Alleviation of copper toxicity in germinating pea seeds by IAA, GA₃, Ca and citric acid

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ABSTRACT

The ameliorating effects of four exogenous effectors were investigated in germinating pea seeds exposed to copper excess. The results showed that the application of IAA, GA₃, Ca or citric acid alleviated Cu-induced inhibition of growth and simultaneously reduced the oxidative stress injury, particularly contents of hydrogen peroxide, malondialdehyde and carbonyl groups. The improving effects can probably be mediated by the decreases in liperoxidation and protein oxidation as evidenced by changes in antioxidant enzyme activities. In addition, the efficiency of this recovery was compared within two types of treatments. Obtained results demonstrated that the stress abruption by the addition of effectors after three days of Cu application (treatment of type II) seems to be more effective than the simultaneous application of ‘Cu + effectors’ at the beginning of germination (treatment of type I). Data could provide some clues to physiological and biochemical mechanisms of the response of germinating seeds to the addition of chemicals under heavy metal stress.

Introduction

Scientists have been more interested in developing potential strategies to promote germination of seeds and growth of plants, in attempt to achieve higher crop production (Sharma et al. 2014; Summar et al. 2015). This goal is getting even more needed to alleviate the adverse effects of environmental pollutants, and the search for molecules mediating stress tolerance is a relevant step toward a better understanding of how lower plants respond to stress (Muhammad et al. 2015).

Subsequent researchers have shown that excess in levels of heavy metals in the environment was hazardous for all organisms (Chaoui and El Ferjani 2005; Al-Busaidi et al. 2015). One of the strategies of heavy metals’ removal from soil is the phytoremediation (Tassi et al. 2008). At the cellular level, plants have developed defense systems to maintain metabolically compatible reactive oxygen species (ROS) levels, particularly those of H₂O₂, via involving the antioxidant enzymes, such as catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and guaiacol peroxidase (GPOX) (Mittler 2002; Schützendübel and Polle 2002; Wang et al. 2011). However, the antioxidant capacity of plants may not be sufficient to minimize the harmful impact after heavy metals’ exposure.

Alternative ways to improve plant resistance and especially to counteract the toxic effects of heavy metals on seed germination and plant growth consist of: (1) the exogenous application of phytohormones (auxins, gibberellins, cytokinins) and polyamines (Choudhary et al. 2012; Piotrowska-Niczyporuk et al. 2012); (2) the co-application of plant growth regulators, including indole-3-butyric acid and 1-naphthalenesacetic acid, as well as indole-3-acetic acid (IAA) with ethylene diamine tetra acetic acid (Gangwar and Singh 2011); (3) the treatment with chemicals, notably nitric oxide (Hu et al. 2007), sulfur (Anjum et al. 2008), β-estradiol (Chaoui and El Ferjani 2013, 2014) and progesterone (Genisel et al. 2013) and (4) the pretreatment with hydrogen peroxide (Hu et al. 2007) and salicylic acid (Belkhadi et al. 2010). In addition, organic acids such as malate, oxalate, citrate and benzoate have been reported to exhibit the capacity to sequester the metallic cations (Gao et al. 2012). Furthermore, calcium was also found to increase the tolerance of plants against stress, to promote growth and development and to enhance protection against heavy metals induced oxidative stress (Wan et al. 2011; Ahmad et al. 2015; Jan et al. 2015; Abd_Allah et al. 2017).

The objective of the present study is to compare the efficiency of two types of treatments with the exogenous effectors (IAA, GA₃, Ca and citric acid) against copper (Cu) toxicity: (I) the simultaneous application of ‘Cu + effectors’ at the beginning of germination (day 0) and (II) the abruption of Cu stress (exogenous effectors are added in the third day).

Materials and methods

Germination and treatments

Pea seeds (Pisum sativum L. var. douce province) were disinfected with 2% sodium hypochlorite for 10 min, then rinsed thoroughly and germinated at 25°C in the dark over two sheets of filter paper moistened with distilled water (control), 200 μM CuCl₂, 1 μM IAA, 1 μM GA₃, 10 mM CaCl₂ and 100 μM Na-citrate, applied alone or in combinations with Cu: Cu + IAA, Cu + GA₃, Cu + Ca and Cu + citrate. This treatment will be referred as treatment of type I.

For the second treatment (abruption of Cu stress), seeds were germinated until the third day in the presence of Cu and then replaced either by distilled water (control) or by Cu added alone or with IAA, GA₃, Ca or citric acid (same concentrations as treatment of type I). This treatment will be referred as treatment of type II.
On day 6, the embryonic axes were harvested and divided into roots and shoots.

**Determination of oxidative stress markers**

Hydrogen peroxide level was measured according to the method of Sergiev et al. (1997). After homogenization in 50 mM potassium phosphate buffer (pH 7.8), the homogenate was mixed with trichloroacetic acid (TCA; 5%, w/v) and centrifuged at 10,000 g for 10 min. One milliliter of 10 mM potassium phosphate buffer (pH 7.0) and 2 mL of 1 M potassium iodide were added to an aliquot of the supernatant. The H$_2$O$_2$ concentration was estimated based on the absorbance of the supernatant at 390 nm.

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA). Samples were homogenized with 0.1% TCA (1/5: w/v), then centrifuged at 15,000 g for 10 min, and the resulting supernatant was treated with 0.5% (w/v) TBA in 20% TCA (w/v). After heating at 90°C for 20 min, the mixture was cooled and centrifuged at 10,000 g for 5 min. The absorbance of the supernatant at 532 nm was recorded and corrected for non-specific turbidity by subtracting the value at 600 nm. The amount of thiobarbituric acid reactive substances was calculated using the extinction coefficient of 155 mM$^{-1}$ cm$^{-1}$.

Carbonyl groups of proteins were determined according to Reznick and Packer (1994), using the spectrophotometric dinitro-phenyl-hydrazine (DNPH) method. Samples were homogenized in 5 mL of 50 mM K-phosphate buffer (pH 7.0) supplemented with 5 mM Na-ascorbate. The homogenate was centrifuged at 20,000 g for 30 min at 4°C. The mixture containing protein extract and the DNPH (10 mM, prepared in 2 N HCl) was left in the dark at room temperature for 1 h, then precipitated with cold TCA (final concentration of 20%) and centrifuged at 20,000 g for 15 min. The protein pellet was washed with 20% TCA, and then three times with ethanol/ acetic acid (v/v). Samples were resuspended in 6 M guanidine hydrochloride (dissolved in 2 N HCl) and incubated at 40°C for 30 min with vortex mixing. Carbonyl content was determined from the absorbance at 480 nm (ε = 22,000 M$^{-1}$ cm$^{-1}$) as described by Levine et al. (1994). The protein-carbonyl content was expressed as nmol mg$^{-1}$ protein.

**Measure of the antioxidant enzymes activities**

Total proteins were extracted in 25 mM potassium phosphate buffer (pH 7.0), containing 5 mM sodium ascorbate, followed by centrifugation at 20,000 g for 30 min and the resulting supernatant was considered as a soluble enzymatic fraction. Protein concentration in the enzymatic extract was evaluated by the method of Bradford (1976), using bovine serum albumin as the standard protein.

CAT (EC 1.11.1.6) activity was measured according to Aebi (1984). The enzymatic assay contained 10 mM H$_2$O$_2$ in 25 mM phosphate buffer (pH 7.0) and enzymatic extract. CAT activity was estimated by monitoring the decrease in the absorbance of H$_2$O$_2$ reduction at 240 nm, using an extinction coefficient of 36 × 10$^{-6}$ M$^{-1}$ cm$^{-1}$.

APX (EC 1.11.1.11) activity was measured according to Nakano and Asada (1981). The reaction mixture contained 0.5 mM ascorbate, 5 mM H$_2$O$_2$, 0.1 mM EDTA and enzymatic extract. APX activity was determined by following the decrease in ascorbate absorbance at 290 nm. The extinction coefficient of 2.8 × 10$^{-3}$ M$^{-1}$ cm$^{-1}$ was used.

GPX (EC 1.11.1.9) activity was measured according to the method described by Nagaralakshmi and Prasad (2001). The reaction mixture is contained in 50 mM phosphate buffer (pH 8.0): 100 mM NaCl, 1 mM reduced glutathione (GSH), 2.5 mM H$_2$O$_2$, 0.5 mM NADPH, 1 U GR and enzyme extract. The oxidation of NADPH was followed by measuring the decrease in absorbance at 340 nm, using an extinction coefficient of 6.22 × 10$^3$ M$^{-1}$ cm$^{-1}$.

GPOX (EC 1.11.1.7) activity was measured according to Fielding and Hall (1978). The reaction mixture contained 10 mM H$_2$O$_2$ in 25 mM potassium phosphate buffer (pH 7.0), 9 mM guaiacol and enzyme extract. GPOX activity was estimated by measuring the increase in absorbance of tetraguaiacol at 470 nm, using an extinction coefficient of 26.6 M$^{-1}$ cm$^{-1}$.

**Statistics**

All data were expressed as the mean values ± SE of several independent bioassays and subjected to a one-way analysis of variance. The averages were separated using Duncan's multiple-range test. Comparisons with $p<.05$ were considered significantly different.

**Results**

The growth of the embryonic axes of pea seeds was evaluated as length and fresh biomass of roots and shoots of six-day germinated seeds under the following treatments: Cu, IAA, GA$_3$, Ca and citric acid applied alone or in combination; Cu + IAA, Cu + GA$_3$, Cu + Ca and Cu + Citrate. Copper treatment decreased the length and fresh weight of root and shoot, as compared to respective controls (Figure 1). The treatments with IAA, GA$_3$, Ca and citrate did not show significant effects on parameters of embryonic growth, as compared with respective controls. However, when applied with Cu (treatment of type I), the inhibitory effects of the metal seem to decrease. In fact, root and shoot fresh weights reached more than twice folds as compared with Cu-exposed ones (Figure 1 (A,B)). The length was also recovered by approximately two folds in ‘Cu + effectors’ treated embryonic axes compared to those treated with Cu (Figure 1 (C,D)). A similar improvement in growth parameters was observed when the Cu treatment was interrupted after 3 days by the addition of the exogenous effectors (treatment of type II; Figure 2). In addition, type II treatment showed higher recovery in biomass and length of roots and shoots compared to type I treatment (Table 1).

Levels of H$_2$O$_2$, MDA and carbonyl groups increased almost twice in Cu-treated roots and shoots in comparison with controls (Figures 3 and 4). However, when Cu was applied in combination with the exogenous effectors at the beginning of germination (treatment of type I), significant decreases in H$_2$O$_2$ contents (Figure 3 (A,B)), MDA (Figure 3 (C,D)) and carbonyl groups (Figure 3 (E,F)) were observed and reached almost control values when the effectors were supplemented after 3 days of Cu exposure (treatment of type II; Figure 4). Indeed, treatment of type II seems to be more interesting to mitigate the levels of indicators of oxidative stress than treatment of type I: the percentages of decrease in Cu treatment (100%) may even exceed twice
when the effects of the second treatment are compared with those of type I (cases of ‘Cu + Ca’ and ‘Cu + Citrate’ for MDA in shoots; 53\% (II) vs. 23\% (I) and 46\% (II) vs. 22\% (I), respectively) (Table 2).

In order to shed more light on the mechanism of the ameliorative effects of exogenous IAA, \( \text{GA}_3 \), \( \text{Ca} \) and citric acid on Cu toxicity, we measured the antioxidant activities in root and shoot tissues of seeds subjected to type I and II...
treatments. In Cu-exposed seeds, a significant increase in the activities of the antioxidant enzymes (CAT, APX, GPX and GPOX) that exceeded two folds in both root and shoot (Figure 5) was recorded. The separate application of the exogenous effectors did not reveal a marked difference with controls, but when applied in combination with Cu (treatment of type I), the activities of CAT (Figure 5 (A,B)), APX (Figure 5 (C,D)), GPX (Figure 5 (E,F)) and GPOX (Figure 5 (G,H)) decreased to about 50%, as compared with Cu-treated samples, thus reaching almost control values. In addition, a similar decrease was observed when IAA, GA3, Ca and citric acid were applied after 3 days of the start of germination in the presence of Cu (treatment of type II; Figure 6). Thus, the comparison between treatments I and II did not reveal better efficiency of the second treatment regarding the restoration of antioxidant enzyme activities to almost control levels (Figures 5 and 6).

### Discussion

Subsequent studies have already shown that Cu imposed a delay in the embryonic axes growth of legume seeds (Sfaxi-Bousbih et al. 2010; Karious et al. 2012; Chaoui and El Ferjani 2013). In the present experiment, a similar decrease in

### Table 1. Extend of improvement of growth of embryonic axes of germinating pea seeds (expressed as percentage of the control): comparison between type I and II treatments.

| Parameters | Treatments | Root | Shoot |
|------------|------------|------|-------|
|            | I          | II   | I     | II   |
| Fresh weight (%) | H2O        | 100  | 100   | 100  |
|              | CuCl2      | 43   | 43    | 44   |
|              | Cu + IAA   | 94   | 108   | 95   |
|              | Cu + GA3   | 92   | 110   | 89   |
|              | Cu + Ca    | 79   | 85    | 91   |
|              | Cu + Citrate | 80   | 83    | 83   |
| Length (%)   | H2O        | 100  | 100   | 100  |
|              | CuCl2      | 39   | 39    | 35   |
|              | Cu + IAA   | 106  | 115   | 91   |
|              | Cu + GA3   | 91   | 108   | 113  |
|              | Cu + Ca    | 94   | 95    | 89   |
|              | Cu + Citrate | 90   | 91    | 82   |

Note: The values are calculated from the data in Figures 1 and 2.

### Figure 3.

Contents of H2O2 (A, B), MDA (C, D) and carbonyl groups (E, F) in root (A, C, E) and shoot (B, D, F) of embryonic axes of pea seeds germinated for 6 days in the presence of distilled water (H2O) or 200 µM CuCl2 applied alone or in combination with 1 µM IAA, 1 µM GA3, 10 mM Ca and 100 µM citrate (treatment of type I). Values ± SE (n = 5) followed by a common letter are not different at the 0.05 level of significance.
root and shoot length and fresh biomass in pea seeds during germination was found. Nevertheless, the application of IAA, GA3, Ca and citric acid recovered embryonic growth to almost control levels. Interestingly, the ameliorating effects of the different effectors assayed appear to be relevant for the treatment of type II, since only 3 days of treatment by the exogenous effectors were able to restore the embryonic normal growth even after 3 days of Cu exposure, which suggests their efficiency in the tolerance toward copper stress.

This finding is in line with other studies that reported the involvement of exogenous effectors to increase plant tolerance against stress (Wang and Song 2009), to promote plant growth and development and to alleviate growth inhibition under adverse environmental conditions (Tassi et al. 2008; Rodriguez-Serrano et al. 2009; Wen et al. 2010; Gangwar et al. 2011). The role of IAA application in alleviating the adverse effects of stress factors including heavy metals was also suggested (Chakrabarti and Mukherji 2003; Gangwar et al. 2011); such as, the decrease in the level of ROS and the increase in seed germination rate, growth and nitrogen metabolism (Gangwar and Singh 2011). Exogenous GA3 is also able to overcome the inhibitory effects of different environmental stresses on seed germination and other physiological parameters; such as dry matter contents, chlorophyll, relative water, proline and mineral nutrients, activities of superoxide dismutase, peroxidase and polyphenol oxidase, as well as extent of electrolyte leakage (Tuna et al. 2008; Alonso-Ramírez et al. 2009; Wen et al. 2010). Similarly, Ca (Rodriguez-Serrano et al. 2009; Wang and Song 2009; Ahmad et al. 2015) and citric acid (Hall 2002) may have protective effects against heavy metals induced oxidative stress in several plant species: Ca is able to compete with metallic cations for uptake through Ca transporters and citrate is able to activate the efflux from the tissues, e.g. by mechanisms

Table 2. Percentages of diminution from Cu treatment (100%) of the oxidative stress indicators in embryonic axes of germinating pea seeds: comparison between type I and II treatments.

| Parameters | Treatments | Root | | | | Shoot | | | |
|------------|------------|-----|-----|-----|-----|-----|-----|-----|-----|
| H2O2       | Cu + IAA   | 49  | 56  | 62  | 71  | Cu + GA3 | 49  | 52  | 57  | 66  |
|            | Cu + Ca    | 46  | 57  | 59  | 61  | Cu + Citrate | 42  | 48  | 53  | 57  |
| MDA        | Cu + IAA   | 30  | 55  | 29  | 49  | Cu + GA3 | 30  | 52  | 25  | 46  |
|            | Cu + Ca    | 41  | 49  | 23  | 53  | Cu + Citrate | 42  | 47  | 22  | 46  |
| Carbonyl   | Cu + IAA   | 46  | 55  | 49  | 61  | Cu + GA3 | 39  | 58  | 46  | 60  |
|            | Cu + Ca    | 44  | 52  | 43  | 56  | Cu + Citrate | 36  | 52  | 41  | 55  |

Note: The values are calculated from the data in Figures 3 and 4.

Figure 4. Contents of H2O2 (A, B), MDA (C, D) and carbonyl groups (E, F) in root (A, C, E) and shoot (B, D, F) of embryonic axes of pea seeds germinated for 3 days in the presence of 200 µM CuCl2, then replaced until day 6 either by distilled water (H2O) or 200 µM CuCl2 added alone or with 1 µM IAA, 1 µM GA3, 10 mM Ca or 100 mM citrate (treatment of type II). Values ± SE (n = 5) followed by a common letter are not different at the 0.05 level of significance.
leading to lower cytoplasmic heavy metal contents. Indeed, citric acid may act via direct intracellular detoxification, by forming a complex, which allows to sequester metallic cations and eliminate them out of the cytoplasm (Gao et al. 2012).

In order to improve our understanding of the role of these exogenous effectors in plant responses to heavy metal toxicity, the cell contents of some oxidative stress indicators and the antioxidant enzyme activities in germinating seeds were determined. Obtained results showed an increase in 

\( H_2O_2 \) levels in Cu-treated embryos, which suggests the rise of ROS production. This hypothesis was also argued by the elevation of the oxidative injury indicators, notably MDA (lipid peroxidation indicator) and carbonyl groups (protein oxidation marker) in roots and shoots in the presence of Cu, as has already been reported in a number of plants (Chaoui and El Ferjani 2005; Posmyk et al. 2009; Wang et al. 2011). Furthermore, Cu is known to cause structural and functional impairments in cell membrane, which were ascribed to the oxidative alterations of macromolecules (Chaoui and El Ferjani 2014; Karmous et al. 2014). However, the data provide evidences that the supplementation of IAA, GA3, Ca and citric acid to Cu-treated embryos is able to

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**Figure 5.** Activities of CAT (A, B), APX (C, D), GPX (E, F) and GPOX (G, H) in root (A, C, E, G) and shoot (B, D, F, H) of embryonic axes of pea seeds germinated for 6 days in the presence of distilled water (H2O) or 200 µM CuCl2 applied alone or in combination with 1 µM IAA, 1 µM GA3, 10 mM Ca and 100 µM citrate (treatment of type I). Values ± SE (n = 5) followed by a common letter are not different at the 0.05 level of significance.
prevent ROS overproduction, maintain cellular ultrastructural integrity and reduce protein oxidations. Besides, these findings were more interestingly obtained with treatment of type II, thus showing a better correction in the oxidative status (decrease in levels of H$_2$O$_2$, MDA and carbonyl groups as compared to Cu-treated samples).

On the other hand, the activities of the antioxidant enzymes CAT, APX, GPX and GPOX increased significantly in the presence of Cu, while the addition of IAA, GA$_3$, Ca or citric acid into the germination medium recovered the antioxidant response of Cu-treated embryos to almost control levels. Hence, the exogenous effectors assayed in the present investigation did not improve the antioxidant enzymatic activities, as reported by other authors (Rodriguez-Serrano et al. 2009; Wang and Song 2009; Gao et al. 2012). Moreover, this hypothesis was evidenced for both types of treatments.

**Figure 6.** Activities of CAT (A, B), APX (C, D), GPX (E, F) and GPOX (G, H) in root (A, C, E, G) and shoot (B, D, F, H) of embryonic axes of pea seeds germinated for 3 days in the presence of 200 µM CuCl$_2$, then replaced until day 6 either by distilled water (H$_2$O) or 200 µM CuCl$_2$ added alone or with 1 µM IAA, 1 µM GA$_3$, 10 mM Ca or 100 µM citrate (treatment of type II). Values ± SE (n = 5) followed by a common letter are not different at the 0.05 level of significance.
Therefore, the suggested mechanisms of the protective effects of IAA, GA₃, Ca and citric acid on pea embryonic growth from Cu toxicity cannot be mediated by a potential effect through the ‘late’ activation of antioxidant response, but rather by the ‘early’ limitation of oxidative stress generation. According to this alternative hypothesis, the decrease in the activities of antioxidant enzymes in the presence of exogenous effectors may also be considered as a marker of an oxidative status attenuation like the other indicators (H₂O₂, MDA and carbonyl contents).

Conclusion
This study investigated the effect of the application of IAA, GA₃, Ca and citric acid on growth impairment by CuCl₂ on germinating pea seeds, applied simultaneously or after the heavy metal was removed. The heavy metal application resulted in the increase in levels of oxidative stress indicators, as well as the induction of enzymes with antioxidant activity. However, the exogenous effectors IAA, GA₃, Ca and citric acid showed a limitation of oxidative damage and a considerable recovery in the growth of seedlings. This ameliorative effect is suggested here to occur even after previous exposure to heavy metal stress.

Disclosure statement
No potential conflict of interest was reported by the authors.

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