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Hg and Cd induced changes in proline content and activities of proline biosynthesizing enzymes in *Phaseolus aureus* and *Triticum aestivum*

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Abstract

The effect of mercury and cadmium, in the form of $HgCl_2$ and $CdCl_2$ respectively, on proline accumulation and two key proline biosynthesizing enzymes, Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and Δ^1-pyrroline-5-carboxylate reductase (P5CR), was investigated in *Phaseolus aureus* Roxb. and *Triticum aestivum* L. The 5-d-old seedlings were exposed to 0.05, 0.1, 0.2 or 0.4 mM concentrations of the metals in Hoagland solution for 12 and 36 h. *T. aestivum* exhibited considerably greater accumulation of proline than *P. aureus* in response to the metal treatment. Among the two metals, Hg induced greater accumulation of proline than Cd. The activity of P5CS increased significantly in response to the metal treatment, particularly in *T. aestivum* in which the activity of the enzyme in the control was much higher than that was in *P. aureus*. The activity of P5CR on the other hand mostly decreased in response to the metal treatment. The study indicated a strong dependence of the metal induced proline accumulation on the constitutive P5CS content of the plants.

Additional key words: bean, heavy metals, Δ^1-pyrroline-5-carboxylate synthetase, Δ^1-pyrroline-5-carboxylate reductase, wheat.

Introduction

Free proline has been reported to accumulate in plants in response to a wide range of environmental stresses (for review see Hare and Cress 1997). The accumulation is also widely spread in plants in response to heavy metals (Alia and Saradhi 1991, 1993, Costa and Morel 1994, Schat *et al.* 1997). The compound has been attributed to a variety of functions of which its function as an osmoprotectant under drought and salinity stress has been widely advocated (Yoshiba *et al.* 1997, and the references therein). The functional significance of proline accumulation in plants under heavy metal stress is, however, yet to be elucidated well. While a few studies (Alia and Saradhi 1991, 1993) in this regard suggest the synthesis of proline and its subsequent accumulation to be a process of non-toxic sink for the excess reductant (NADPH/NADH) accumulating as a result of disturbances in the metabolic processes upon exposure of the plants to heavy metals, other studies (Costa and Morel 1994, Schat *et al.* 1997) suggest the accumulation of the compound to be simply a consequence of development of metal-induced water deficit without having any role to play in over-coming the metal-induced toxicity. Hence, understanding the reason of accumulation of the compound in plants in response to heavy metal treatment needed further investigation.

Few studies were carried out to see the species-specific response of plants to heavy metals, and also metal specific response of a plant species, in terms of accumulation of proline. Reports on the effect of heavy metals on the activity of proline biosynthesizing enzymes were also scant. Hence, the present work was designed to study changes in the dry matter content, the concentration of proline and the activities of two crucial enzymes of the proline biosynthesis pathway from glutamate, Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and Δ^1-pyrroline-5-carboxylate reductase (P5CR), in *Phaseolus aureus* Roxb. (a dicot) and *Triticum aestivum* L. (a monocot), in response to mercury and cadmium.

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Abbreviations: P5CR - Δ^1-pyrroline-5-carboxylate reductase; P5CS - Δ^1-pyrroline-5-carboxylate synthetase

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Materials and methods

The seeds of *P. aureus* and *T. aestivum* were soaked in distilled water overnight and then germinated and grown on a nylon net over 100 cm^3 Hoagland solution in a growth chamber (temperature of 25 ± 2 °C, irradiance of 200 μmol m^{-2} s^{-1}, and 12-h photoperiod).

Analytical grade mercuric chloride ($HgCl_2$) and cadmium chloride ($CdCl_2$) were used for the treatment. In our earlier study (Shaw and Rout 1998) it was observed that Cd at concentration 30 μM or greater was lethal to the seedlings of *P. aureus* when applied after the 5th day of germination, but not when applied on or before the 5th day. Hg had no such age-dependent effect, and the seedlings survived irrespective of whether treated at an early or late stage of germination. An initial test on the seedlings of *T. aestivum* revealed no lethal effect of either Cd or Hg applied (in concentration as high as 1 M) on any day from the 4th to 9th day of germination. Based on the above information, the seedlings of the two plant species were exposed to 4 sub-lethal concentrations, 0.05, 0.1, 0.2 and 0.4 mM, of either Hg or Cd on the 5th day of germination. The seedlings not treated with the metals served as the control.

The seedlings from both treated and untreated sets were harvested after 12 and 36 h of exposure. The portions above the cotyledons in case of *P. aureus* and above the seed in case of *T. aestivum* were considered for the dry matter determination. The analysis of proline and the enzymes involved in its synthesis were done only in the leaves considering them as the seat of maximum metabolic activities. The dry mass of the seedlings was obtained after drying them properly for 24 h in an oven at 140 °C. The leaves meant for proline and enzyme analysis were frozen in liquid N_2 and stored at -70 °C.

Proline contents of the leaves were determined following the procedure of Bates *et al.* (1973). For the enzyme analysis the leaves were homogenized in an extraction buffer (pH 7.5) containing 100 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM β-mercaptoethanol, 4 mM dithiothreitol, 2 mM PMSF (phenylmethylsulfonyl fluoride) and 2 % polyvinylpolypyrrolidone (Chilson *et al.* 1992) in pre-chilled mortars and pestles in a cold room. The homogenates were centrifuged twice at 4 °C for 20 min at 20 000 *g*. The activity of P5CR was assayed as proline-dependent reduction of NAD^+ (the reverse reaction) following Chilson *et al.* (1992). The reaction was carried out in a final volume of 1 cm^3 sodium glycinate buffer (200 mM, pH 10.3) containing 20 mM proline, 15 mM NAD^+ and the enzyme extract. The activity was determined at 25 °C by monitoring the formation of NADH at 340 nm on an spectrophotometer (*DU-68*, *Beckman*, Fullerton, USA), and expressed as unit (U) mg^{-1}(protein); 1 U is defined as the amount of the enzyme required to generate 1 μmol of NADH in 1 min.

The activity of P5CS in the same enzyme extract was determined as γ-glutamyl kinase by monitoring the formation of γ-glutamyl hydroxamate (Hayzer and Leisinger 1980). The enzyme mixture in a final volume of 0.5 cm^3 contained 50 mM Tris-HCl (pH 7.0), 50 mM L-glutamate, 20 mM $MgCl_2$, 100 mM hydroxamate-HCl, 10 mM ATP and the enzyme extract. After addition of the extract, the reaction mixture was incubated at 37 °C for 15 min. The reaction was stopped by adding 1 cm^3 of the stop buffer (2.5 g $FeCl_3$ and 6 g trichloroacetic acid in a final volume of 100 cm^3 of 2.5 M HCl). The precipitated proteins were removed by centrifugation, and the absorbance of the clear supernatant was read at 535 nm against a blank identical to the above but lacking ATP. The activity was expressed in U mg^{-1}(protein); 1 U represented the amount of the enzyme required to produce 1 μmol of γ-glutamyl hydroxamate in 1 min.

The protein in the enzyme extract was quantified by the Coomassie brilliant blue dye binding method of Bradford (1976).

The data presented are the means of ten determinations for the dry matter content, five analyses for the proline content and six analyses for the enzyme activity. The difference between the means was tested by Duncan's multiple range test (Blis 1967).

Results

The dry matter contents of the seedlings of both *P. aureus* and *T. aestivum* were reduced significantly upon exposure for 36 h to both Cd and Hg (Table 1). However, the effect of different concentrations of the metals was not significantly different from each other. 12-h exposure of the seedlings to the metals did not result in any significant reduction in their dry mass when compared to control.

The constitutive (control) content of proline was more or less same in both *P. aureus* and *T. aestivum*. However, the two species differed greatly in the amount of proline accumulated upon exposure to the metals. *P. aureus* did not show any significant change in proline content upon exposure to either Hg or Cd for 12 h (Fig. 1*A*). Upon 36-h exposure also the proline content increased significantly only in response to 0.1 mM or higher Hg concentration. In contrast *T. aestivum* exhibited significant accumulation of proline upon exposure for even 12 h to Hg as well as to Cd (Fig. 1*B*). The accumulation increased even more upon exposure for 36 h. The increase in the content of

proline in response to 0.1 mM Hg was maximal; proline content was nearly 2-fold and 3-fold to that of the control value after 12 h and 36 h of exposure, respectively. 0.2 and 0.4 mM Hg resulted in significant accumulation of proline only after 36 h of exposure. Unlike Hg, Cd after 36 h resulted in a concentration-dependent increase in the accumulation of proline in *T. aestivum*. After 12-h exposure the increase was nearly the same in all

Table 1. Dry matter contents [mg seedling^{-1}] of the seedlings of *P. aureus* and *T. aestivum* exposed to various concentrations of Hg or Cd on the 5th day of germination for 12 h and 36 h. Means ± SD of 10 determinations. The values of the control and Hg and Cd treatment sets of the 36-h exposure marked by the same letter are not significantly different from each other at $P \leq 0.05$ as determined by the Duncan's multiple range test. The values of the dry matter content of the seedlings of the 12-h exposure sets are statistically not different significantly from each other.

Hg/Cd [mM]	*Phaseolus aureus*				*Triticum aestivum*			
	12-h exposure		36-h exposure		12-h exposure		36-h exposure	
	Hg	Cd	Hg	Cd	Hg	Cd	Hg	Cd
Control	11.77 ± 1.57		14.23 ± 1.96a		10.45 ± 1.44		12.88 ± 2.22a	
0.05	10.70 ± 1.54	10.09 ± 1.69	10.45 ± 2.12b	10.59 ± 1.35b	9.15 ± 1.29	9.63 ± 1.51	9.77 ± 1.55b	10.70 ± 1.69b
0.1	10.80 ± 1.77	10.02 ± 1.80	10.93 ± 2.43b	9.73 ± 2.53b	8.65 ± 2.09	9.00 ± 2.04	9.08 ± 2.08b	9.77 ± 2.56b
0.2	9.76 ± 2.07	10.15 ± 2.16	9.97 ± 1.19b	9.88 ± 1.48b	8.78 ± 1.42	8.84 ± 1.63	8.94 ± 1.74b	10.00 ± 2.21b
0.4	9.96 ± 1.77	9.10 ± 0.99	9.86 ± 1.17b	9.44 ± 1.56b	8.51 ± 1.86	8.98 ± 2.16	9.28 ± 2.11b	9.82 ± 1.97b

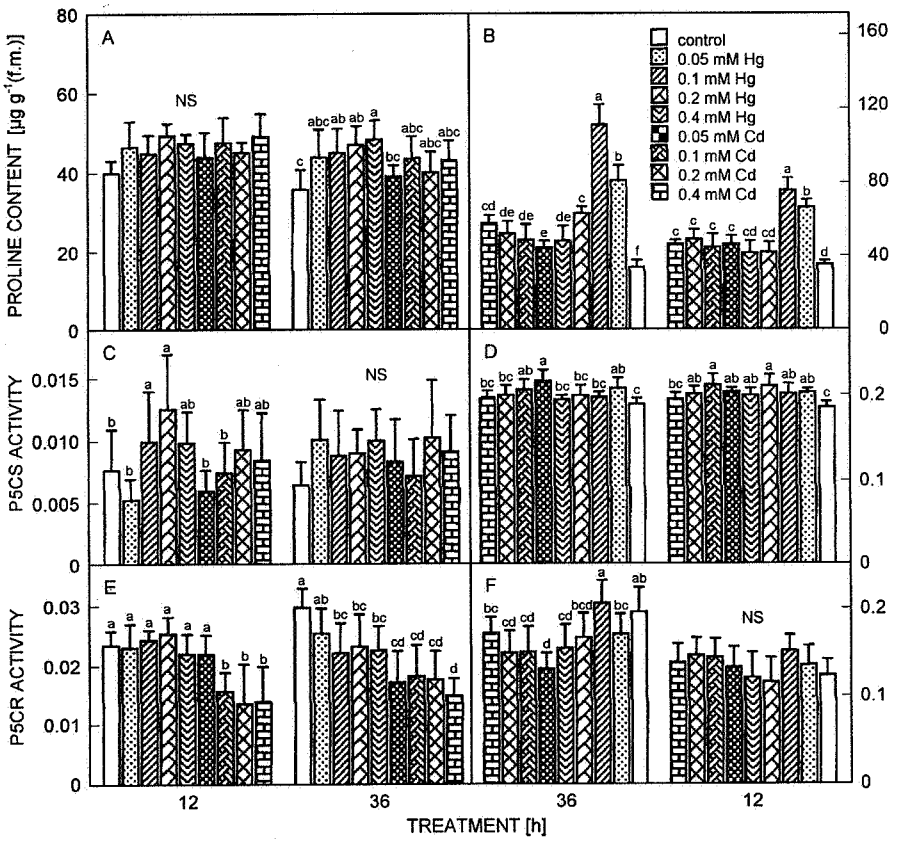

Fig. 1. Proline contents [µg g^{-1} (f.m.)] (*A,B*), P5CS (Δ^1-pyrroline-5-carboxylate synthetase) activities [U mg^{-1}(protein)] (*C,D*) and P5CR (Δ^1-pyrroline-5-carboxylate reductase) activities [U mg^{-1}(protein)] (*E,F*) in the leaves of *P. aureus* (*A,C,E*) and *T. aestivum* (*B,D,F*) seedlings exposed to 0 (control), 0.05, 0.1, 0.2 or 0.4 mM concentrations of Hg (as $HgCl_2$) or Cd (as $CdCl_2$) on the 5th day of germination for 12 h and 36 h. Each column is a mean value with the *vertical bar* representing standard deviation. The means (of the parameter) of the control and various treatment sets in an exposure period (12 h or 36 h) marked by the same letter are not significantly different from each other at $P \leq 0.05$. NS - the difference between the values of the parameter obtained for the control and treated sets in an exposure period is statistically not significant.

concentrations. The seedlings of *P. aureus* exposed to 0.05 and 0.1 mM Hg or Cd for 12 h on the 7th day of germination did not show any significant accumulation of proline (data not shown), similar to that when exposed on the 5th day of germination.

The activity of P5CS differed greatly in the two species (Fig. 1*C,D*): the activity was more than 20-fold in *T. aestivum* when compared to that in *P. aureus*. The activity of the enzyme was enhanced in both the test species upon exposure to Hg as well to Cd. In *P. aureus*, however, significant enhancement was observed only after 12-h exposure to 0.1 and 0.2 mM Hg and 0.2 and 0.4 mM Cd (Fig. 1*C*). In *T. aestivum* the increase in the activity of the enzyme was observed in response to all the concentrations and both the exposures. After 36-h exposure, however, significant increase in the activity of the enzyme was observed only in response to 0.05 mM Hg and 0.05 and 0.1 mM Cd (Fig. 1*D*).

The activity of P5CR exhibited significant decrease in response to the metal treatment. In *P. aureus* the decrease was significant upon 12-h exposure to Cd, and upon 36-h exposure to both Hg and Cd (Fig. 1*E*). The decrease was more in response to Cd than in response to Hg. In *T. aestivum* a significant decrease in the activity of the enzyme was observed only after 36-h exposure to the metals (Fig. 1*F*); while Cd decreased the activity of the enzyme at all the concentrations, Hg resulted in a significant decrease only at 0.4 mM concentration. The decrease in the activity of P5CR in response to the metal treatments in general was more in *P. aureus* than in *T. aestivum*.

Discussion

The suggestion of involvement of proline in abating heavy metal stress stems from its enhanced accumulation in the plants exposed to metals (Alia and Saradhi 1991, 1993, Wu *et al.* 1998). The present result, however, does not suggest any protective role of proline against heavy metal toxicity. The accumulation rather appears to be a species-specific response; both the test species were equally affected by the two metals in terms of reduction in the dry mass (Table 1) and had similar constitutive content of proline, but *T. aestivum* showed a much higher accumulation of the compound in response to the metal treatment than *P. aureus* (Fig. 1*A,B*). Such a great difference in the accumulation of the compound by the plants is unlikely to be due to any difference in uptake of the metals by the two plants. This is because *1*) proline accumulation is not linearly related to the internal metal content of plant (Schat *et al.* 1997), *2*) both the metals have been reported to be taken up by plants, both monocot and dicot, in a somewhat concentration dependent manner (Landberg and Greger 1994, Suszcynsky and Shann 1995), and *3*) although Hg resulted in significant increase in the content of proline in *P. aureus* exposed on the 5th day of germination, it did not result in so when applied on the 7th day of germination.

The accumulation of proline also appeared to be metal specific as in both the test species Hg induced greater accumulation of proline than Cd. This seems to justify the suggested role of accumulation of the compound as a non-toxic sink for energy for the regulation of redox potential (Alia and Saradhi 1991, 1993, Hare and Cress 1997); Hg being a more effective inhibitor of enzymes than Cd probably induced greater accumulation of proline than Cd. However, if this physiological significance is conceivable then both the species should have accumulated more or less similar amount of the compound in response to exposure to a particular metal. The study also does not support the generalized view of Schat *et al.* (1997) *in toto* that the accumulation is induced due to water-deficit created upon exposure of the plant to a metal. This is because Hg resulted in much less accumulation of the compound at 0.2 and 0.4 mM concentrations than at 0.05 and 0.1 mM concentrations, but the water deficit induced by a metal cannot be more at lower concentration than at higher concentration.

P5CS catalyzes the first two steps of the proline biosynthesis pathway from glutamate. It is a bifunctional enzyme with apparent activities of γ-glutamyl kinase (γ-GK) and glutamic acid-5-semialdehyde (GSA) dehydrogenase. During the process glutamate is phosphorylated by γ-GK to γ-glutamyl phosphate, which is then reduced to GSA by GSA dehydrogenase. GSA spontaneously changes to Δ^1-pyrroline-5-carboxylate (P5C). The increase in the activity of the enzyme observed (Fig. 1*C,D*) is in agreement with that reported for terrestrial plants (Hu *et al.* 1992, Zhang *et al.* 1995). The accumulation of proline in the test plants, however, appears to be linked to the constitutive content of the enzyme rather than being dependent upon increase in its activity in response to the metal treatment. This stems from the fact that the increase in the activity of P5CS was only marginal in *T. aestivum* while proline accumulated many folds, particularly in response to Hg treatment (Fig. 1*D*), and that the content of proline in *P. aureus* did not increase in proportion to the increase in the activity of the enzyme (Fig. 1*C*). This indicates that the regulation of the biosynthesis of proline at the level of P5CS suggested by Kishor *et al.* (1995) and Liu and Zhu (1997) may not be universal. In fact plant having high constitutive activity of P5CS has been reported to accumulate much greater amount of proline than that having not even in response to salt stress (Rout and Shaw 1998).

The observed decrease in the activity of P5CR, which reduces P5C to proline, in response to the metal treatment is in contrast to the reports of significant increase in its activity in response to salt treatment (LaRosa *et al.* 1991, Williamson and Slocum 1992, Rout and Shaw 1998). Significant decrease in the activity of the enzyme in response to Cd (Fig. 1*E,F*) could be the reason of *1*) absolutely no increase in the level of proline in *P aureus* upon exposure to the metal (Fig. 1*A*), and *2*) much lesser accumulation of the compound in *T. aestivum* (Fig. 1*B*) exposed to Cd (for 36 h) than that exposed to Hg (for 36 h) despite significant increase in the activity of P5CS (Fig. 1*D*). Furthermore, significantly less accumulation of proline in *T. aestivum* at 0.2 and 0.4 mM Hg treatments than that at 0.05 and 0.1 mM treatments could be due to the decrease in the activity of P5CR at the higher metal exposure concentrations (Fig. 1*F*). Thus although the accumulation of proline in plants under heavy metal stress could be primarily dependent on their constitutive P5CS content, a certain minimal activity level of P5CR appears to be highly necessary for the increase in biosynthesis of the compound.

From the study it appears that the accumulation of proline in plants under heavy metal stress is totally a species-specific response. The physiological significance of accumulation of the compound in plants under heavy metal stress, however, still remains unclear. Furthermore, the accumulation of the compound in a plant under heavy metal stress appears to be largely dependent upon the constitutive P5CS activity of the plant rather than on the increase in its activity in response to the stress. The enzyme P5CR, however, because of decrease in its activity *in vivo* upon the metal treatment, could regulate the accumulation of the compound in plants under heavy metal stress.

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