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Phytochelatin as a Biomarker for Cu-toxicity in Soybean Callus Cultures

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Abstract: Soybean (*Glycine max* L.) Cell suspension cultures were exposed to excess copper, in liquid MS-medium. Viability, growth rate and phytochelatin concentration were monitored in treated and untreated cultures. The viability of Cu-treated cultures as reduced and more affected with the time of Cu-exposure, while the O₂-uptake was slightly raised in treated cultures and decreased with the time of Cu-exposure. The growth rate was more affected at 10 and 15 days of treatment and reduced by about 76% and 83% below that of the control culture, respectively. The production of PCs in treated cultures markedly increased with the time of Cu-exposure. The glutathione depletion is coincident with PCs production. A marked increase in the level of GSSG in treated and untreated cultures with the time of Cu-exposure. The accumulation of Cu in treated cultures was raised as the time of exposure increased. The accumulation of copper is coincident with PCs levels. Phytochelatin detection seem to be an important biomarker technique in systems contaminated with heavy metals.

Key words: Soybeans, Copper, Glutathione, Phytochelatin, Tissue culture

Introduction

Contamination of soils with heavy metals becomes more and more a problem in many countries all over the world. In areas where metal contaminated soils are used for food crop production, metals relatively mobile within the plant, can easily come into the food chain with great risks for human health. The bioavailability of heavy metals in soils varies with soils and plant characteristics, e.g., mineralogical and organic matter properties and pH (De Haan *et al.*, 1990), though the metals can also interact mutually. A clear example is the strong Zn-Cd interaction in soils with increasing Zn levels, the availability of Cd in the soil and its uptake by plants are generally inhibited and vice versa (Abdel-Sabour *et al.* 1988; El-Enany, 1995).

Copper is required by biological systems as a structural and catalytic component of proteins and enzymes and as cofactor (Clijsters and van Assche, 1985). However, excessive concentrations of copper are highly toxic (Woolhouse, 1983; Verkleij and Schat, 1990). Copper in the soil operates as a stress factor that it caused physiological constraints that decrease the vigor of the plants and even totally inhibit plant growth (Ouzounidou *et al.*, 1994). Glutathione (GSH) identified as a precursor for the formation of PCs in plants (Scheller *et al.*, 1987; Steinkamp and Rennenberg, 1985; Schaefer *et al.*, 1997). Treatment of plant cells with buthionine sulfoximine, an inhibitor of γ -Glu-Cys synthetase, reduces the synthesis of PCs and increases sensitivity to metal (Steffens *et al.*, 1986; Reese and Wagner, 1987; Scheller *et al.*, 1987; Salt *et al.*, 1989; Gupta *et al.*, 1998; Gussarsson *et al.*, 1996).

Plants grown under heavy metals stress had increased the levels of sulfhydryl-rich peptides (Phytochelatin), non-protein thiols with the general structure (γ -Glu-cys)_n Gly, n varying between 2 and 11 (Grill *et al.*, 1987; Scheller *et al.*, 1987; Steffens, 1990; De Knecht, 1994; Chen *et al.*, 1997). Phytochelatin induction is observed only in plants, algae, yeast and fungi grown under conditions of excess heavy metals and have been described as sequester and detoxifiers of heavy metal ions (Zenk, 1996; Skowronski *et al.*, 1998). The phytochelatin compounds were able to detoxify metals inside the cytoplasm of plants cells by in situ chelation (Becher and Hofner, 1994; Stroinski and Zielezinska, 1997; Gupta *et al.*, 1998; Maserti *et al.*, 1998). Phytochelatin also, served as metal carriers during tonoplast transport (Voegel-Lange and Wagner, 1990). Consequently, there is a direct relationship between the PCs levels in plants and elevated metal tolerance (Salt *et al.*, 1989; Min *et al.*, 1997).

The objective of the present work was to study the effect of excess copper on the viability, respiration, growth rate and the

usefulness of phytochelatin as a biomarker for copper toxicity in soybean callus cultures.

Materials and Methods

Soybean (*Glycine max* L. Clark) suspension cell culture were cultured in aseptic conditions in 250 mL conical flasks, containing 50 ml of MS-medium, supplemented with 0.5 mg/L naphthalene acetic acid (NAA), 105 mg/L benzyladenine (BA), 170 mg/L NaH₂PO₄ · H₂O, 100 mg/L inositol, 0.1 mg/L thiamine-HCl, 0.5 mg/L nicotinic acid, 30 g/L sucrose. Half the MS-medium received no additional Cu (control), where the total concentration in the other cultures was adjusted to 5 ppm Cu (as Cu SO₄). Treatments were given in three replicates. The pH was adjusted to 5.5 ± 0.2 before autoclaving. Cultures were kept on a rotatory shaker at 1000 rpm in an illuminated incubator at temperature 27°C with a light intensity of 376.8 μ mol m⁻² S⁻¹ provided by fluorescent tube a 16-day/8- night hour photoperiod.

The cells were collected at 2, 5, 15 and 21 days after culture inoculation by vacuum and weighted to determine the fresh weight of each culture.

Viability of cultures: About 200 mg of cells were incubated in five fraction (20-30 mg) in MS-medium containing Cu with 0.8% TTC as 2:1 mixture in 50 mM Na-phosphate buffer (pH = 7.4). After 20 hours, the cells were washed with distilled water, the red color was extracted for 1 h at 70°C with 95% heated ethanol. After cooling down, the solution was filtered and measured at 485 nm against 95% methanol as a reference (Towill and Mazur, 1975).

Respiration rate: Respiration rate were measured as μ MO₂/g fresh weight/min. using a Clark electrode (Type E 1 6WTW, GmbH, Weilheim) in a 3 ml containing temperature constant glass acrylic cuvette, in which the suspension was agitated by a magnetic stirrer.

Extraction and assays of non-protein thiols: Non-protein thiols were extracted as De Vos *et al.* (1992) by grinding 1g callus fresh weight in 1 ml 5% (W/V) sulfosalicylic acid plus 6.3 mM EDTA at 4°C in a mortar. The homogenate was centrifuged at 10.8 xg for 15 min at 4°C. The clear supernatants were collected and immediately used for the determination of thiols. The level of total-SH was determined with Ellman's reagent (Ellman, 1959). One-hundred microliters of supernatant was mixed with 0.7 mL of 0.5 M K₂PO₄ (pH = 7.5) and 0.1 ml of 10 mM 5,5-dithiobis(2-

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nitrobenzoic) acid. The absorbency was measured spectrophotometrically after 5 min., at wavelength 412 nm against a blank with 100 μ l H₂O. GSH and GSSG were assayed by the GSSG-recycling method (Anderson, 1985). The final concentration of GSH reductase was 0.5 U/ml in the assay medium of total glutathione (GSH + GSSG) and 1 U/ml in the assay of GSSG. GSSG was used as a standard. Phytochelatin concentrations in supernatant were determined indirectly by subtracting, the amount of total glutathione (GSH + GSSG) from the amount of total-SH. Phytochelatin content was expressed as PC-SH in μ mol/g f.wt. (De Vos *et al.*, 1992).

Metal measurements: Copper content was determined by atomic absorption spectrophotometry, after digest the cells of soybean suspension culture by the method adapted by Vymazal (1984) using acid mixture (36% HCl:20% HClO₄: H₂O = 2:1:1 vlv).

Results and Discussion

The viability of soybean cultures treated with toxic level of Cu (5 ppm) and untreated culture (control) was shown in (Fig. 1a). Viability was more affected with the time of Cu-exposure. After 5, 7 days of treatment, the viability of cultures was reduced after 2 and 5 days by about 21.0 and 63% of control, respectively. It was found that the viability depend on the concentration of the metal ion in the culture medium as well as on the period of exposure (El-Enany, 1992). Domazlicka and Opatrny (1989) found that 100 μ M Cd resulted in a steep decrease in cell viability of tobacco cells. Jackson *et al.* (1984) reported that metals inhibited cell division and DNA biosynthesis.

The rate of O₂-uptake (dark respiration) was slightly raised in treated cultures (Fig. 1b). A severe inhibition of O₂-uptake in treated culture was observed at 15 and 21 days of growth in treated and untreated culture. These results are in agreement with many investigators. The fresh weight of soybean cell suspension culture was shown in (Fig. 1c). The data revealed that Cu reduced the growth rate of the soybean cell suspension, growth was more affected at 2 and 5 days and reduced by about 76.9% and 83% below that of control, respectively. While the growth rate after 10 and 15 days were reduced by about 59, 52% and 55% of control, respectively.

Singh *et al.* (1989) reported that the addition of Ni, Hg and Cu inhibited the O₂ uptake in the cells of *Cylindrospermum*. In this respect Issa *et al.* (1995), reported that the response of respiration was quite similar to that of photosynthesis and was enhanced proportionally to metal toxicity on growth, i.e., enhanced respiration with retarded growth. Bittel *et al.* (1974) and Lee *et al.* (1976) concluded that heavy metals inhibit phosphorylation mechanisms and interfered with SH groups of respiratory enzymes.

The effect of the toxic level of Cu (5 ppm) on the levels of acid soluble SH compounds (total SH) was studied in a 21-day experiment (Fig. 2a). Copper treatment resulted in a marked increase of total-SH level as the time of Cu-exposure increased. The level of total SH increased by about 58% of control during the time of Cu- treatment. While, in control cultures the levels of total-SH varied during the subsequent of growth.

In contrast reduced glutathione (Fig. 2b) show a marked depletion with the time of Cu-exposure. Although the GSH was increased in control culture especially after 5 days of treatment. The GSH depletion is coincident with the increase in PC-SH synthesis (Fig. 3a) especially at 15-days of Cu-treatment. PCs synthesis was lowered at 21-day of Cu-treated cultures. In control cultures the level of PCs was lowered at 15 and 21 day of growth. The oxidized glutathione (GSSG) level was also followed (Fig. 4b).

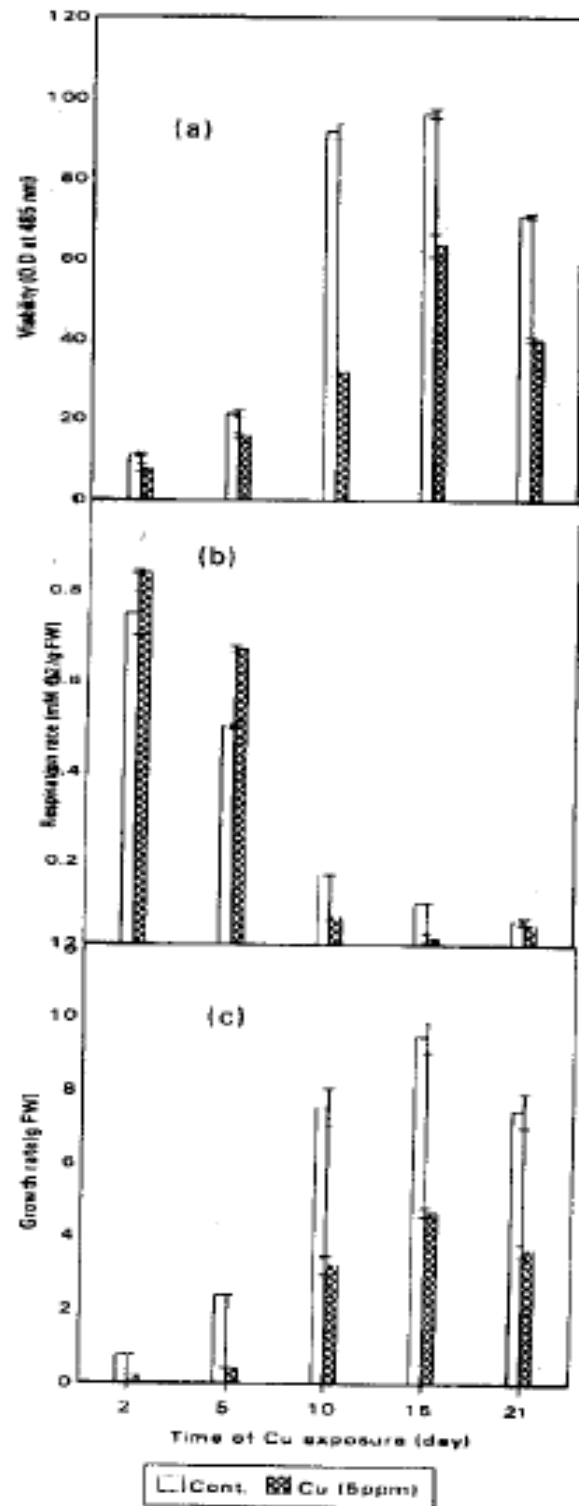


Fig. 1: Viability, respiration and growth rate of soybean suspension cultures exposed to 0 and 5 ppm Cu for 2, 5, 15 and 21 days. Values are means with SD (n = 3)

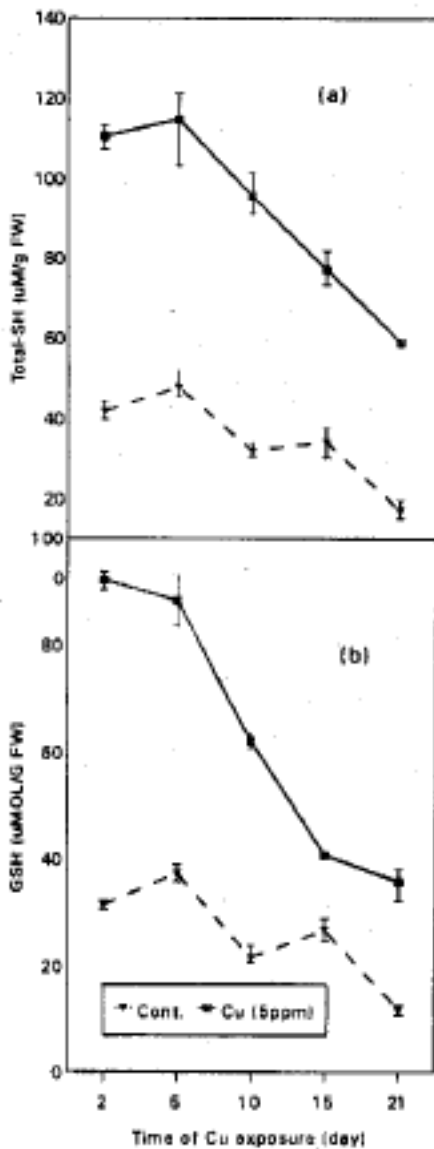


Fig. 2: Total -SH and GSH in soybean suspension cultures exposed to 0 and 5 ppm Cu for 2, 5, 10, 15 and 21 days. Values are means with SD = in = 3)

GSSG contents of suspension cultures of soybean were increased in treated and untreated cultures, The accumulation of GSSG was obviously raised at 21-day of growth.

Glutathione is the most important non-protein thiol present in plants. It is obvious that GSH plays a central role in the removal of toxins generated during cellular metabolism (Halliwell, 1978). In plant cells the glutathione is present predominantly in reduced form and its function are connected with thiol group, reactivity of which has important reductive properties. It is generally assumed that GSH maintain cysteine, homocysteine and proteins in biologically active form.

Our results indicate that GSH depletion is coincident with the increase in PCs levels. This is in agreement with many investigators (Scheller *et al.*, 1987; Grill *et al.*, 1988; Mendum *et al.*, 1990). GSH has also been identified as a precursor for the formation of PCs in plants. Treatment of plant

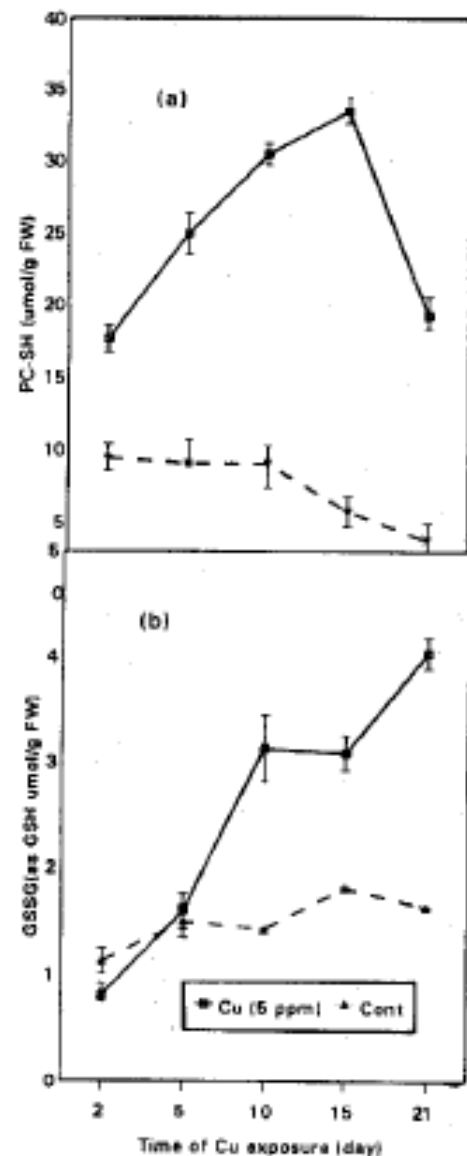


Fig. 3: Phytochelatins (a) and GSSG (b), in soybean suspension cultures exposed to 0 and 5 ppm Cu for 2, 5, 10, 15 and 21 days. Values are means with SD = (n = 3)

cells with buthionine sulfoximine, an inhibitor of γ -GluCys synthetase, reduces the synthesis of PCs and increases sensitivity to Cd (Steffens *et al.*, 1986; Reese and Wagner, 1987; Scheller *et al.*, 1987). Chen *et al.* (1997) found that PC synthase, isolated from tomato cell culture, requires GSH or PCs as a substrates but can not utilize γ -GluCys or GSSG. They also 'Concluded that PCs synthase is activated *in vitro* and *in vivo* by heavy metals.

In a number of previous studies of PC-SH levels has been assessed by subtracting the GSH concentration from the total acid soluble concentrations (Scheller *et al.*, 1987; De Vos *et al.*, 1992). Our results indicate that PCs production increased with the time of Cu-exposure. This result suggest that copper tolerance in the cells is dependent on an elevated production of PCs. In this respect (Grill *et al.*, 1987; Salt *et al.*, 1989; Mendum *et al.*, 1990) observed that BSO increased the sensitivity of tolerant plant to copper. They also concluded that PCs play a crucial role in copper tolerance.

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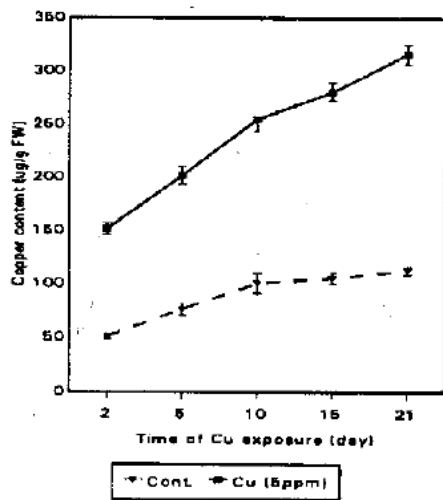


Fig. 4a: Copper accumulation in soybean callus cultures exposed to (0 and 5 ppm Cu) for 21-days. Values are means with SD (n = 3)

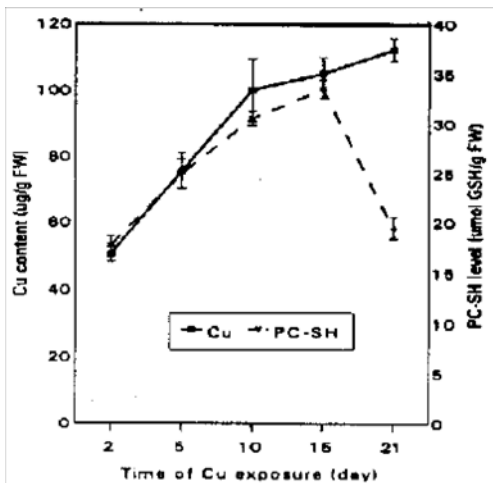


Fig. 4b: The relationship between PC-SH levels and copper accumulation in soybean callus cultures exposed to (5 ppm) Cu for 21-days. Values are means with SD (n=3)

Copper in PCs is bound as Cu-thiolate complex depend on the chain length of PC-SH (Reese *et al.*, 1988; Mehra and Winge, 1988). The decline of PC-SH in the cell soybean cultures after 15-day of Cu-treatment may be due to oxidation of. GSH. Phytochelatinas released Cu ions, which may result in an increased toxicity. Chen *et al.*, (1997) found that the level of PCs synthase activity was regulated during the cell culture cycle, with the highest activity occurring 3-days after subculture. Our results in Fig. 3b show a marked increase in the level of oxidized glutathione (GSSG) with the time of culture growth in treated and untreated cultures. The GSH/GSSG status is sensitive to the Redox State of a cell. The increase of GSSG content of cells indicates an increased of oxidation of GSH *in vivo*. Copper is known to catalyze not only the oxidation of GSH, but also that of other cellular thiols (Miller *et al.*, 1990; Salhany *et al.*, 1978). De Vos *et al.* (1992) found that, copper capable of catalyzing the oxidation of PC-SH group, as well, which explain the depletion in the level of PCs with the time of Cu-exposure. Stroinski and Zielezinska (1997) found that oxidative stress produced by Cd-stress in potato tuber, activated GSH synthesis and thus suggest a possibility of redox type regulation.

Figure 4a show the accumulation of Cu in treated cultures. Copper was raised in the cells as the time of growth increased. There is a relation between the internal PC-SH and metal accumulation. This may be the result of a continuous Cu accumulation in storage compartment e.g. (vacuoles) as postulated by Rauser and Ackerley (1987), Vasquez *et al.* (1992), Voegel-Lange and Wagner (1990). The actual metal concentration in the cytosol rather than the total metal concentration will determine the actual PC-SH level in plant tissue (Keltjens and van Beusichem, 1998). Organisms grow on environment polluted with heavy metal are often not stressed to the extent of severe growth reduction. PCs are induced in detectable levels, therefore of PCs consider as a good biomarker technique for systems contaminated with heavy metals.

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