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# Copper toxicity in expanding leaves of *Phaseolus vulgaris* L.: antioxidant enzyme response and nutrient element uptake

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ABSTRACT

Bioaccumulation and toxicity of copper ($CuSO_4$) were assessed in expanding leaves of 14-day-old bean seedlings. $CuSO_4$ was administrated in the growth medium for three days and changes in the activities of the antioxidant enzymes guaiacol peroxidase (GPX) and catalase (CAT), and in the $H_2O_2$ production and mineral element contents were measured. Copper accumulated in exposed plants caused severe symptoms such as chlorosis and necrosis as well as a dramatic reduction in dry weight production. Simultaneously, concentrations of iron, zinc and potassium were reduced significantly suggesting that a change in nutrient homeostasis may be responsible for the observed symptoms. Contrary to mature tissues, the expanding leaves did not display significant oxidative stress, since malondialdehyde (MDA) content was unchanged, the activities of GPX and CAT were lowered or unaltered, and endogenous $H_2O_2$ only increased at high copper concentrations. Our results suggest that while excess copper slightly alters the activity of the antioxidative enzyme system in young expanding leaves of bean plants, it exerts its toxicity primarily through causing a disturbance in the nutrient balance.



## 1. Introduction

Copper is a trace element necessary for plant nutrition. It plays an important role in various metabolic processes, but it can become toxic at high concentrations both for plants and animals (Gaetke and Chow, 2003). The typical concentration of this metal in plant tissues is between 1 and 5 $\mu g\ g^{-1}$ dry weight (Marschner, 1995), whereas leaves show a slightly higher concentration with 5–20 $\mu g\ g^{-1}$ dry weight (Baker and Senef, 1995). These values vary depending on the studied species and varieties. Smelting, mining, land applications of sewage sludge and discharge of untreated urban and industrial residues and other human activities, lead to widespread soil contamination with copper. In addition, during past decades copper has also been extensively released into the environment by agricultural activities, since it is used as an antifungal agent.

Copper is crucial to the functioning of numerous proteins, such as superoxide dismutase, ascorbate oxidase, polyphenol oxidase, cytochrome oxidase and plastocyanin (Marschner, 1995). The metal plays an essential role in cell wall metabolism, it acts as a structural element in regulatory proteins, and it participates in photosynthetic electron transport and mitochondrial respiration (Sandmann, 1985; Yruela, 2005). It plays an essential role in signaling to the transcription and protein trafficking machinery and it is involved in hormone signaling (Yruela, 2005; Pilon et al., 2006; Krämer and Clemens, 2006).

At elevated concentrations, copper becomes toxic for plants. This may be due to the fact that $Cu^{2+}$ induces oxidative stress by catalyzing the formation of harmful reactive oxygen species (ROS) (Hall, 2002; Schützendübel and Polle, 2002). Furthermore, it can alter membrane integrity and permeability, and it affects enzyme activities, photosynthesis and respiratory processes (Chen et al., 2000; Yruela, 2005; Jouili et al., 2008). Moreover, excess Cu can affect the uptake of other nutrient elements (Ke et al., 2007; Puig et al., 2007). By consequence, nutrient imbalance can be a symptom of heavy metal toxicity in plants. A direct or indirect consequence of Cu toxicity is a reduction in the mitotic index, since the metal inhibits cell division and induces chromosomal aberrations (Liu et al., 2009; Souguir et al., 2008).

Previous studies on the effect of Cu on plants focused mostly on roots and mature leaves. These investigations revealed important information on Cu accumulation, Cu deficiency and on the protective role of Cu in plants. However, the effect of Cu stress on young and expanding tissues is poorly understood. It is known that Cu can inhibit cell division (Liu et al., 2009), but it is largely unknown how this effect is mediated. In order to find out

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whether the induction of oxidative stress is involved in this process, we determined the effect of Cu on the activities of antioxidant enzymes guaiacol peroxidase (GPX) and catalase (CAT), on $H_2O_2$ production, and on mineral element contents.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Bean seeds (*Phaseolus vulgaris* L. var. Belna) were disinfected with 1% sodium hypochlorite for 15 min, then washed thoroughly with distilled water and placed between wet paper towels at 25 °C in the dark for three days for germination. Seedlings were transferred to plastic beakers filled with 6 L of the nutrient solution (pH 5.7) containing $Ca(NO_3)_2$ (2.5 mM), $KNO_3$ (2 mM), $KH_2PO_4$ (1 mM), $MgSO_4$ (1 mM), Fe–K–EDTA (50 μM), $H_3BO_4$ (30 μM), $MnSO_4$ (10 μM), $ZnSO_4$ (1 μM), $CuSO_4$ (1 μM) and $(NH_4)_6Mo_7O_{24}$ (0.03 μM). Plants were grown in a growth chamber (16 h light–8 h dark) under mercury lamps, providing a light intensity of 150 μmol/m$^2$ pers, day/night temperature of 25/20 °C and 65 (±5)% relative humidity. After 14 days, treatments were performed by adding 50 or 75 μM $CuSO_4$ to the nutrient solution. Samples were analyzed after 72 h.

### 2.2. Estimation of total copper amount and element uptake

Dried leaf material was ground to powder and wet-digested in 65% nitric-perchloric acid (1 mL per 0.1 g of dry matter). The digested material was resuspended in distilled water and total concentrations of Cu, Mn, Fe, K, Ca and Zn were determined by atomic absorption spectrometry (Perkin Elmer-model 2380, C.R.G.R.).

### 2.3. $H_2O_2$ measurement

Hydrogen peroxide levels were determined according to Sergiev et al. (1997). Leaf tissues (500 mg) were homogenized on an ice bath with 5 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000*g* for 15 min and 0.5 mL of the supernatant was added to 0.5 mL potassium phosphate buffer (10 mM, pH 7.0) and 1 ml potassium iodide (KI) (1 M). The absorbance of the supernatant was measured at 390 nm, the content of $H_2O_2$ was obtained using a standard curve.

### 2.4. Lipid peroxidation

The level of lipid peroxidation was determined by the thiobarbituric acid (TBA) reaction. Fresh tissue was homogenized in 0.5% (w/v) TBA prepared in 30% (w/v) TCA (Alia et al., 1995). The homogenate was incubated at 95 °C in a water bath for 30 min and then cooled in an ice bath. After centrifugation at 10,000*g* for 10 min, the absorbance of the supernatant was measured at 532 nm and corrected by subtracting the non-specific absorbance at 600 nm. Malondialdehyde (MDA) concentration was calculated using an extinction coefficient of 155 mM$^{-1}$ cm$^{-1}$.

### 2.5. Enzyme extraction

Plant material was extracted in 50 mM K–Phosphate buffer (pH 7.0) containing 5 mM sodium ascorbate and 0.2 mM EDTA. The homogenate was centrifuged at 13,000*g* for 15 min. The resulting supernatant was considered as soluble enzymatic fraction. Extraction was performed at 4 °C. Protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard protein.

### 2.6. Antioxidant enzyme assays

Catalase activity was determined spectrophotometrically by monitoring the absorbance of changes caused by $H_2O_2$ reduction at 240 nm according to Aebi (1984).

Guaiacol peroxidase activity was determined following the increase in absorbance at 470 nm by adding the enzymatic preparation to 2 ml of 9 mM guaiacol and 10 mM hydrogen peroxide in 25 mM K–phosphate buffer (pH 7.0) (Fielding and Hall, 1978).

### 2.7. Statistical analysis

The results presented are obtained from at least six replicates. Statistical comparisons between treated and control plants were performed by analysis of variance (ANOVA). Subsequent pair-wise comparisons were performed with the HSD Tukey test using statistical software (Statsoft, France). Results are expressed as mean ± SE. $P < 0.05$ is taken as a significant probability.

## 3. Results

### 3.1. Growth inhibition and copper accumulation in expanding bean leaves under copper stress conditions

In order to characterise the toxic effects of excess copper on growth and development in expanding leaves, we exposed fourteen day-old bean seedlings to growth medium containing 50 or 75 μM $CuSO_4$ for 3 days. These concentrations were chosen, because they had been shown earlier to cause distinct, dose-dependent effects in this species on both root and shoot development (Bouazizi et al., 2008, in press, 2010). Our data show that expanding bean leaves of seedlings exposed to the ion exhibit chlorotic symptoms and necrosis at both copper sulfate concentrations, but more dramatically at the higher concentration (Fig. 1). This was accompanied by a significant reduction in dry weight (Fig. 2a). Due to the exposure, copper content in the leaves of treated plants was significantly higher than in the control, but this effect was not dose-dependent at the concentrations tested (Fig. 2b).

### 3.2. Alterations in nutrient element content

In order to investigate the effect of copper exposure on the uptake of other nutrients, we analyzed the nutrient composition of expanding leaves. Fe, Zn and K concentrations decreased significantly upon Cu exposure, but this effect was not dose-dependent at the concentrations tested (Table 1). The concentration of Ca decreased, but this effect was only significant at 50 μM $CuSO_4$. On the other hand, the abundance of Mn in leaves of treated plants was not significantly affected by copper exposure.

### 3.3. Effect of cupric stress on $H_2O_2$ and MDA content

Measurement of malondialdehyde (MDA) level is routinely used as an index of lipid peroxidation under stress conditions. The quantification of MDA content in leaves from plants exposed to copper revealed that the metal stress did not change this parameter significantly (Fig. 3a). Endogenous $H_2O_2$ on the other hand was more abundant in stressed leaves, but this effect was only significant at 75 μM $CuSO_4$ (Fig. 3b).

### 3.4. Response of antioxidants to Cu exposure

Plant responses to metal stress are often accompanied by a change in the activities of antioxidant enzymes. We quantified the

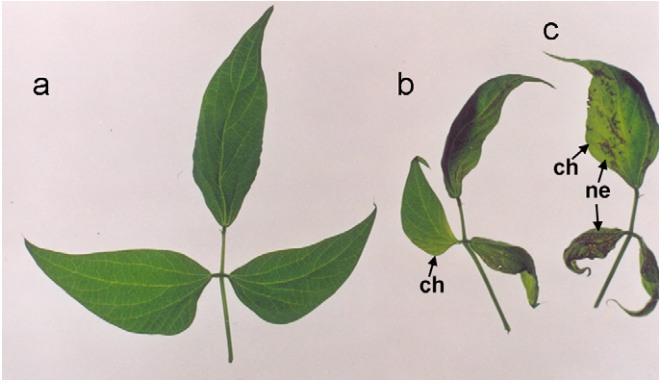


**Fig. 1.** Morphological effect of cupric stress on 14-day-old expanding bean leaves treated with 50 and 75 μM of $CuSO_4$ (b and c) during 3 days compared to control (a). ne: necrosis and ch: chlorosis.

activities of GPX and CAT to demonstrate whether this applies to expanding bean leaves. Neither of the enzymes were stimulated, however. At 75 μM $CuSO_4$ their activities were unchanged compared to the control values and at 50 μM $CuSO_4$, they were even reduced (Fig. 3c, d).

## 4. Discussion

The toxicity of Cu on plants has been documented in numerous studies (Luna et al., 1994; Aloui-Sossé et al., 2004; Yruela, 2005; Upadhyay and Panda, 2009), but most of these have focused on adult plants and tissues. Our data showed that exposure to copper had dramatic effect on dry matter production in expanding leaves. This phenomenon was accompanied by severe macroscopic symptoms such as chlorosis and necrosis. The effects on expanding bean leaves shown here were significantly more pronounced than those on mature leaves of the same plants (Bouazizi et al., 2010). This is consistent with the studies on other species (Sayar, 2005).

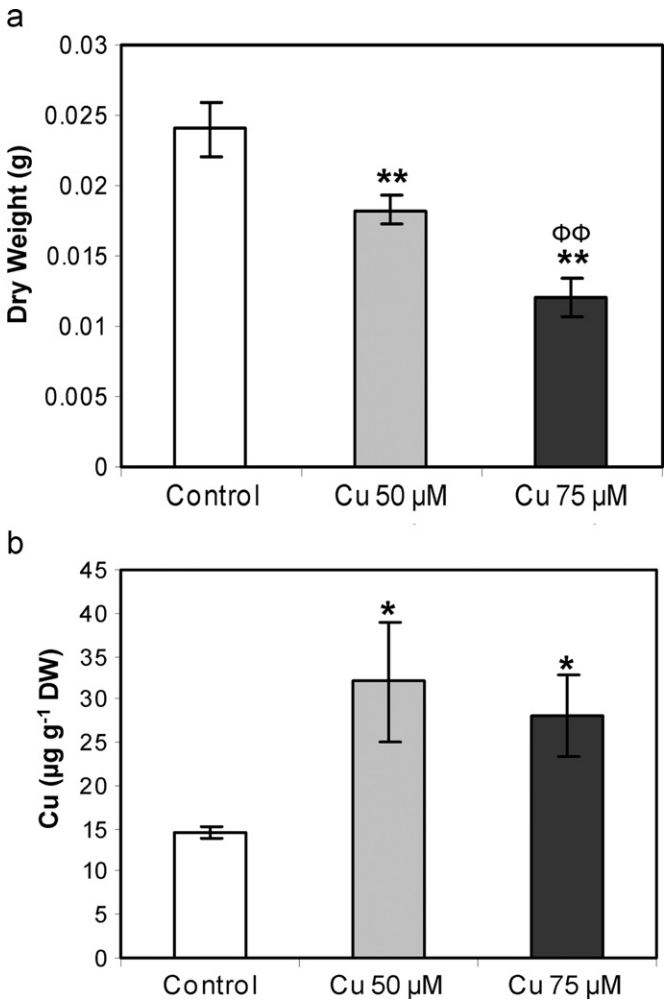


**Fig. 2.** Total copper content (a) and dry weight production (b) in expanding bean leaves exposed to 50 and 75 μM of $CuSO_4$ for 3 days. Data represent the mean ± SE of at least six independent assays. SE are indicated by vertical bars. $^{*}P < 0.05$ compared to control, $^{**}P < 0.01$ compared to control and $^{\Phi\Phi}P < 0.01$ compared to Cu50-treated plants.

Expanding organs have a different metabolism from that of mature organs, since they display intense mitotic activity. In some species, high Cu sensitivity of plant growth is related to disturbances of mitosis or to damage of cell membranes. Copper can lower the mitotic index, inhibit cell division and induce chromosomal aberrations (Liu et al., 2009; Souguir et al., 2008). The dramatic effects of copper on the morphology of expanding leaves can therefore be explained with the fact that cell division is much more active in these organs than in mature leaves.

The inhibition of growth in expanding leaves is clearly caused by the bioaccumulation of copper in these organs. However, interestingly, the concentration of copper accumulated in expanding leaves did not differ between the two administered concentrations tested here. By contrast, in root tissues of plants treated identically to the samples presented here, copper accumulates in dose-dependent manner. Furthermore, the overall accumulation of copper was significantly higher in the root tissues than in the young leaves tested here (Bouazizi et al., 2008). According to Yurekli and Porgali (2006), the higher accumulation of copper in roots results from a tolerance mechanism developed by the plant in order to reduce the effect of heavy metal stress. Some copper-tolerant plants prevent copper from reaching stems and leaves by keeping it in their roots.

It is unclear how the effects of copper toxicity on plant growth are mediated. The appearance of chlorotic symptoms accompanied by the higher accumulation of Cu in leaves suggests that chlorophyll degradation occurs. It was shown that heavy metals can induce changes in chlorophyll content associated with the modification in the chlorophyll a/b ratios (Mysliwa-Kurdziel et al., 2004; Dazy et al., 2008). These modifications seem to depend on the biological model and the nature of the metal stress. Chlorophyll a/b ratios decrease in tomato or bean exposed to Cd and increase in spinach, sugar beet and pea exposed to Cu (Mysliwa-Kurdziel et al., 2004). Copper induced chlorosis might be the result of the effect of the metal on chlorophyll synthesis through interference with enzymes such as ALA deshydratase, an enzyme implicated in the porphyric pathway (Scarponil and Perucci, 1984). Excess copper can also cause a decrease in carotenoids level (Luna et al., 1994). The effect of heavy metals on chlorophyll content would be expected to result in a reduction in photosynthetic activity. This phenomenon has been suggested to be one of the primary causes responsible for the reduction of growth (Upadhyay and Panda, 2009).

Alternatively, the inhibitory effect of copper on growth could be related to the disruption of nutrient element uptake in young leaves. (Rouphael et al., 2008). Chlorosis could be caused by a perturbation of the general mineral element homeostasis, in particular that of iron. Iron is a cofactor in one step of the

**Table 1**
Effect of copper treatment on mineral element content in expanding bean leaves.

| | **Fe** (mg $g^{-1}$ DW) | **Zn** (mg $g^{-1}$ DW) | **K** (mg $g^{-1}$ DW) | **Mn** (mg $g^{-1}$ DW) | **Ca** (mg $g^{-1}$ DW) |
|---|---|---|---|---|---|
| **Control** | 1.10 ± 0.23 | 1.62 ± 0.34 | 91.0 ± 1.8 | 0.243 ± 0.015 | 16.2 ± 1.6 |
| **50 μM** | 0.35 ± 0.09* | 0.65 ± 0.15* | 49.3 ± 2.8* | 0.267 ± 0.023 | 10.0 ± 1.8* |
| **75 μM** | 0.45 ± 0.08* | 0.74 ± 0.17* | 63.9 ± 5.4* | 0.203 ± 0.071 | 15.6 ± 1.1 |

Data represent the mean ± SE of at least six independent assays.

* $p < 0.05$, compared to control.

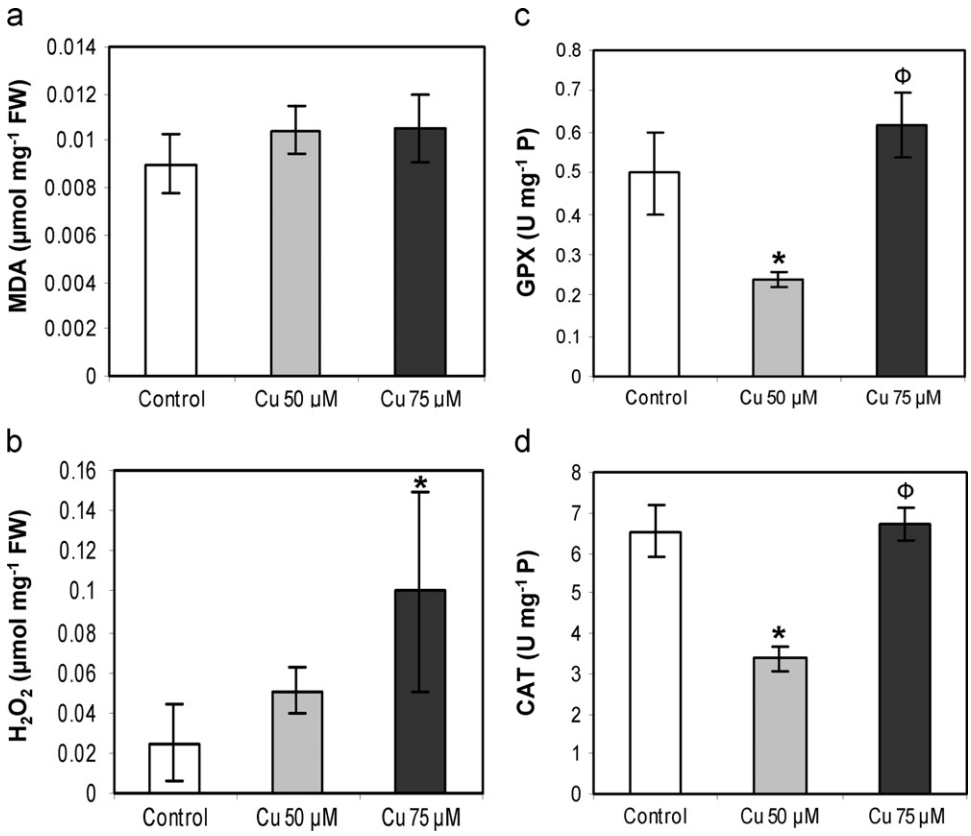


**Fig. 3.** MDA accumulation (a), $H_2O_2$ level (b), activities of guaiacol peroxidases (c) and catalase (d) in expanding bean leaves exposed to 50 and 75 µM of $CuSO_4$ for 3 days. Data represent the mean ± SE of at least six independent assays. SE are indicated by vertical. $^{*}P < 0.05$ compared to control and $^{\Phi}P < 0.05$ compared to Cu50-treated plants.

chlorophyll synthesis pathway and copper has an inhibitory effect on the absorption and translocation of iron (Ke et al., 2007). Copper induced chlorosis could thus be mediated by its effect on iron mobility within the plant. Consistent with this hypothesis, our results show a reduction in iron, potassium and zinc ion contents in expanding leaves from seedlings exposed to copper.

While the observed reduction in potassium content could be a secondary effect mediated by the decrease in iron content (Hall, 2002), the reduction in zinc content on the other hand may be explained by the fact that the $Zn^{2+}$ and $Cu^{2+}$ ions are transported by the same type of membrane transporters. A saturation of these transporters by excess copper could result in decreased absorption of zinc (Luo and Rimmer, 1995) or its reduced transport (Reichman, 2002). Since zinc plays an important role in the biosynthesis of several growth hormones, its reduced availability could be partly responsible for the observed reductions in growth and dry weight production (Cakmak et al., 1989).

The response of mature plant tissues to excess copper includes metabolic changes that are indicative of the activation of antioxidative defense mechanisms. Oxidative stress occurs as a result of the formation of reactive oxygen species such as superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide. These radicals cause lipid peroxidation and increased levels of malondialdehyde in plant cells, and they typically entail a stimulation of enzymatic antioxidant systems. The antioxidant protection in plant cells is complex and highly compartmentalized. Superoxide radicals ($O_2^{\bullet -}$) are dismutated to $H_2O_2$ by superoxide dismutase (SOD) which has at least three different isoforms, CuZn–SOD, Mn–SOD and Fe–SOD. $H_2O_2$ is decomposed in peroxisomes by catalases, and the ascorbate (ASC)–glutathione (GSH) cycle is localized in different cell compartments (Jiménez et al., 1997). This cycle comprises of the enzymes ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), as well as ascorbate (ASC) and reduced glutathione (GSH) (Cobbett and Goldsbrough, 2002).

In previous studies performed under the same conditions as those used here, root and mature leaf tissues of *Phaseolus vulgaris* L. plants exposed to copper showed dramatically increased levels of hydrogen peroxide and MDA (Bouazizi et al., 2008, 2010). Peroxidase activities in these tissues were significantly stimulated. By contrast, in the present study, expanding leaves of stressed plants do not seem to display oxidative stress. The increase in the amount of MDA was not significant, and a significant increase in $H_2O_2$ levels occurred only at higher copper concentrations. The activity of GPX and CAT decreased in the case of 50 µM-treatments and remained identical to the control in the case of 75 µM-treatments. The decrease in the activity of GPX and CAT by 50 µM $CuSO_4$ can be explained by a lack of effect on $H_2O_2$ levels at this concentration. In 75 µM-treatments we have noted an increase in the level of endogenous $H_2O_2$ in comparison with the control plants. However, this increase was not accompanied by the stimulation in the activity of GPX and CAT which remained unchanged compared to the control. We, therefore, suspect that the detoxification of $H_2O_2$ in plants treated with 75 µM $CuSO_4$ may be performed by other antioxidant enzymes whose activities were not measured in this study. Previous studies showed that ROS could be eliminated through the SOD–CAT pathway or by the ascorbate–glutathione cycle which are considered to be ROS-scavenging systems (Dazy et al., 2009). This idea of a "two-way defense system" was proposed by Mittler (2002), who supported his hypothesis by the differences in affinities for $H_2O_2$ between APX and CAT.

The overproduction of $H_2O_2$ in leaves treated with 75 µM $CuSO_4$ can explain the differences in dry weight observed in

expanding leaves. Overproduction of $H_2O_2$ is known to decrease cell wall extensibility due to the formation of cross-links between cell wall polymers (Lin et al., 2005). Also, Maksymiec and Kupa (2007) showed that the increased $H_2O_2$ accumulation may result from jasmonate (JA) activity indicating a close relationship between these two signaling molecules. Stimulation of the jasmonate pathway has been shown to reduce the growth mediated by increases in ethylene concentration through stimulation of the activity of ACC synthase and oxidase (Kruzmane et al., 2002). The high level of ethylene can increases senescence and growth inhibition.

## 5. Conclusions

Taken together, these data show that in expanding leaves, copper toxicity does not act through oxidative stress, but that an imbalance in the nutrient homeostasis seems to be involved in causing the dramatic morphological changes resulting in inhibition of photosynthetic activity and reduction of growth.

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