

## Effect of cadmium on soluble sugars and enzymes of their metabolism in rice

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### Abstract

The effect of cadmium on the content of starch and sugars, and changes in the activities of the enzymes of sugar metabolism were studied in growing seedlings of rice (*Oryza sativa* L.) cultivars Ratna and Jaya. During a 5- to 20-d exposure at 100  $\mu$ M or 500  $\mu$ M Cd(NO<sub>3</sub>)<sub>2</sub> in the growth medium an increase in the content of total soluble sugars and reducing sugars, and decrease in the content of non-reducing sugars was observed. Cd-induced increase in the sugar content was greater in shoots than in roots. No definite pattern of changes in starch content or in  $\alpha$ -amylase activity was observed. Presence of 100 or 500  $\mu$ M Cd(NO<sub>3</sub>)<sub>2</sub> increased the activities of sucrose degrading enzymes, acid invertase and sucrose synthase, whereas the activity of sucrose phosphate synthase declined.

*Additional key words:*  $\alpha$ -amylase, acid invertase, cadmium toxicity, *Oryza sativa*, sucrose phosphate synthase, sucrose synthase.

### Introduction

Cadmium is one of the heavy metal pollutants and its concentration in soil environment is increasing with time, because its addition becomes greater than its removal through leakage and plant harvesting (Naidu *et al.* 1994, Shah and Dubey 1998a). It is readily absorbed by plants and is retained mainly in roots and up to some extent it is also translocated to the shoots and even into the seeds (*e.g.* Chardonens *et al.* 1998). Cd inhibits plant growth, induces synthesis of specific polypeptides (Shah and Dubey 1995), leads to accumulation of certain metabolites (Alas *et al.* 1996, Shah and Dubey 1998b) and alters the behaviour of many key enzymes of various metabolic pathways (Vallee and Ulmer 1972, Shah and Dubey 1995, 1998c).

Photosynthesis and the rate of import of photosynthates into individual sink organs are important components of crop productivity. Photosynthetically fixed carbon is ultimately converted into two main carbohydrates, sucrose and starch in the photosynthetic cells. During active periods of photosynthesis, starch is formed as a temporary storage form of fixed carbon and is deposited as starch granules in the chloroplasts while the sucrose is transported to different organs and is the most commonly used photoassimilate by plants.

The enzyme  $\alpha$ -amylase hydrolyzes starch producing D-glucose and oligosaccharide units. The enzyme liberates D-glucose units from reducing ends of amylaceous polymers (Zwar *et al.* 1995). Sucrose phosphate synthase (SPS) catalyzes the synthesis of sucrose in photosynthetic as well as in nonphotosynthetic plant tissues (Geigenberger and Stitt 1993) and is an important control point in the biosynthesis of sucrose. The enzyme is highly active in source leaves (Huber *et al.* 1996).

Cleavage of sucrose is catalyzed by sucrose synthase, a cytosolic enzyme or by invertase which could be localized in cell wall, vacuole or cytosol (Ranwala and Miller 1998). Sucrose synthase catalyzes reversible reaction and is thought to play a primary role in sucrose breakdown *in vivo* (Geigenberger and Stitt 1993). It plays important role in energy metabolism by metabolising sucrose into diverse pathways relating to metabolic, structural and storage function of plant cell (Ranwala and Miller 1998). Invertase hydrolyzes sucrose to glucose and fructose. Invertases are a group of ubiquitous enzymes with different pH optima and subcellular localization. Acid invertase is located either in vacuoles or bound to cell walls (Obenland *et al.* 1993). Enzyme activity

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*Abbreviations:* BSA - bovine serum albumin, EDTA - ethylenediamine tetraacetic acid, PMSF - phenyl methyl sulfonyl fluoride, SPS - sucrose phosphate synthase, SS - sucrose synthase.

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is mainly found in immature plant organs and rapidly declines in mature organs (Iwatsubo *et al.* 1992). In several instances, direct correlation between acid invertase activity and hexose contents has been observed (Zhou *et al.* 1994, Zrenner *et al.* 1995).

Various stressful conditions of the environment adversely affect metabolism of sugars and partitioning of photosynthates in growing plants (Dubey 1997, Foyer *et al.* 1998). Contents of soluble sugars increase under salinity (Dubey 1997), water-stress (Foyer *et al.* 1998), and chilling (Guy *et al.* 1992, Hurrey *et al.* 1995). Sugar

accumulation possibly contributes for regulation of internal osmolarity and protection to the biomolecules and membranes (Hayashi *et al.* 1997, Sinniah *et al.* 1998).

The present study was undertaken to examine the quantitative changes in the content of starch and sugars and the activity of enzymes of their metabolism  $\alpha$ -amylase, sucrose-phosphate synthase, sucrose synthase and acid invertase in different parts of rice seedlings growing under increasing concentrations of cadmium in the growth medium.

## Materials and methods

**Plants:** Seeds of two rice (*Oryza sativa* L.) cultivars Ratna and Jaya were surface sterilized with 1 % sodium hypochlorite solution and then imbibed in water for 24 h at  $28 \pm 1$  °C. Seedlings were raised in plastic pots in sand saturated with Hoagland nutrient solution (Hoagland and Arnon 1938) which served as control, and nutrient solution supplemented with 100  $\mu$ M and 500  $\mu$ M  $\text{Cd}(\text{NO}_3)_2$  which served as treatment solutions. Seedlings were raised in a growth chamber at  $28 \pm 1$  °C, 80 % relative humidity and 12-h light/dark cycle (irradiance of 40 - 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Pots were maintained at field saturation capacity by irrigation with double distilled water when required. Seedlings were uprooted at 5-d intervals and experiments were performed in triplicate.

**Starch and sugar estimation:** Root/shoot samples were oven dried at 70 °C for 24 h. About 100 mg dried samples were homogenized in 10  $\text{cm}^3$  of 80 % ethanol and placed in a water bath at 80 °C for 30 min. Contents were centrifuged at 22 000 g for 20 min. Samples were thrice extracted, supernatants were pooled together and their volumes reduced to 3  $\text{cm}^3$  by evaporation and then diluted up to 25  $\text{cm}^3$  with water. Sugars were estimated in ethanolic extracts. For starch extraction, residues left in the centrifuge tubes after sugar extraction were oven dried at 80 °C for 24 h. After addition of 2  $\text{cm}^3$  of water, tubes were placed in boiling water bath for 15 min and after cooling 2  $\text{cm}^3$  of 9.2 M perchloric acid (PCA) was added. Contents were stirred for 15 min and volumes were made up to 10  $\text{cm}^3$  with water. Supernatants were collected after centrifuging the contents at 3 000 g for 20 min. Residues were reextracted twice with 2  $\text{cm}^3$  of 4.6 M PCA. After centrifugation supernatants were combined, volumes were made to 50  $\text{cm}^3$  with water. Total sugars and starch were estimated colorimetrically using phenol-sulphuric acid method of Dubois *et al.* (1956) and reducing sugars by Nelson-Somogyi method as described by Oser (1971). Amount of non-reducing sugars was calculated by subtracting the values of reducing sugars from total sugars.

**$\alpha$ -Amylase assay:**  $\alpha$ -amylase (EC 3.2.1.1) was assayed following the method of Shain and Mayer (1968). Fresh samples (100 mg) were homogenized with chilled mortar and pestle in 0.1 M sodium acetate buffer (pH 4.8) containing 5  $\mu$ M cysteine. Homogenate was centrifuged at 22 000 g and 4 °C for 15 min and dialyzed against the extraction buffer at 4 °C for 4 h with 3 changes of the buffer. Dialyzed extract was heated at 70 °C for 5 min in presence of 3 mM  $\text{CaCl}_2$ , which destroyed the activity of  $\beta$ -amylase. For assay of  $\alpha$ -amylase, reaction mixture in a total volume of 4  $\text{cm}^3$  contained 100 mM sodium acetate buffer (pH 4.7), 1  $\text{cm}^3$  of 1 % soluble starch in 0.15 M NaCl and enzyme extract. After incubation at 30 °C for 30 min, reaction was terminated with the addition of 1  $\text{cm}^3$  of 6 M HCl. One  $\text{cm}^3$  of aliquots were transferred to 25  $\text{cm}^3$  volumetric flasks, 15  $\text{cm}^3$  of distilled water was added followed by addition of 0.5  $\text{cm}^3$  of IKI solution (0.2 %  $\text{I}_2$  in 2 % KI). Volume was finally made up to 25  $\text{cm}^3$  with distilled water and absorbance was read at 660 nm.

**Acid invertase assay:** Acid invertase (EC 3.2.1.26) was extracted and assayed following the method of Borkowska and Szczerba (1991). Fresh samples (100 mg) were homogenized using chilled mortar and pestle in 5  $\text{cm}^3$  of 10 mM sodium acetate buffer (pH 4.6) containing 3.3 mM  $\text{MgCl}_2$ , 1 mM EDTA and 1 mM PMSF. The homogenate was centrifuged at 22 000 g and 4 °C for 10 min. The supernatant was dialyzed with 3 - 4 changes of extraction buffer in cold for 4 h. In the dialyzed extract invertase activity was assayed. Reaction mixture in a total volume of 1  $\text{cm}^3$  contained 10 mM sodium acetate buffer (pH 4.6), 0.4 M sucrose and enzyme extract. After incubation at 30 °C for 30 min, reaction was terminated with the addition of 0.5  $\text{cm}^3$  of 0.5 M  $\text{Na}_2\text{HPO}_4$ . The resulting reducing sugars were estimated by the Nelson-Somogyi method (Oser 1971).

**Assay of sucrose phosphate synthase and sucrose synthase:** Sucrose phosphate synthase (SPS, EC 2.4.1.14) and sucrose synthase (SS, EC 2.4.1.13) were

extracted following the method of Hubbard *et al.* (1989). About 100 mg of fresh root/shoot samples were washed with water and extracted in 5 cm<sup>3</sup> of 50 mM HEPES-NaOH buffer (pH 7.5) containing 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 2.5 mM DTT and 0.05 % (v/v) Triton X-100, at 4 °C. After centrifugation at 22 000 g and 4 °C for 10 min, SPS and SS were assayed in the supernatant according to the method of Miron and Schaffer (1991). Assay mixture for SPS in a total volume of 0.07 cm<sup>3</sup> contained 50 mM HEPES-NaOH buffer (pH 7.5), 15 mM MgCl<sub>2</sub>, 25 mM fructose-6-phosphate, 25 mM glucose-6-phosphate, 25 mM UDP-glucose and 0.04 cm<sup>3</sup> enzyme extract. After incubation at 30 °C, for 30 min, reaction was terminated with the addition of 0.07 cm<sup>3</sup> of 30 % KOH. Controls were terminated at 0 min. The reaction mixture for SS assay was similar to SPS but it contained

25 mM fructose instead of fructose-6-phosphate and was devoid of glucose-6-phosphate. The sucrose formed during SPS and SS catalyzed reactions was estimated following the method of Vassey *et al.* (1991). Hexoses were destroyed by placing the reaction tubes in boiling water for 10 min. After cooling, 1 cm<sup>3</sup> of anthrone reagent (prepared by mixing 76 cm<sup>3</sup> of concentrated H<sub>2</sub>SO<sub>4</sub>, 36 cm<sup>3</sup> of water and 150 mg of anthrone) was added to each tube. Contents were incubated at 38 °C for 20 min and absorbance was recorded at 620 nm in a Bausch and Lomb (USA) spectrophotometer. Sucrose served as standard.

**Protein estimation:** In all enzyme preparations protein was estimated by method of Lowry *et al.* (1951) using BSA as standard.

## Results

In 10- and 20-d-old grown seedlings of cv. Ratna, an increase in starch content both in roots and shoots with

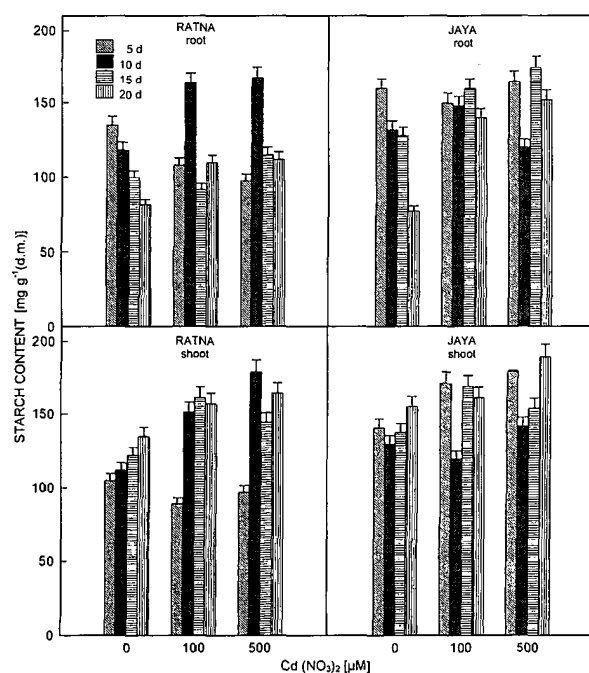


Fig. 1. Starch content in shoots and roots of rice cvs. Ratna and Jaya at 5, 10, 15, and 20 d of growth under 0 (control), 100, and 500 μM Cd(NO<sub>3</sub>)<sub>2</sub>. Means of three independent determinations and bars indicate standard deviations.

increase in Cd concentration was noted, however, this trend was missing in seedlings at 5- or 15-d old (Fig. 1). Similarly, in 5- or 15-d old seedlings of cv. Jaya, an increase in starch content was observed under Cd-treatments, however, no such trend was noticed in shoots of 10- or 20-d-old seedlings and in roots of 10-d-old

seedlings.

With increasing concentrations of Cd(NO<sub>3</sub>)<sub>2</sub> in the growth medium a steady increase in the content of total as well as reducing sugars was observed in roots as well as in shoots, whereas the content of non-reducing sugars declined (Figs. 2, 3). Cadmium induced increase in the content of sugars was greater in shoots compared to roots in seedlings of both the rice cultivars.

The activity of α-amylase declined steadily in control seedlings of cv. Jaya during 5 to 20 d growth period but not in cv. Ratna (Fig. 4). In cv. Jaya both in roots and shoots, 500 μM Cd treatment led to a significant inhibition of enzyme activity whereas in cv. Ratna no definite pattern of change could be noticed.

The acid invertase activity increased during 5 to 15 d of growth and thereafter it declined or remained at almost similar level by day 20 and a higher enzyme activity was noticed in cv. Ratna compared to cv. Jaya (Fig. 5). Acid invertase showed a significant increase in activity, with increasing concentration of Cd(NO<sub>3</sub>)<sub>2</sub> and this increase was more pronounced in shoots than in roots.

Due to increasing concentration of Cd treatment sucrose phosphate synthase (SPS) activity decreased in both roots and shoots of the two rice cvs. Ratna and Jaya except in shoots of cv. Ratna at day 10 of growth, where at 100 μM Cd an increase in SPS activity was observed (Fig. 6). Inhibition of SPS activity due to Cd was higher in roots than in shoots.

The activity of sucrose synthase (SS) increased steadily in shoots during a 5 to 20 d growth period, however in roots almost similar level of SS activity was noted throughout the growth period under study (Fig. 7). Seedlings growing in presence of Cd in the medium showed higher SS activity in roots as well as in shoots compared to controls. Cadmium induced increase in SS activity was greater in roots than in shoots.

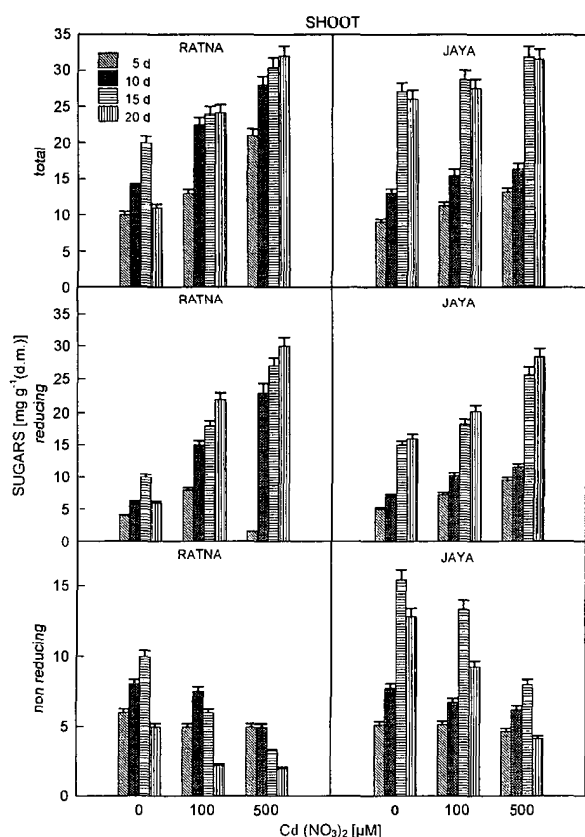


Fig. 2. Content of sugars (non-reducing, reducing and total) in shoots of rice cvs. Ratna and Jaya at 5, 10, 15, and 20 d of growth under 0 (control), 100, and 500  $\mu\text{M}$   $\text{Cd}(\text{NO}_3)_2$ . Means of three independent determinations and bars indicate standard deviations.

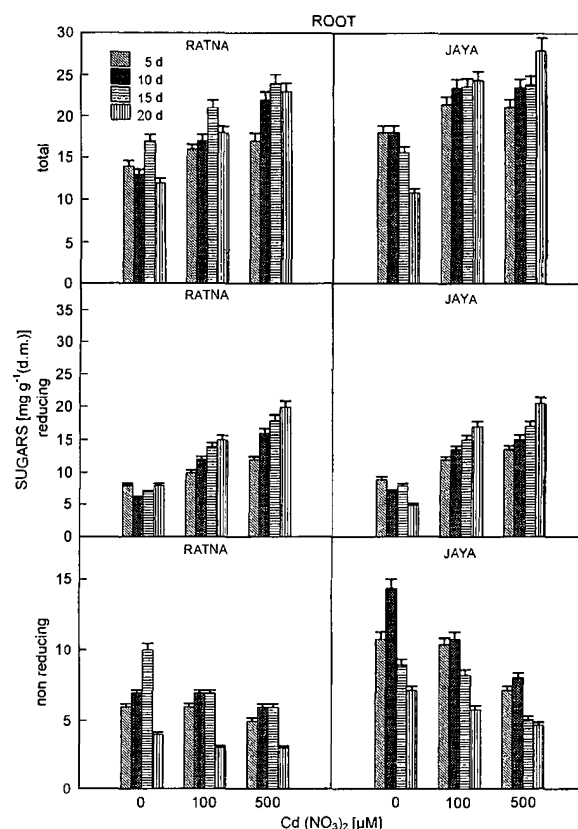


Fig. 3. Content of sugars (non-reducing, reducing and total) in roots of rice cvs. Ratna and Jaya at 5, 10, 15, and 20 d of growth under 0 (control), 100, and 500  $\mu\text{M}$   $\text{Cd}(\text{NO}_3)_2$ . Means of three independent determinations and bars indicate standard deviations.

## Discussion

Cadmium adversely affects plant growth by decreasing water transport to leaves impairing transpiration rate, causing ultra-structural changes in cell organelles and altering the behaviours of key enzymes of various metabolic pathways (Shah and Dubey 1998a,b). Earlier studies conducted in our laboratory have indicated that the heavy metal Cd alters the activities of proteolytic, nucleolytic and phosphorolytic enzymes in growing rice seedlings and induces synthesis of specific amino acids and proteins (Shah and Dubey 1995, 1997, 1998a,b).

Our results indicate an increase in the content of soluble sugars, decreased content of non-reducing sugars accompanied with increased activity of acid invertase and sucrose synthase and decrease in activity of sucrose phosphate synthase in rice seedlings growing in presence of Cd in the medium.

As Cd decreases water transport to the leaves (Chardonnens *et al.* 1998) the accumulating sugars in rice plants growing in presence of Cd could possibly provide

an adaptive mechanism in maintaining a favourable osmotic potential under adverse conditions of Cd toxicity. It is known that Cd also induces accumulation of free amino acids and specially proline which serves as compatible cytoplasmic solute (Shah and Dubey 1997/1998). In addition to the role of sugars in osmoregulation, the soluble sugars allow the plants to maximize sufficient carbohydrate storage reserves to support basal metabolism under stressed environment (Hurry *et al.* 1995, Dubey and Singh 1999).

In our study, though in certain cases an apparent high content of starch and inhibition in  $\alpha$ -amylase activity was noted, such response was not uniform. This suggests that Cd in rice seedlings does not have a significant impact on alteration in starch content or on its mobilisation due to  $\alpha$ -amylase. When the role of acid invertase and sucrose synthase in mobilisation of non-reducing sugars (predominantly sucrose) was investigated, a decrease in the level of non-reducing sugars accompanied with

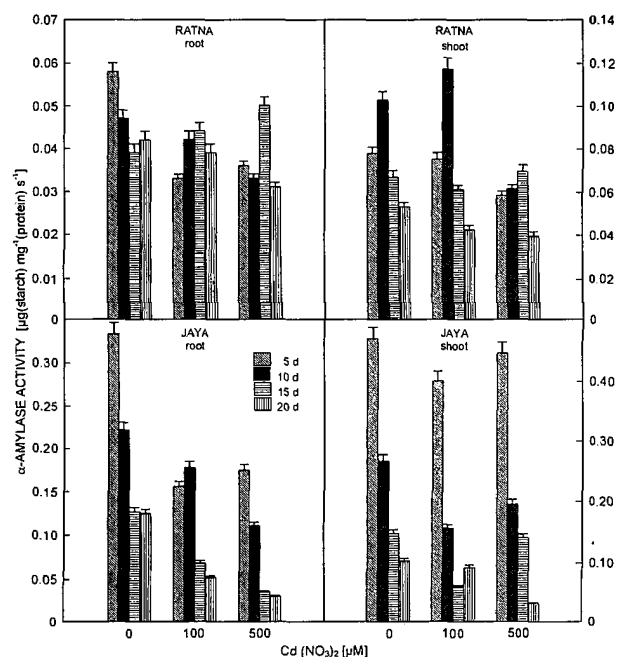


Fig. 4. Specific activity of  $\alpha$ -amylase in roots and shoots of seedlings of rice cvs. Ratna and Jaya at 5, 10, 15, and 20 d of growth under 0 (control), 100, and 500  $\mu\text{M}$   $\text{Cd}(\text{NO}_3)_2$ . Means based on three independent determinations and bars indicate standard deviations.

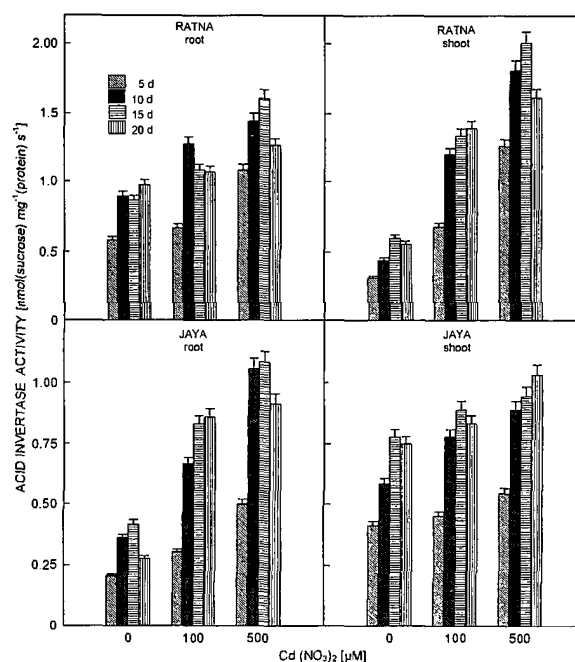


Fig. 5. Specific activity of acid invertase in roots and shoots of seedlings of rice cvs. Ratna and Jaya at 5, 10, 15, and 20 d of growth under 0 (control), 100, and 500  $\mu\text{M}$   $\text{Cd}(\text{NO}_3)_2$ . Means based on three independent determinations and bars indicate standard deviations.

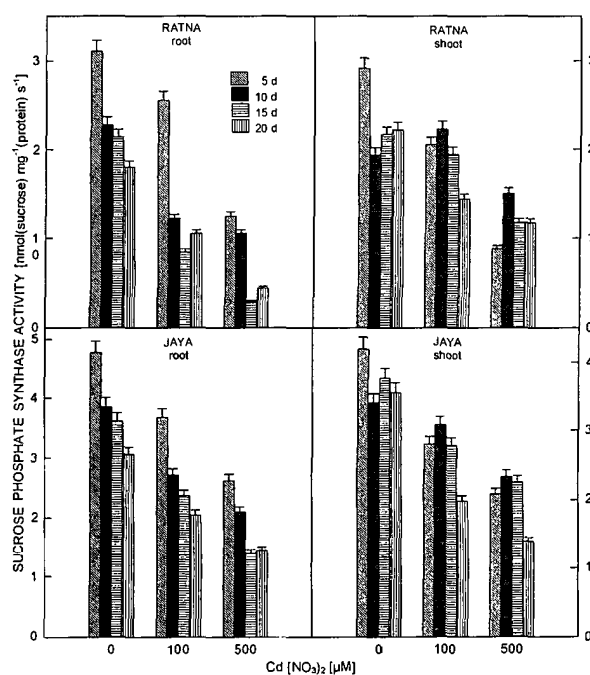


Fig. 6. Specific activity of sucrose phosphate synthase in roots and shoots of seedlings of rice cvs. Ratna and Jaya at 5, 10, 15, and 20 d of growth under 0 (control), 100, and 500  $\mu\text{M}$   $\text{Cd}(\text{NO}_3)_2$ . Means based on three independent determinations and bars indicate standard deviations.

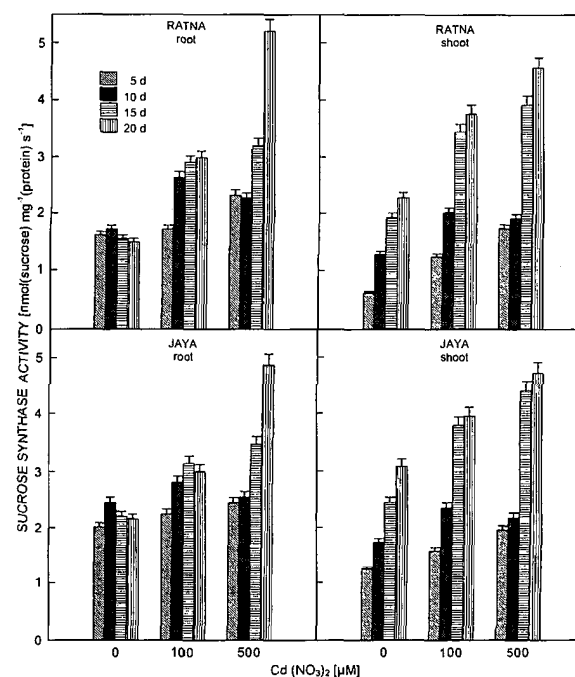


Fig. 7. Specific activity of sucrose synthase in roots and shoots of seedlings of rice cvs. Ratna and Jaya at 5, 10, 15, and 20 d of growth under 0 (control), 100, and 500  $\mu\text{M}$   $\text{Cd}(\text{NO}_3)_2$ . Means based on three independent determinations and bars indicate standard deviations.

increased activities of sucrose degrading enzymes acid invertase and sucrose synthase was noticed in rice seedlings under cadmium treatment. The enzymes SS and acid invertase play important role in phloem loading/unloading by maintaining steep sucrose concentration gradient (Lohaus *et al.* 1995). Sucrose is regarded as a principal C source in growing seedlings and its level in tissues is strongly influenced by the activities of SS and SPS (Ricard *et al.* 1998). Similar to our observations in the cases of anoxia of rice plants an increase in SS activity (Ricard *et al.* 1991) and in cold hardened winter oat plants an increase in invertase activity (Livingston and Henson 1998) have been noticed. Increase in acid invertase and SS activities accompanied with decreased level of non-reducing sugars (sucrose) in Cd-grown rice seedlings suggests that Cd-toxicity in rice limits availability of sucrose in the cells by favouring its enhanced degradation due to both invertase and sucrose

synthase activities. Cadmium inhibited the activity of sucrose synthesizing enzyme sucrose phosphate synthase in rice seedlings. Such decrease in activity paralleled with decreased level of non-reducing sugar (sucrose) and increased levels of reducing sugars. Unlike our studies the activity of SPS has been shown to be induced under low temperature and osmotic stresses (Ingram *et al.* 1997, Krause *et al.* 1998). SPS has been regarded as key enzyme in the regulation of sucrose metabolism and the overall regulation of its activity is strongly influenced by the osmotic potential of the cytoplasm (Ingram *et al.* 1997) which is liable to change under Cd-toxicity.

Our observations suggest that the presence of Cd in the growth medium of rice plants causes marked perturbations in sugar metabolism. Such events might impair carbohydrate metabolism ultimately leading to impaired growth of rice seedlings in Cd-polluted soils.

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