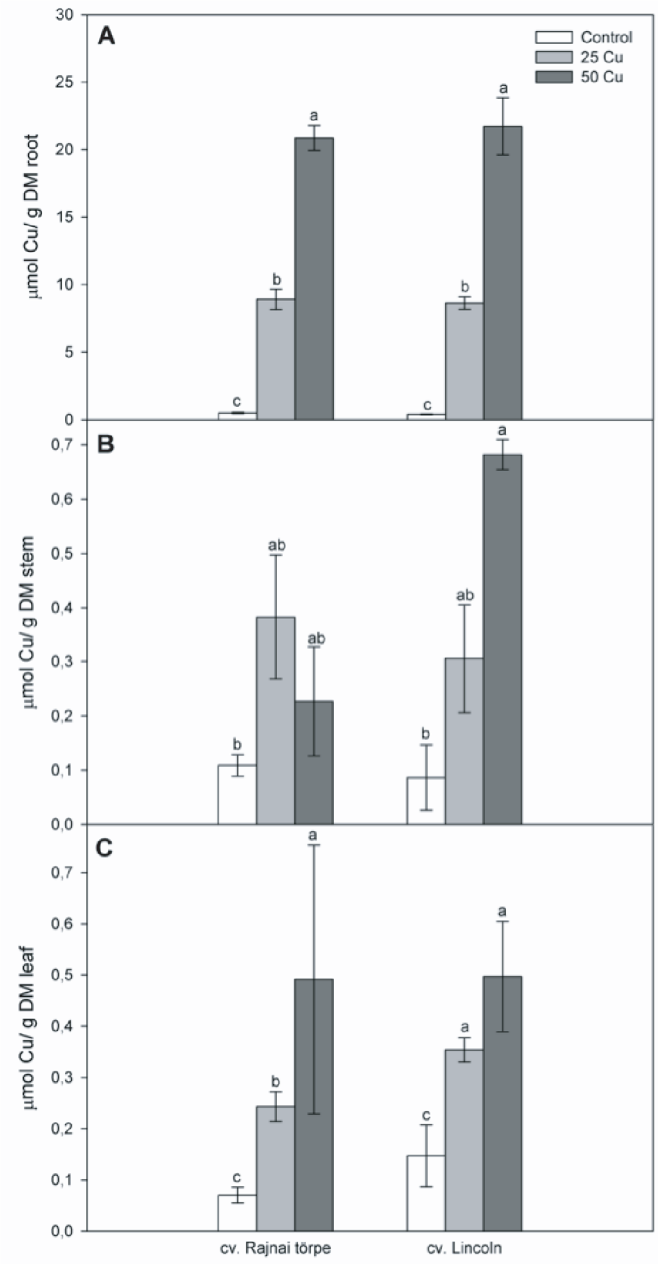

Figure 2. Copper concentration (µmol/g DM) in the root (A), stem (B) and leaf (C) of 0, 25 or 50 µM copper-treated cv. Rajnai törpe and cv. Lincoln. Values are means of 10 plants ±SE. Different letters indicate significant differences (P<0.05) according to Duncan's test.

Detection of superoxide anion was carried out by nitroblue tetrazolium (NBT) staining. Root samples were dyed for 2 h with 0.1 mg/mL NBT (in 0.2 M phosphate buffer, pH 7.6) in the dark. Finally, they were washed once with phosphate buffer. Schiff reagent was applied for the detection of lipid peroxidation and Evans blue was used for the determination of cell death according to Lehotai et al. (2011).

Fluorescent microscopy

For the fluorescent detection of NO, $ONOO^-$ and $O_2^{\cdot-}$ Zeiss Axiowert 200M microscope (Carl Zeiss, Jena, Germany) equipped with a high resolution digital camera (Axiocam HR, HQ CCD, Carl Zeiss, Jena, Germany) and filter set 10 (exc.: 450-490 nm, em.: 515-565 nm) or filter set 9 (exc.: 450-490 nm, em.: 515-∞ nm) was used. FLUAR 5x/0.12 NA objective lens was applied for the investigations. Nitric oxide levels in root tips of pea were visualized by a NO-specific fluorescent dye, 4,5-diaminofluorescein diacetate (DAF-FM DA) according to Pető et al. (2011). Root segments of 1.5- 2 cm length were dyed with 10 µM DAF-FM DA (in 10 mM Tris-HCl buffer, pH 7.4) for 20 min in the dark at 25±2°C and were washed 4 times within 20 min with MES/KCl buffer. Peroxynitrite generation was monitored using aminophenyl fluorescein (APF) according to Lehotai et al. (2011). This fluorophore is suitable for the detection of highly reactive oxygen species (e.g. $ONOO^-$, $OH^{\cdot}$ or OCl^-), but it does not react with NO, $O_2^{\cdot-}$ or H_2O_2. Segments of pea root tips were incubated in 10 µM APF (in 10 mM Tris-HCl, pH 7.4) for 60 min in darkness at room temperature. Samples were washed twice within 30 min with Tris-HCl and were prepared on microscopic slides. Superoxide radicals were detected in segments of pea root, which were incubated at 37°C in darkness for 30 min with 10 µM dihydroethidium (DHE in 10 mM Tris–HCl, pH 7.4) as described by Corpas et al. (2009). Then the root segments were washed twice in the same buffer for 15 min. The intensity of NO-, $ONOO^-$- and $O_2^{\cdot-}$-dependent fluorescence was measured within area of circles with 0.5 mm radii 0.5 mm from the root tip with the help of Axiovision Rel. 4.8 software. The radii of circles were not modified during the experiments.

Statistical analysis

Results are expressed as mean ± SE. Statistical analysis was performed applying SigmaStat 11. software using analysis of variance (ANOVA, P<0.05) and Duncan's test for multiple comparison analyses. All experiments were carried out at least two times. In each treatment at least 10 samples were measured.

Results

The effect of copper on growth and development of pea cultivars

Under control conditions, significant differences were observed in shoot height, root fresh weight and primary root length of the pea cultivars. In the case of control *Pisum sativum* L. cv. Lincoln, longer PR and larger fresh weight was measured compared to cv. Rajnai törpe. In both cultivars 50 µM copper resulted in a serious inhibition of the stem and leaf, while 25 µM Cu^{2+} had no effect on the growth of them. In cv. Lincoln, both copper concentrations significantly de-

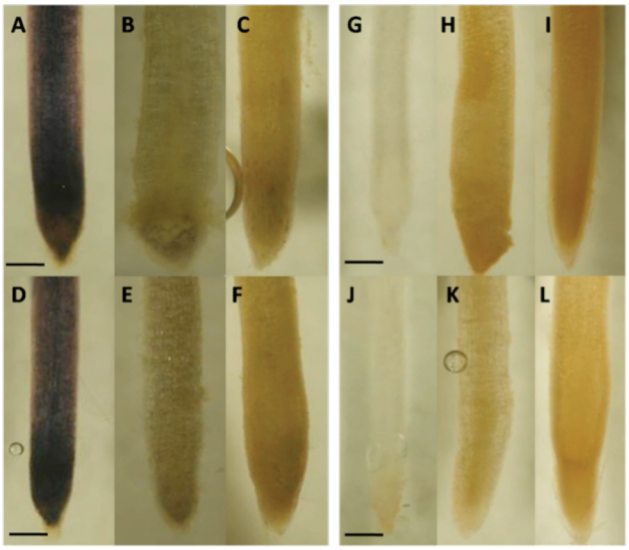

Figure 3. Superoxide (A-F) level in root tips of 0, 25 or 50 µM copper-treated cv. Rajnai törpe (A-C) and cv. Lincoln (D-F). Hydrogen peroxide (G-L) level in root tips of 0, 25 or 50 µM copper-treated cv. Rajnai törpe (G-I) and cv. Lincoln (J-L). Bars = 1 mm.

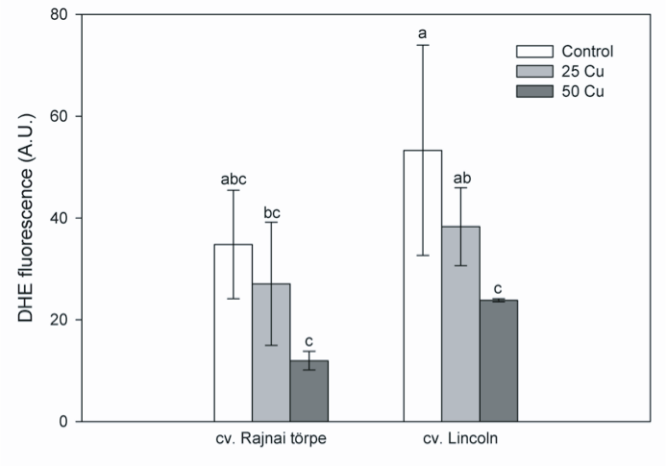

Figure 4. Intensity of superoxide-specific fluorescence (DHE) in root tips of 0, 25 or 50 µM copper-treated cv. Rajnai törpe and cv. Lincoln. Values are means of 10 plants ±SE. Different letters indicate significant differences (P<0.05) according to Duncan's test.

creased PR length, but only 50 µM Cu^{2+} caused PR shortening of cv. Rajnai törpe (Fig. 1).

Copper accumulation in the root and stem system of pea plants

In the case of both cultivars copper concentration within the plants enhanced as the effect of treatments. The largest proportion of the copper uptaken accumulated in the root system, hence 10- and 20-fold increase in Cu^{2+} concentration was

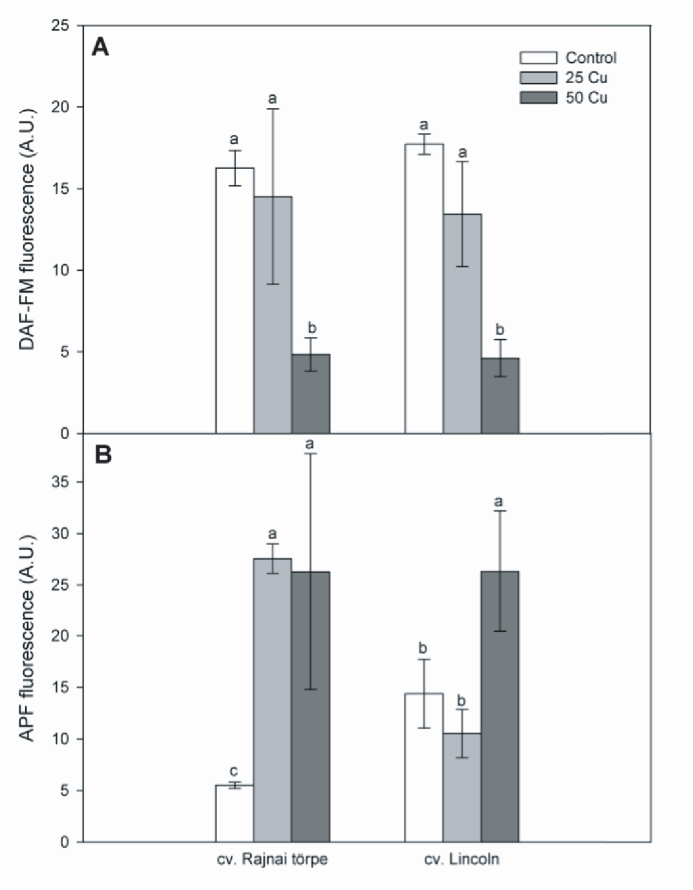

Figure 5. Intensity of nitric oxide- (DAF-FM, A) and peroxynitrite- (APF, B) specific fluorescence in root tips of 0, 25 or 50 µM copper-treated cv. Rajnai törpe and cv. Lincoln. Values are means of 10 plants ±SE. Different letters indicate significant differences (P<0.05) according to Duncan's test.

measured in the roots of 25 and 50 µM copper-treated plants, respectively (Fig. 2A). In the stem of cv. Rajnai törpe Cu^{2+} concentration enhanced moderately compared to cv. Lincoln, where notable (~7-fold) Cu^{2+} accumulation was observed (Fig. 2B). In the leaves of the cultivars, copper accumulated to a similar extent (Fig. 2C).

The effect of copper on ROS and RNS levels of pea root tips

In both cultivars, 25 and 50 µM copper treatment caused the complete elimination of superoxide from root apices, which was indicated by the lack of NBT staining (Fig. 3A-F). Similar results were obtained by dihydroethidium, where the fluorescence intensities significantly decreased as the effect of 50 µM Cu^{2+} in both cultivars (Fig. 4). Concurrently with superoxide elimination, an extensive H_2O_2 accumulation was observed by DAB staining in root tips of cv. Rajnai törpe (Fig 3 G-I), while in cv. Lincoln the degree of copper-induced H_2O_2 formation seemed to be lower (Fig. 3J-L).

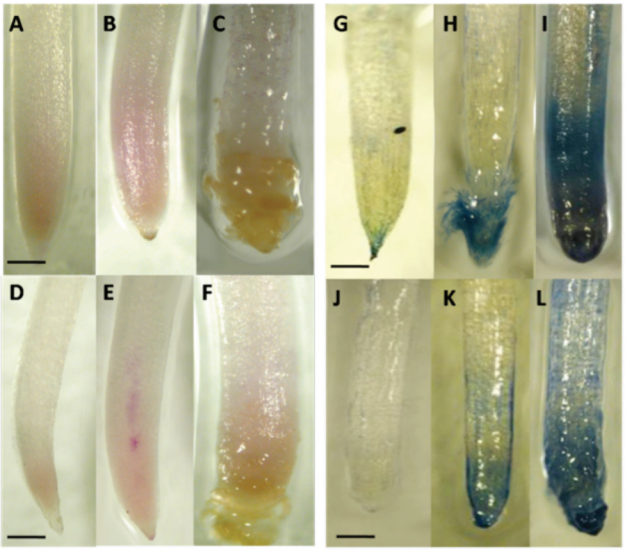

Figure 6. Lipid peroxidation (Schiff reagent staining, A-F) in root tips of 0, 25 or 50 µM copper-treated cv. Rajnai törpe (A-C) and cv. Lincoln (D-F). Cell death (Evans blue staining, G-L) in root tips of 0, 25 or 50 µM copper-treated cv. Rajnai törpe (G-I) and cv. Lincoln (J-L). Bars= 1 mm.

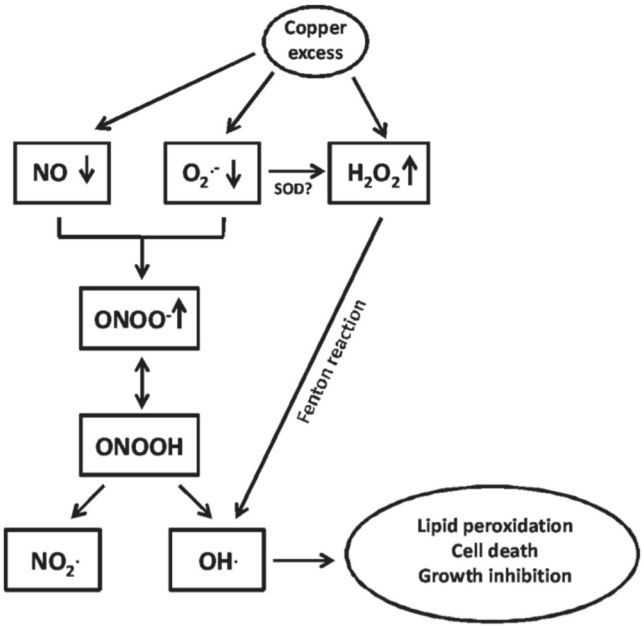

Figure 7. Schematic representation of possible pathways leading to gowth inhibition under copper exposure.

Nitric oxide levels of root apexes were also modified by long-term copper exposure. Interestingly, 25 µM Cu^{2+} had no effect on NO generation, but higher copper concentration (50 µM) resulted in a significant decrease of NO level in cv. Rajnai törpe and cv. Lincoln root tips (Fig. 5A). Furthermore, $ONOO^-$ ($+OH^{\cdot}$) –dependent fluorescence significantly increased in cv. Rajnai törpe treated with 25 or 50 µM CuSO4, although in cv. Lincoln roots ONOO- (+OH.) level did not enhance as the effect of 25 µM copper (Fig. 5B). In 50 µM copper-treated root samples of cv. Lincoln 1.6-fold increase of APF-fluorescence was detected compared to control.

Copper exposure induces lipid peroxidation and death of root tip cells

As the effect of 25 and 50 µM Cu^{2+}, lipid peroxidation (detected by Schiff reagent) and cell death (detected by Evans blue) intensified in the root tips, and the damage of 50 µM-copper treated roots were visible. In root tips of cv. Rajnai törpe the Evans blue staining was more pronounced, indicating the strong cell death (Fig. 6).

Discussion

The first physiological process during copper exposure is the uptake of the metal from the environment. Our results showed that copper is accumulated in the root system of both pea cultivars and only a slight increase of Cu^{2+} concentration was observed in the aerial parts of the plant bodies. The inhibition of copper translocation into the leaves (metal exclusion from the shoot) provides an important defence mechanism for the plant against toxicity (Baker 1981). Among the symptoms of copper toxicity the growth inhibition is one of the most characteristic. Long-term copper exposure resulted in a significant decrease in growth parameters of pea plants, and the root system proved to be more sensitive to copper compared to the shoot, which can be explained by the significant copper accumulation within the root system (Lequeux et al. 2010). In the stem of cv. Rajnai törpe less copper accumulated compared to cv. Lincoln, which correlates the slighter decrease of elongation. The root system of cv. Lincoln suffered more serious growth inhibition than cv. Rajnai törpe, which implies that cv. Lincoln is more sensitive to copper exposure. This finding seems to be contrary to that of Palma et al. (1987), where cv. Lincoln was found to be more sensitive compared to cv. Granada. The alterations of ROS and RNS levels were established by *in vivo* and *in situ* staining methods. The complete elimination of superoxide anion from the root tip tissues was detected by NBT staining and was verified by the significant decrease of DHE fluorescence in both cultivars. The concurrent accumulation of H_2O_2 in the root apex suggests the spontaneous or enzymatic dismutation of $O_2^{\cdot-}$ to H_2O_2 under copper exposure. Whereas, in root tips of Cu-treated soybean the significant activation of superoxide dismutase (SOD) was observed (Chongpraditnun et al. 1992), this enzyme may have a role in superoxide detoxification under copper stress. Nitric oxide levels in root tips were significantly decreased in both cultivars as the effect of long-term copper exposure. Heavy metal-induced decrease of NO levels was observed, inter

alia, in pea leaves and roots (Rodriguez-Serrano et al. 2009); however, it must be noted, that the concentration of the applied metal, the treatment condition, the age of the plant and the variety of tissue examined all determine the effects on NO production (Xiong et al. 2010). Related to the decrease of the NO content, it was attractive to hypothesize that superoxide radicals eliminate NO by the reaction yielding peroxynitrite. Aminophenyl fluorescein was applied for the detection of $ONOO^-$ ($+OH^\cdot$) and significant enhance of the fluorescent intensities was found in root tips of copper-treated cv. Rajnai törpe and cv. Lincoln, which suggests the occurrence of the reaction between $O_2^{\cdot-}$ and NO producing $ONOO^-$. The peroxynitrite anion is in pH-dependent protonation equilibrium with peroxynitrous acid (ONOOH), which decompose resulting nitrogen dioxide ($NO_2^\cdot$) and hydroxyl radical ($HO^\cdot$) (Virag et al. 2003). Hydroxyl radical can originate also from H_2O_2, because copper as a transition metal is able to catalyze the Fenton-reaction (Rowley and Halliwell 1983). The elevation of $OH^\cdot$ level within the root tip cells (which was partly demonstrated by APF) is responsible for lipid peroxidation process as it was shown by Schiff reagent staining in both cultivars. The damage of membrane lipids leads to cell death in root apex, which results in growth inhibition of the whole organ. The hypotetical pathways leading to growth inhibition under copper exposure are shown in Fig. 7.

Based on our results it can be concluded, that the general responses of the examined pea cultivars to copper stress are similar. However, cv. Lincoln proved to be more sensitive to copper than cv. Rajnai törpe, which can be explained by e.g. potential hormonal disturbances. An important defence mechanism of pea plants is the exclusion of the toxic copper from the aerial organs and the accumulation of it in the root system. At the back of superoxide elimination can be the spontaneous or SOD-catalyzed dismutation of it or the reaction of it with NO to produce peroxynitrite. Nitric oxide is considered to be an antioxidant, because it is able to convert the highly reactive $O_2^{\cdot-}$ to the less toxic $ONOO^-$. However, from the protonated form of $ONOO^-$ (ONOOH) the reactive hydroxyl radical can generate, which contributes to cell death induction and growth inhibition of pea plants.

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