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MORPHOLOGICAL AND PROTEIN PROFILE ALTERATIONS IN *Withania somnifera* L. WITH RESPONSE TO IRON STRESS

JYOTI RANJAN ROUT[a1] AND SANTI LATA SAHOO[b]

Biochemistry and Molecular Biology Laboratory, P. G. Department of Botany, Utkal Univ., Vani Vihar, Bhubaneswar, Odisha, India

[a]E-mail: routjr@gmail.com

[b]E-mail: santi_bot_uu@yahoo.co.in

ABSTRACT

In order to access the role of Fe-toxicity towards growth and protein profile of *Withania somnifera* L., the plants were exposed to increasing concentrations (Control, 25, 50, 100 and 200 µM) of Fe for a period of 7 days under *in vitro* condition. Present study has demonstrated that the exposure of plants to Fe, resulted in the reduction of growth parameters, even at low concentration of Fe (25 µM). The total protein content (11.33 mg g^{-1}) suddenly increased in leaf samples at 25 µM of Fe in comparison to control (8.42 mg g^{-1}) and then declined. Similar results were observed in case of root samples that the maximum protein was found at 25 and 50 µM (7.82 and 8.92 mg g^{-1}, respectively) than that of control (6.42 mg g^{-1}). To enhance the understanding of the molecular mechanisms, comparison of protein profile changes due to Fe stress is done by SDS-PAGE analysis and found that several new protein bands like 98.14, 38.16, 23.27, 21.25 and 17.49 kDa are newly synthesized and some (87.53 and 66.70 kDa) are expressed more, in leaf samples of treated plants. In root samples, two different polypeptides (147.82 and 131.55 kDa) have appeared in both the 25 and 50 µM of treatment whereas, peptide like 26.88 kDa completely disappeared under Fe stress. The work suggests the relationship between Fe excess and alterations of protein patterns which will provide a new insight for better understanding of the molecular basis of nutritional stress responses to medicinal plants.

KEYWORDS : Protein profile, *Withania somnifera*, Fe-toxicity, SDS-PAGE, nutritional stress

Among all toxicants, iron (Fe) is considered as one of the major pollutants in the current environment which is being happening due to the increasing intensity of domestic sewage, industrial waste, agricultural runoff, heavy metals and other toxic waste materials. Fe is an essential nutrient for plants. It functions to accept and donate electrons and plays important roles in the electron-transport chains of photosynthesis and respiration. Especially in plant tissues, up to 80 % of iron is found in the chloroplasts (Hansch and Mendel, 2009). But iron is toxic when it accumulates to high levels. It can act catalytically via the Fenton reaction to generate hydroxyl radicals, which can damage lipids, proteins and DNA, all of which could lead to growth inhibition (Li et al., 2012).

The word 'proteomics' defined as the systematic study of the proteins expressed by the genome, is not only a powerful molecular tool for describing complete proteomes at the organelle, cell, or tissue level, but also for comparing proteins under different stressful environmental factors like metal stress. Even though the various physiological and biochemical analysis have been investigated towards the effect of iron on *Triticum aestivum* (Li et al., 2012), the proteomic approach has remained limited on effect of iron on medicinal plant. The expression of different polypeptides under heavy metal excess and less are detected by SDS-PAGE analysis because metals are required by plants as components in protein synthesis. In excess, plants have developed both a strategy of avoiding uptake of these metals and ability to synthesize proteins and peptides then can tightly bind and seize these metals (El-Aref and Hamada, 1998). By grouping proteomic, genomic and metabolic, it is possible to get a comprehensive and exhaustive analysis of the mechanisms of plant defense against metal stress.

The present research aimed to study the effects of Fe stress on growth response and changes in protein patterns on *W. somnifera* by using SDS-PAGE analysis, which provides additional information on the proteomic basis of iron tolerance in Ashwagandha.

MATERIALS AND METHODS

Plant Material and Culture Condition

The mature seeds of Ashwagandha (*Withania somnifera* L.) were harvested from Botanical garden of Post Graduate Department of Botany, Utkal University, Bhubaneswar, Odisha, India and air dried at 25 ± 3°C and stored in brown bottle at room temperature (18-25°C). For the experiment, the seeds were washed thoroughly under

[1]**Corresponding author**

running tap water for 30 min followed by treatment with an aqueous solution of 5 % teepol for 10 min and rinsed five times with double distilled water. After that, the surface of selected seeds were disinfected with an aqueous solution of 0.1 % $HgCl_2$ for 5 min and rinsed five times with autoclaved double distilled water. The disinfected seeds were inoculated in 150 ml Erlenmeyer flasks (Borosil, Bangalore, India) containing half-strength MS basal medium (Murashige and Skoog, 1962), with 0.7 % (w/ v) agar. The pH of the medium was adjusted to 5.8 ±0.2 before autoclaving at 121°C for 15 min. The seeds were germinated at 25±1°C, 60 % relative humidity and 16 h photoperiod (2000 photon flux intensity) provided by cool white fluorescent tubes (Philips, Bangalore, India). The germinated plants were subcultured for four weeks in the same medium. The healthy and uniformly grown plantlets were transferred to full-strength MS liquid medium. After three weeks, the plantlets were transferred to MS liquid medium (control) or MS liquid medium with different concentrations (Control, 25, 50, 100 and 200 µM) of Fe in the form of $FeSO_4$ for 7 days. No plant growth regulators were added in any medium during the whole experiment.

Growth Parameters

By the end of the 7 days under Fe treatment, plants were gently removed from the MS liquid medium, washed with distilled water, soaked in blotting papers and then various growth parameters like plant height, root length, shoot length, leaf length and total number of leaves per plant were recorded.

Extraction and Estimation of Total Protein

For estimation of total protein, leaf and root tissues were collected from control as well as Fe treated plants after 7 days of the treatment. Samples (500 mg) were macerated in a pre-cold mortar and pestle by adding 1.5 ml of 50 mM potassium phosphate buffer (pH 7.8), 50 mM EDTA, 2 mM PMSF and 10 % (w/ v) PVP to a fine slurry followed by centrifugation at 14000 g for 15 min at 4°C (Remi Instruments, India). The supernatants were stored at -20°C in small aliquots for analysis of protein estimation and protein profile by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Quantitative estimation of protein was done according to the method of Lowry et al., (1951) by measuring absorbance at 750 nm, using bovine serum albumin as a standard and expressed as mg g^{-1} fresh weight (f.w.).

Analysis of Protein Profile by SDS-PAGE

The supernatant containing 50 µg of protein was mixed with equal volume of solubilizing buffer [62.5 mM Tris-HCl, pH 6.8, 20 % (w/ v) glycerol, 2 % (w/ v) SDS, 5 % (v/ v) 2-mercaptoethanol and 0.01% bromophenol blue] and heated for 4 min at 95°C, cooled on ice before loading on 10 % polyacrylamide slab gels. Gels were made according to Laemmli (1970). A 10 % separating gel containing 375 mM Tris- HCl, pH 8.8, 0.1 % (w/ v) SDS, 0.05 % (w/ v) ammonium persulfate and 0.4 µl ml^{-1} TEMED was used for resolving the polypeptides whereas a 5 % stacking gel containing 125 mM Tris-HCl, pH 6.8, 0.1% (w/ v) SDS, 0.05 % (w/ v) ammonium persulfate and 0.5 µl ml^{-1} TEMED was used to concentrate (stock) the polypeptides. The electrophoresis running buffer consisted of 25 mM Tris, 192 mM glycine and 0.1 % SDS, pH 8.3. Electrophoresis was accomplished at 35 mA for 4 h using a GeNei electrophoresis system (Bangalore, India). The gels were stained by silver staining method until the background was clear (Switzer, 1979). The gel was then scanned and photographed by gel documentation system and analyzed with quantity one software from Bio-Rad (Bio-Rad, Italy). For all the SDS-PAGE, standard protein markers [phosphorylase b (97.4 kDa); bovine serum albumin (66.0 kDa); ovalbumin (43.0 kDa); carbonic anhydrase (29.0 kDa); lactoglobulin (18.4 kDa)] was used as control.

Statistical Analysis

The physiological parameters and protein contents were the mean of independent experimental replicates (n = 3). Means and Standard errors were carried out for each treatment.

RESULTS AND DISCUSSION

Growth Parameters

The young and healthy plants were exposed to increasing concentration of $FeSO_4$ in MS liquid medium for 7 days. In Table,1 the variation of changes of growth parameters in control as well as treated plants are presented. Data showed the growth of *Withania* plants reflect the

Table 1: Growth parameters of treated and non-treated (control) plants of *Withania somnifera* L. under Fe stress

Treatment (µM)	Plant height (cm)	Root length (cm)	Shoot length (cm)	Leaf length (cm)	No. of leaves/ plant
Control	19.41±0.32	6.44±0.22	12.97±0.33	1.92±0.11	14.00±0.10
25	19.03±0.61	6.11±0.34	12.92±0.21	1.77±0.63	13.00±0.65
50	17.51±0.55	5.70±0.19	11.81±0.43	1.21±0.34	10.00±0.20
100	15.77±0.95	4.55±0.22	11.22±0.14	0.91±0.35	8.00±0.85
200	14.88±0.71	4.06±0.26	10.82±0.30	0.88±0.20	8.00±0.45

The data represents mean ± SE of replicates (n = 3).

toxicity effects of iron. In low concentration of Fe (25 µM), slight inhibition of growth were noticed, but an overall inhibition was observed in higher concentration. The maximum reduction was occurred in 200 µM of treatment. However, the parameters like plant height, root length, shoot length, leaf length and number of leaves per plant decreased by different degree of percent (76.66, 63.04, 83.42, 45.83 and 57.14 %, respectively) in 200 µM of Fe when compared to control plants (Table,1). The result is in conformity with the result of Nenova, (2006), who observed major changes of morphological parameters in pea plant occurred with an increasing concentration of Fe and concluded that the growth indexes are concentration dependant. The percentage of inhibition is more in root growth than the shoot as the roots are easily affected region to metal toxicity. The present investigation was also supported with the findings of Snowden and Wheeler,

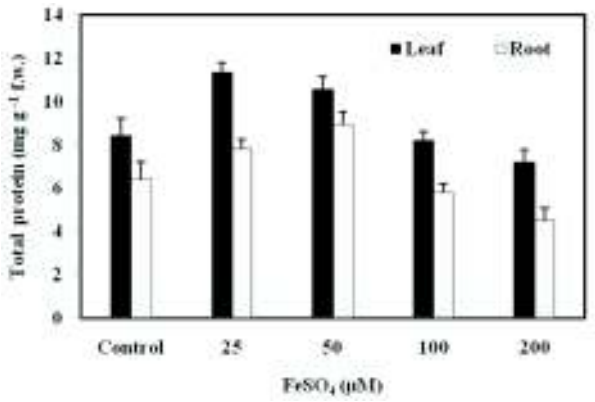

Figure 1: Effect of iron (Control, 25, 50, 100 and 200 µM $FeSO_4$) on total protein estimation in leaves and roots of *Withania somnifera* L. after 7 days of treatment. The data represents mean ± SE of replicates (n = 3)

(1993) that root enlargement of most investigated dicotyledonous fen species was more affected than shoot growth. However, in a plant cell, after up-taking excess amount of Fe, the generation of active oxygen species is increased, which caused the toxicity and the growth parameters that are the ever seen procedure to take the necessary action to prevent the environmental stress like metals.

Changes in Protein Content

Comparbly, more amount of proteins were found in leaf samples (8.42 mg g^{-1}) than roots (6.42 mg g^{-1}) in control plants (Fig., 1). The total protein content of leaves increased at 25 µM (11.33 mg g^{-1}) and 50 µM (10.54 mg g^{-1})) treatment, which was 1.34 and 1.25 times higher over the control plants (8.42 mg g^{-1}) . But at 100 and 200 µM of Fe, amount of protein content (8.20 and 7.21 mg g^{-1}, respectivelly) decreased. In root tissues, the highest protein content was observed in 50 µM Fe treated plants and was significantly higher (8.92 mg g^{-1}) than that of control (6.42 mg g^{-1}). The maximum negative effect of protein content (4.50 mg g^{-1}) was observed in roots, at higher concentration (200 µM) of treatment and was 1.42 time less as compared to control (6.42 mg g^{-1}). Protein content in leaves and roots increased significantly up to a certain limit and then decreased. The proteomes in an organism acts as an indicator of reversible and irreversible changes in metabolism, is known to respond to a wide variety of stress materials such as metals and xenobiotics (Sing and Tewari, 2003). However, early evidence suggests that combined effect of metals such as Al, Cu and Cd induce the detrimental effect of soluble protein in barley plants (Guo et

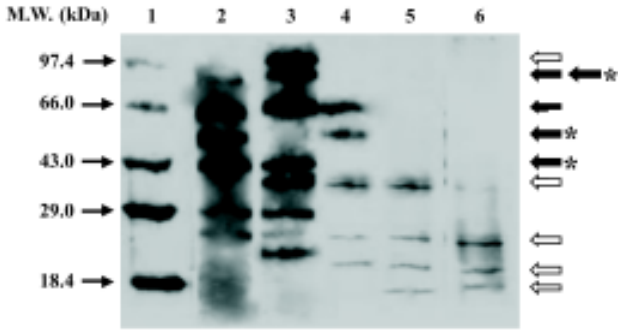

Figure 2: Changes in protein profile in leaf samples of *Withania somnifera* L. after 7 days treatment with iron. Lane 1, molecular weight marker; lane 2, control; lane 3, 25 µM; lane 4, 50 µM; lane 5, 100 µM; lane 6, 200 µM of iron-treated samples. The white and black arrows indicate newly synthesized and increased expression of polypeptides respectively. Whereas, the black arrows with asterisk are identified as polypeptides disappeared upon Fe stress

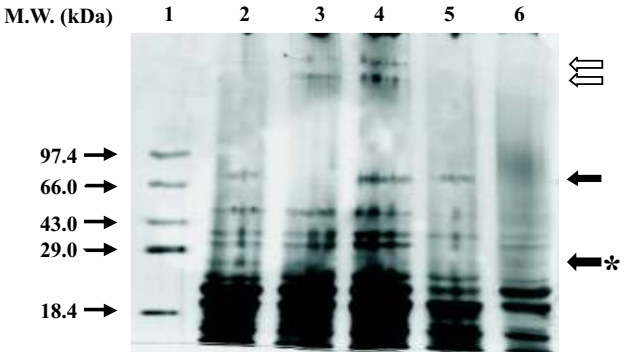

Figure 3: Changes in protein profile in root samples of *Withania somnifera* L. after 7 days treatment with iron. Lane 1, molecular weight marker; lane 2, control; lane 3, 25 µM; lane 4, 50 µM; lane 5, 100 µM and lane 6, 200 µM of iron-treated samples. The white and black arrows indicate newly synthesized and increased expression of polypeptides respectively. Whereas, the black arrow with asterisk is identified as polypeptides disappeared upon Fe stress

al., 2007). Sometimes, the increasing trends of total protein content was also observed in Cd and Pb stress in *Brassica juncea* (John et al., 2009). Elevated concentration of Fe may effect the other nutrients uptake by plants and causes toxicity. The toxicological effect hampers the alternation of protein content in meta treated plants. The mechanism by which Fe affects protein content is still unknown and needs a further analytical study.

SDS-PAGE Analysis

The SDS-PAGE analysis of the protein profile in leaf and root samples of Fe-treated plants revealed major changes than control. The silver stained one dimensional banding pattern of proteins shown in Figs,2 and 3, indicates the differences between control and treated plants. After day 7 treatment, the control leaf samples synthesized six polypeptides (87.53, 66.70, 54.56, 43.12, 30.03 and 25.56 kDa) but in 25 µM of Fe, three new polypeptides of molecular weight 98.14, 38.16 and 23.27 kDa were newly appeared. The polypeptides like 87.53, 66.70, 43.12, 30.03, and 25.56 kDa were completely disappeared in higher concentration (100 and 200 µM) of Fe. However, protein band of 87.53 kDa expressed more in response to 25 µM treatment in comparison to control (Fig., 2). The treated as well as control root samples comparatively yielded more number of polypeptides than leaves. In response to Fe, the 7 days treated (25 and 50 µM) roots able to develop two new polypeptides (147.82 and 131.55 kDa, respectively). Peptide like 75.36 kDa expressed slightly in 50 µM of Fe treatment and 26.88 kDa of protein totally disappeared from all treated plants. Similar results were obtained in higher concentration that, a group of polypeptides were completely disappeared in 100 and 200 µM of treatment (Fig., 3). The disappearance and reappearance of some proteins and de novo synthesis of others in response to Fe exposure indicated a direct relationship of metal stress induced proteomics. Similar observations were also reported by El-Aref and Hamada, (1998) under copper stress in tomato plants. Since proteins were newly synthesized under Fe-stress, it appears to have a role in the mechanism of Fe tolerance which allows making biochemical and structural adjustments that enable the plant to cope with stress conditions.

CONCLUSION

The findings lead us to conclude that there is a relationship between Fe-excess and alternation of protein patterns. Thus the analysis of protein profile seems to be a good indicator to estimate the level of nutritional stress to medicinal plants. Molecular genetics studies should complement the morphological and proteomic approaches concerning the role of iron in protein expression for increased plant resistance to metal stress.

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REFERENCES

El-Aref H. M. and Hamada A. M., 1998. Genotypic differences and alternations of protein patterns of tomato explants under copper stress. Biol. Planta, **41**(4): 555-564.

Guo T. R., Zhang G. P. and Zhang Y. H., 2007. Physiological changes in barley plants under combined toxicity of aluminum, copper and cadmium. Colloids Surfaces B: Biointerfaces, **57**(2): 182-188.

Hansch R. and Mendel R. R., 2009. Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). Curr. Opin. Plant Biol., **12**: 259-266.

John R., Ahmad P., Gadgil K. and Sharma S., 2009. Heavy metal toxicity: effect on plant growth, biochemical parameters and metal accumulation by *Brassica juncea* L. Int. J. Plant Product, **3**(3): 65-75.

Laemmli U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**: 680-685.

Li X., Ma H., Jia P., Wang J., Jia L., Zhang T., Yang Y., Chen H. and Wei X., 2012. Responses of seedling growth and antioxidant activity to excess iron and copper in *Triticum aestivum* L. Ecotoxicology Environ. Saf., **86**: 47-53.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., **193**: 265-275.

Murashige T. and Skoog F., 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant, **15**: 473-497.

Nenova V., 2006. Effect of iron supply on growth and photosystem II efficiency of pea plants. Gen. Appl. Plant Physiol.: 81-90.

Sing P. K. and Tewari R. K., 2003. Cadmium toxicity induced changes in plant water relations and oxidative metabolism of *Brassica juncea* L. plants. J. Environ. Biol., **24**: 107-112.

Snowden R. and Wheeler B. D., 1993. Iron toxicity to fen plant-species. J. Ecology, **81**: 35-46.

Switzer R. C., 1979. A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal. Biochem., **98**: 231-237.