BIOLOGIA PLANTARUM 53 (4): 737-740, 2009

BRIEF COMMUNICATION

Responses of *Camellia sinensis* cultivars to Cu and Al stress

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Abstract

The response of *Camellia sinensis* (L.) O. Kuntze cultivars Chinary and Assamica to Cu and Al stresses was investigated. Exposure to 100 μM $CuSO_4$ or 100 μM $AlCl_3$ led to accumulation of reactive oxygen species (ROS) more in Assamica than in Chinary. Proline content was higher in Chinary compared to Assamica, while chlorophyll and protein contents decreased upon Cu and Al exposure in both the cultivars. Expression of glutathione biosynthetic enzymes γ-glutamylcysteinyl synthetase (γ-ECS) and glutathione synthetase (GSHS) was elevated. Phytochelatin synthase (PCS), an enzyme involved in phytochelatins synthesis by using glutathione as a substrate was up-regulated at its transcript level more in Chinary than in Assamica. These results suggest that Chinary could be more tolerant than Assamica.

Additional key words: gene expression, glutathione metabolic enzymes, oxidative stress, proline, tea.

Tea plants exposed to high levels of heavy metals showed reductions in photosynthesis, and water and nutrient uptake (Sanita di Toppi and Gabbrielli 1999). Plants grown in such soils containing high levels of these ions show visible symptoms of injury (chlorosis, growth inhibition, browning of root tips), and finally died. Such stresses also lead to the induction of various changes on cellular level, including gene expression, antioxidant capacity and redox balance (Kumar *et al*. 2003, Singla-Pareek *et al*. 2006).

Among metals stress, high uptake of copper causes destruction of chloroplast structure and considerable modification of their lipid and protein composition. Copper ions can readily oxidize the thiol bonds present in the proteins, causing disruption of their structure and functions (Quartacci *et al*. 2001, Mouratao *et al*. 2009). Excess copper inhibits root and leaf growth (Maksymiec and Krupa 2007). In germinating rice seeds copper stress caused a differential expression of 25 proteins and majority of these proteins were antioxidants or stress-related proteins (Ahsan *et al*. 2007). Quantitative trait loci (QTLs) affecting the copper tolerance in wheat have also been mapped recently (Bálint *et al*. 2007).

Aluminium shows toxic effect when absorbed by plants in Al^{3+} form. Its high uptake causes inhibition of cell division and cell elongation. However, the primary effect of aluminum was recognized on root growth (Shamsi *et al*. 2007). In *Arabidopsis*, 256 genes have been reported as aluminium-responsive (Goodwin and Sutter 2009). Exposure of tea to high contents of Cu, Hg and Ni causes decrease in chlorophyll and increase in proline content (Basak *et al*. 2001). Reduction in chlorophyll content of tea has also been reported during Cd stress (Mohanpuria *et al*. 2007).

Glutathione (GSH), a tripeptide (Glu-Cys-Gly) is one of the major endogenous antioxidants in plants known to play an important role in plant defense mechanism. Phytochelatins (PCs) are small peptides derived from GSH and represent one of the major metal chelator and detoxifier in plants (Singla-Pareek *et al*. 2006). Glutathione biosynthesis comprises of two step reaction catalyzed by γ-glutamylcysteine synthetase (γ-ECS, EC 6.3.2.2) and glutathione synthetase (GS, EC 6.3.2.3) (May *et al*. 1998), while PCs synthesis is catalyzed by phytochelatin synthase (PCS). Enhanced GSH contents along with ascorbic acid ameliorated oxidative stress

Received 5 November 2007, *accepted* 30 April 2008.

Abbreviations: γ-ECS - γ-glutamylcysteinyl synthetase; GSH - glutathione; GSHS - glutathione synthetase; PCs - phytochelatins; PCS - phytochelatin synthase; ROS - reactive oxygen species.

Acknowledgements: We thank our Director, Dr. P.S. Ahuja for valuable suggestions and guidance during the pursuance of this research work. This work was gratefully supported by grants from Council for Scientific and Industrial Research (CSIR), Govt. of India.

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induced by the excess of Cu and Cd in *Arabidopsis* (May *et al*. 1998, Maksymiec *et al*. 2007).

The objective of this study was to determine the relative response of two tea cultivars Chinary and Assamica to high content of Cu and Al. Their influence was monitored on ascorbate, chlorophyll, proteins and proline contents. Additionally, the influence of Cu and Al on the expression level of γ-ECS, GS and PCS genes was investigated.

Apical buds of *Camellia sinensis* (L.) O. Kuntze cvs. Chinary and Assamica were treated with 100 μM $CuSO_4$ or 100 μM $AlCl_3$ for 24 h and used for various biochemical and molecular studies. For control, buds were kept in water for the same time period. However, for histochemical detection of hydrogen peroxide, superoxide radical and ascorbate first leaf (next to apical bud) instead of bud was used. H_2O_2 and superoxide radicals were detected using diaminebenzene and nitro-blue tetrazolium staining, respectively. To detect ascorbate the round discs were incubated with 5 % silver nitrate solution as described earlier (Liso *et al.* 2004). Chlorophyll (Chl) was extracted in 80 % chilled acetone and Chl *a*+*b* content was estimated by the method used earlier (Mohanpuria *et al*. 2007). Protein content was estimated by Bradford (1976) method. Proline content was estimated according to Bates *et al*. (1973). The data are means ± standard deviation of three independent measurements. A least significant difference (LSD) test was employed to test the effect of Cu and Al.

For expression analysis, 200 mg of bud tissues were used for RNA isolation. cDNA was synthesized using 1 μg of RNA in the presence of 200 U reverse transcriptase *Superscript*™ III (*Invitrogen*, Carlsbad, USA), 0.001 cm^3 of 10 mM dNTPs and 250 ng oligo $(dT)_{12\text{-}18}$. Resulting cDNA was used to carryout the PCR reactions with γ-ECS gene primers (forward 5'-GACATACCM AYMATGCCYAAGG-3' and reverse 5'-CCATCAGC RCCTCTCATCTC-3'), GSHS gene primers (forward 5'-GAAGARMGVWAYATGTATGACC-3' and reverse 5'-GTKCKCATCAARTAACCACACTG-3') and PCS gene primers (forward 5'-CAGACRCAGTCKGARCCG-3' and reverse 5'-CAATYGTGTTCATGGCTTCCC-3'). After standardizing the optimal amplification at exponential phase, PCR was carried out under the conditions of 94 °C - 4 min for 1 cycle, 94 °C - 30 s, 50 °C - 40 s, 72 °C - 1 min for 30 cycles and fractionated on agarose gel electrophoresis and visualized with ethidium bromide staining. 26S rRNA based gene primers were used as internal control for expression studies (Singh *et al*. 2004).

Several biotic and abiotic factors ultimately impose oxidative stress in various plants (*e.g.* Rao and Sresty 2000, Singla-Pareek *et al*. 2006). To check whether Cu and Al exposure induces generation of reactive oxygen species (ROS) in tea, H_2O_2 and superoxide radical were detected histochemically. An increase in intensity of brown colour in leaf discs of both tea cultivars upon Cu and Al treatment indicated the generation of ROS. ROS generation was found to be more expressive after Cu than Al exposure in both cultivars. Further, higher ROS generation was observed in Assamica than Chinary (data not shown). Ascorbate is known antioxidant protecting cells from oxidative stress either by direct interactions with ROS or in reaction catalyzed by ascorbate peroxidase. Both of these activities led to the generation of ascorbate free radical and dehydroascorbate (Shigeoka *et al*. 2002). To assess the influence of Cu and Al on ascorbate in tea, its content was also monitored histochemically, and higher increase in ascorbate was observed in Chinary than Assamica. Furthermore, Cu induced more ascorbate than Al (data not shown).

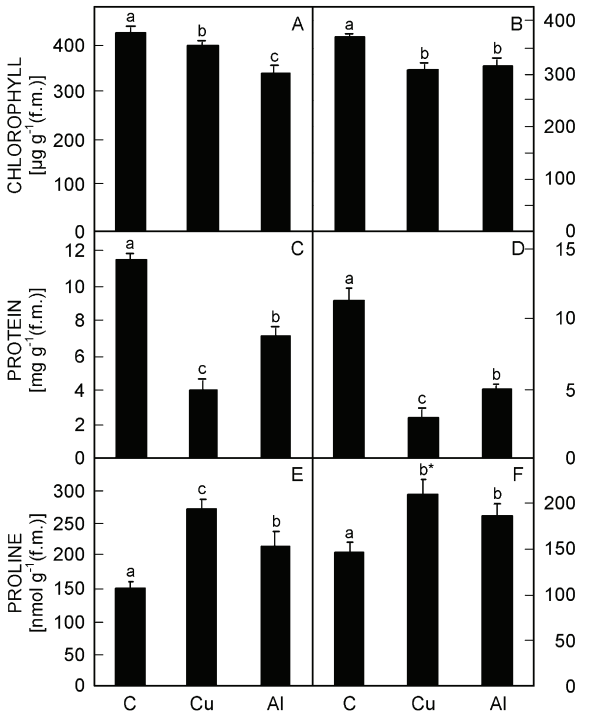

Fig. 1. Chlorophyll (*A*,*B*), protein (*C*,*D*) and proline (*E*,*F*) content in young leaf of Chinary (*A*,*C*,*E*) and Assamica (*B*,*D*,*F*) tea cultivars after 24 h treatment with 100 μM $CuSO_4$ and $AlCl_3$. Values are the mean of three independent measurements and bars represent SD. Data with the same letter are not significantly different from each other at 5 % level according to LSD test, while same letters having * as superscript are significantly different at 1 % level.

In general, decrease in chlorophyll content under stress conditions in plants is considered as direct indicator of damage. In this investigation, Cu treatment decreased Chl *a*+*b* content by 8 % in Chinary and 18 % in Assamica, while Al treatment decreased chlorophyll content by 20 % in Chinary and 16 % in Assamica (Fig. 1*A*,*B*). Decrease in Chl content of tea leaves has also been observed earlier after exposure to Ni^{2+}, Hg^{2+} and Cu^{2+} (Basak *et al*. 2001). Recently, we have also observed the decrease in Chl content of tea after exposure to high Cd concentration (Mohanpuria *et al*. 2007). Similarly protein content decreased after exposure to Cu

by 65 and 72 % in Chinary and Assamica, respectively, as compared to control (Fig. 1*C*,*D*), while due to Al stress, the protein content was decreased by 39 and 52 % in Chinary and Assamica, respectively (Fig. 1*C*,*D*). However, proteins in *Arabidopsis thaliana* showed both up and down regulation after exposure to different concentrations of Cd (Sarry *et al*. 2006). Similarly, Cu stress induced changes in the protein concentration of bean (Cuypers *et al*. 2005).

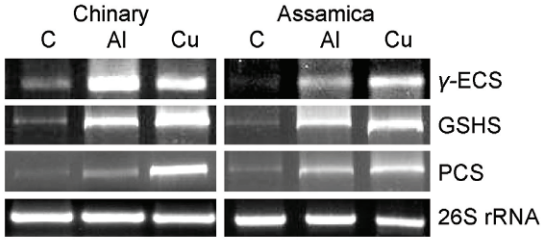

Fig. 2. Steady state transcript levels of GSH metabolism genes in young leaf of Chinary and Assamica tea culivars in response to Cu and Al stress for 24 h. C - control sample.

Proline is widely considered as an osmoregulatory solute. It accumulates in plant when exposed to wide variety of environmental stresses and provide stress tolerance (Alia *et al*. 1997, Mossor-Pietraszewska *et al*. 1997). Proline also stabilizes cellular structures and acts as a free radical scavenger (Hare and Cress 1997). In Chinary, Cu exposure increased proline content by 80 %, while Al exposure by 50 % (Fig. 1*E*). However, in Assamica Cu increased proline content to similar extent as in Chinary but Al increased its content only by 20 % (Fig. 1*F*). Significant increase in proline content has been reported earlier in tea under Ni^{2+}, Hg^{2+} and Cu^{2+} stresses (Basak *et al*. 2001).

Glutathione (GSH) plays several roles in cellular metabolism of plants such as redox maintenance, oxidative stress control, and protection against xenobiotics and heavy metals. Phytochelatins (PCs) involved in metal chelation, are synthesized by the polymerization of GSH that is catalyzed by phytochelatin synthase (PCS). The enhanced contents of GSH and/or phytochelatins led to the increased tolerance to high metal contents (Singla-Pareek *et al*. 2006, Blum *et al*. 2007). In view of this, here we assessed the transcript expression of γ-ECS, GSHS and PCS in two tea cultivars upon exposure to Cu and Al. As compared to control, Al and Cu stress up-regulated the transcript levels of all three glutathione metabolic genes in both Assamica and Chinary. However, more increase was observed in Chinary than Assamica (Fig. 2). The increase in the contents of GSH and PCs was observed in many plant species in response to metal stresses (Grill *et al*. 1987). The *Arabidopsis* accumulated PCs when exposed to Cd^{2+} and Cu^{2+} (Wang and Oliver 1996) and upregulated transcript levels of γ-ECS and GSHS (Xiang and Oliver 1998). The induced expression of more than 20 genes by Al stress have been reported in many plant species including wheat (Delhaize *et al*. 1999), rye (Gallego and Benito 1997), rice (Nguyen *et al*. 2001), and *Arabidopsis* (Richards *et al*. 1998). It was also proposed that there are common mechanisms for gene induction by Al and oxidative stress.

In conclusion, this study documents the generation of oxidative stress in tea upon exposure to Cu and Al. However, more increase in ascorbate and proline contents and less degradation in chlorophyll and protein contents of Chinary than Assamica suggested Chinary to be more tolerant to such stresses. Additionally, greater increase in transcript expression levels of glutathione metabolic genes in Chinary than Assamica upon exposure to Cu and Al strengthen its tolerance capacity.

References

Ahsan, N., Lee, D.G., Lee, S.H., Kang, K.Y., Lee, J.J., Kim, P.J., Yoon, H.S., Kim, J.S., Lee, B.H.: Excess copper induced physiological and proteomic changes in germinating rice seeds. - Chemosphere **67**: 1182-1193, 2007.

Alia, Saradhi, P.P., Mohanty, P.: Involvement of proline in protecting thylakoid membranes against free radical-induced photodamage. - J. Photochem. Photobiol. B **38**: 253-257, 1997.

Bálint, A.F., Röder, M.S., Hell, R., Galiba, G., Börner, A.: Mapping of QTLs affecting copper tolerance and the Cu, Fe, Mn and Zn contents in the shoots of wheat seedlings. - Biol. Plant. **51**: 129-134, 2007.

Basak, M., Sharma, M., Chakraborty, U.: Biochemical response of *Camellia sinensis* (L.) O. Kuntze to heavy metal stress. - J. environ. Biol. **22**: 37-41, 2001.

Bates, L.S., Waldren, R.P., Teare, I.D.: Rapid determination of free proline for water-stress studies. - Plant Soil **39**: 205-207, 1973.

Blum, R., Beck, A., Korte, A., Stengel, A., Letzel, T., Lendzian, K., Grill, E.: Function of phytochelatin synthase in catabolism of glutathione-conjugates. - Plant J. **49**: 740-749, 2007.

Bradford, M.N.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Cuypers, A., Koistinen, K.M., Kokko, H., Kärenlampi, S., Auriola, S., Vangronsveld, J.: Analysis of bean (*Phaseolus vulgaris* L.) proteins affected by copper stress. - J. Plant Physiol. **162**: 383-392, 2005.

Delhaize, E., Hebb, D.M., Richards, K.D., Lin, J.M., Ryan, P.R., Gardner, R.C.: Cloning expression of a wheat (*Triticum aestivum* L.) phosphatidylserine synthase cDNA: overexpression in plants alter the composition of phospholipids. - J. biol. Chem. **274**: 7082-7088, 1999.

Gallego, F.J., Benito, C.: Genetic control of aluminum tolerance in rye (*Secale cereale* L.). - Theor. appl. Genet. **95**: 393-399, 1997.

Goodwin, S.B., Sutter, T.R.: Microarray analysis of *Arabidopsis* genome response to aluminum stress. - Biol. Plant. **53**: 85-99, 2009.

Grill, E., Winnaker, E.L., Zenk, M.H.: Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothionineins. - Proc. nat. Acad. Sci. USA **34**: 439-443, 1987.

Hare, P.D., Cress, W.A.: Metabolic implications of stress induced proline accumulation in plants. - Plant Growth Regul. **21**: 79-102, 1997.

Kumar, S., Singla-Pareek, S.L., Reddy, M.K., Sopory, S.K.: Glutathione: biosynthesis, homeostasis and its role in abiotic stresses. - J. Plant Biol. **30**: 179-187, 2003.

Liso, R., De Tullio, M.C., Ciraci, S., Balestrini, R., La Rocca, N., Bruno, L., Chiappetta, A., Bitonti, M.B., Bonfante, P., Arrigoni, O.: Localization of ascorbic acid, ascorbic acid oxidase, and glutathione in roots of *Cucurbita maxima* L. - J. exp. Bot. **55**: 2589-2597, 2004.

Maksymiec, W., Krupa Z.: Effects of methyl jasmonate and excess copper on root and leaf growth. - Biol. Plant. **51**: 322-326, 2007.

Maksymiec, W., Wojcik, M., Krupa, Z.: Variation in oxidative stress and photochemical activity in *Arabidopsis thaliana* leaves subjected to cadmium and excess copper in the presence or absence of jasmonate and ascorbate. - Chemosphere **66**: 421-427, 2007.

May, M.J., Vernoux, T., Sanchez-Fernandez, R., Van Montagu, M., Inze, D.: Evidence for posttranscriptional activation of gamma-glutamylcysteine synthetase during plant stress responses. - Proc. nat. Acad. Sci. USA **95**: 12049-12054, 1998.

Mohanpuria, P., Rana, N.K., Yadav, S.K.: Cadmium induced oxidative stress influence on glutathione metabolic genes of *Camellia sinensis* (L.) O. Kuntze. - Environ. Toxicol. **67**: 368-374, 2007.

Mossor-Pietraszewska, T., Kwit, M., Legiewicz, M.: The influence of aluminium ions on activity changes of some dehydrogenases and aminotransferases in yellow lupine. - Biol. Bull. Poznan **34**: 47-48, 1997.

Mouratao, M.P., Martins, L.L., Campos-Andrada, M.P.: Physiological responses of *Lupinus luteus* to different copper concentrations. - Biol. Plant. **53**: 105-111, 2009.

Nguyen, V.T., Burow, M.D., Nguyen, H.T., Le, B.T., Le, T.D., Paterson, A.H.: Molecular mapping of genes conferring aluminum tolerance in rice (*Oryza sativa L.*). - Theor. appl. Genet. **102**: 1002-1010, 2001.

Quartacci, M.F., Cosi, E., Navari-Izzo, F.: Lipids and NADPH-dependent superoxide production in plasma membrane vesicles from roots of wheat grown under copper deficiency or excess. - J. exp. Bot. **52**: 77-84, 2001.

Rao, K.V.M., Sresty, T.V.S.: Antioxidative parameters in the seedlings of pigeonpea (*Cajanus cajan* (L.) Millspaugh) in response to Zn and Ni stresses. - Plant Sci. **157**: 113-128, 2000.

Richards, K.D., Schott, E.J., Sharma, Y.K., Davis, K.R., Gardner, R.C.: Aluminum induces oxidative stress genes in *Arabidopsis thaliana*. - Plant Physiol. **116**: 409-418, 1998.

Sanita di Toppi, L., Gabbrielli, R.: Response to cadmium in higher plants. - Environ. exp. Bot. **41**: 105-130, 1999.

Sarry, J.E., Kuhn, L., Ducruix, C., Lafaye, A., Junot, C., Hugouvieux, V., Jourdain, A., Bastien, O., Fievet, J.B., Vailhen, D., Amekraz, B., Moulin, C., Ezan, E., Garin, J., Bourguignon, J.: The early responses of *Arabidopsis thaliana* cells to cadmium exposure explored by protein and metabolite profiling analyses. - Proteomics **6**: 2180-2198, 2006.

Shamsi, I. H., Wei, K., Jilani, G., Zhang, G.P.: Interactions of cadmium and aluminum toxicity in their effect on growth and physiological parameters in soybean. - J. Zhejiang Univ. Sci. **8**: 181-188, 2007.

Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takeda, T., Yabuta, Y., Yoshimura, K.: Regulation and function of ascorbate peroxidase isoenzymes. - J. exp. Bot. **53**: 1305-1319, 2002.

Singh, K., Raizada, J., Bhardwaj, P., Ghawana, S., Rani, A., Singh, H., Kaul, K., Kumar, S.: 26S rRNA-based internal control gene primer pair for reverse transcription-polymerase chain reaction-based quantitative expression studies in diverse plant species. - Anal. Biochem. **335**: 330-333, 2004.

Singla-Pareek, S.L., Yadav, S.K., Pareek, A., Reddy, M.K., Sopory, S.K.: Transgenic tobacco overexpressing glyoxalase pathway enzymes grow and set viable seeds in zinc-spiked soils. - Plant Physiol. **140**: 613-623, 2006.

Wang, C., Oliver, D.J.: Cloning of the cDNA and genomic clones for glutathione synthetase from *Arabidopsis thaliana* and complementation of a gsh2 mutant in fission yeast. - Plant mol. Biol. **31**: 1093-1104, 1996.

Xiang, C., Oliver, D.J.: Glutathione metabolic genes co-ordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. - Plant Cell **10**: 1539-1550, 1998.