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Zinc dependant polypeptides in different compartments of *Lathyrus- Rhizobium* sp. Symbiosomes

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SUMMARY

To analyze the effect of zinc deficient condition on N_2 – fixing root nodules, the proteins were analyzed in different compartments of *Lathyrus – Rhizobium* sp. Symbiosomes as a model symbiotic system. *Lathyrus sativus* plants were grown in pots containing *Rhizobium* – inoculated acid washed river sand irrigated by nitrogen free plant growth medium with and without zinc. Symbiosomes, isolated from N_2- fixing root nodules were sub-fractionated and protein profiles in the different fractions (host plant cytoplasm, peribacteroid space and bacteroid cytosol) were analyzed through SDS-PAGE. In all different fraction high protein content was observed in plants grown at optimal zinc concentration of 1.0mg/L. The increase in protein was due both to a general increase in abundance of the most dominant polypeptides and to a pronounced increase in the abundance of specific polypeptides. Besides the unique pattern of protein in each compartment, polypeptides of same molecular size were also observed in the symbiotic interface PBS.

Key words : *Lathyrus sativus* - *Rhizobium*, Symbiosomes, Zinc, Polypeptides

Zinc has a unique place in the biology of planet earth. About 50% zinc deficient soil of the developed and developing world is recognized as a serious threat to both crop production and human health globally. Across all phyla from bacteria to humans, more proteins bind or require zinc for their function than those binding all other biologically essential cations combined (Gladshev *et al.*, 2004 ; Cakmak, 2008).

Zn is an essential catalytic component of over 300 enzymes and metalloenzyme complexes. It also plays a critical structural role in many proteins. For example, several motifs found in transcriptional regulatory proteins are stabilized by Zn, including the Zn finger, Zn cluster, and RING finger domains. Inside cells, Zn is neither oxidized nor reduced; thus, the essential roles of Zn in cells is based largely on its behavior as a divalent cation that has a strong tendency to form stable tetrahedral complexes. The Zn deficiency in plants has been recognized as a serious problem. Zn deficiency remarkably depresses the protein content and affects their polypeptides composition in the cells. Zn deficiency may be diagnosed by a combination of visual symptoms and soil analysis.

Despite the importance of Zn as an essential micronutrient for plant growth, it acts as environmental toxic factor and is known to affect the nodulation and dinitrogen fixation. The toxic effects of zinc and other heavy metals are explained by their interaction with sulphahydryl groups and concomitent inactivation of proteins (Assche and Clijesters, 1990). Therefore, it is generally accepted that tolerance towards zinc stress is based on mechanisms that maintain optimum and low free concentrations in the cytoplasm In addition, ressearchers have already suggested a role of zinc in maintaing the membrane integrity and H^+ - ATPase dependent pH gradient across the membrane. It is essential to investigate the role of zinc in the symbiotic dinitrogen fixation process because of the importance of the relationship established across the membrane between the legume and *Rhizobium*.

Root nodulation starts with a molecular dialogue between two partners and takes place through a series of developmental stages. During nodule development the rhizobia are released from the infection thread into the host cortical cell as bacteroids in a process resembling endocytosis, forming a new compartment within the host cell, the symbiosome (Roth *et al.*, 1989).We still do not know the detail mechanisms underlying Zn dependency and N_2-fixation of grasspea. Therefore, present study was intented as an effort in this direction.

MATERIALS AND METHODS

Seeds of *Lathyrus sativus* (Grass pea) were procured from local farmers of Doon valley and germinated seedlings were used for nodule isolated

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Rhizobium inoculation assays. The seeds were rinsed in 95% ethanol followed by immersion in 0.2% mercuric chloride ($HgCl_2$) acidified with 0.5% HCl for 3 minutes The seeds were then washed thoroughly with at least 5 changes of sterile water and directly spread on 1% agar Petriplates. TY broth were inoculated with isolated bacterial strains for 5 days to late exponential phase (O.D 600= 1) on a rotary shaker (100 r.p.m) at $28^0 - 30^0$C. The broth medium contained from 10^7- 10^9 cell/ml as determined by serial dilution and plate counting on the same day was used in the pot culture experiment. TY broths were diluted using N- free plant growth medium with and without zinc to obtain the appropriate cell number for experiments. The seedlings about 2-5 days old were transplanted in 1.5 L plastic pots filled with sand containing fine granular $CaCO_3$ in the ratio of 2:1 to control the rhizosphere acidification and frequently irrigated by N-free plant growth medium (pH 6.8) with and without zinc to maintain a maximum water holding capacity of nearly 70%. After 8 weeks nodules were harvested from the plants for the isolation and fractionation of symbiosomes. The purification of symbiosomes was done according to the methods followed by Rosendahl *et al.* (1992) with a slight modifications of Gordon *et al.* (1999). The plant cytoplasmic proteins were extracted at this stage. The procedure used to sub-fractionation of symbiosomes was modified from the method of Verma *et al.* (1978). The bacteroids and peribacteroids space proteins were recovred. The nodulation potential was evaluated and the protein estimation was done according to the method of Lowry *et al.* (1951). Separation of Proteins was done by 12% sodium dodecyl sulphate-polyacrylmide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed on the protein samples according to Laemmli (1970) using Hoeffer Scientific Electrophoresis unit. Molecular weight of different protein bands were determined by constructing a plot of $\log_{10}$ polypeptide molecular mass versus relative mobility (R_f).

RESULTS AND DISCUSSION

The results obtained from the present investigation are presented below:

Symbiotic effectiveness of Rhizobium leguminosarum isolates:

The potential of *rhizobium- Leguminosarum* isolates to nodulate the *Lathyrus sativus* plant was determined by nodulation assay in pot culture experiments. The results of the nodulation assay are indicated in Table 1. Under normal or deficient conditions, the isolates were able to produce nodules in *Lathyrus sativus* seedlings in about 8 weeks. The response of isolates was observed maximum in normal plants growing in 1.0mgL^{-1} concentration of zinc as compared to zinc deficient plants. Zinc deficient condition suppressed the formation of the nodules in plants.

Table 1 : Nodulation response of *Rhizobium Leguminosarum* isolates in *Lathyrus sativus* plants treated with and without zinc

Zinc treatment (mgL^{-1})	No. of nodules per plant inoculated with Rhizobium isolates
1.0	7
00	2

Purity of symbiosomes and their sub fractions:

In order to examine the proteins in the symbiotic interface, previous procedure for isolation of symbiosomes were optimized .(Rosendahl *et al.*, 1992; Verma *et al.*, 1978). Optimized procedure results in highly purified symbiosomes from root nodules with no detectable contamination with plant organelles. Plastids and mitocondria are potential contaminants. Similarly, sub-fractionation of symbiosomes was performed by optimizing the procedure described by Simonsen and Rosendahl (1999) and Gorden *et al.* (1999) in order to minimize cross contamination of one compartment fraction by another. The vortex mixing of the symbiosomes releases the content of PBS and may at the same time release proteins loosely associated with peribacteroid membrane and outer bacteroid membrane.

Protein patterns of inoculated nodules:

Table 2 and 3 and Fig. 1, 2 and 3 show the significant changes in cytoplasmic host plant proteins and symbiosomic (PBS and Bacteroid cytosol) proteins at normal and zinc deficient conditions. Results indicate zinc dependant changes in both qualitative or quantitative patterns of proteins.

Quantitative changes:

The protein content in host plant cytoplasm and both symbiosomic sub-fractions (PBS and Bacteroid cytosol) of zinc treated nodules was higher as compared to zinc deficient nodules.

Qualitative changes:

Table 2 : Protein content of zinc treated nodules

Zinc treatment mgL^{-1}	Protein content (mg g^{-1} weight of nodules)		
	Cytoplasmic	PBS	Bacteroid
1.0	42.87	22.13	32.25
00	40.25	19.87	29.25

Table 3 : Profile of proteins in nodules treated with and without zinc

Zinc treatment mgL^{-1}	Molecular weight of proteins (kDa)		
	Nodule Cytoplasm	Symbiosomic PBS	Bacteroid cytosol
1.0	45, 43, 32, 29, 25, 23, 18, 17.5, 14, 7 and 4	45, 43, 29, 25, 18, 17.5, 14, 7 and 4	43, 38, 29, 22,14, 7 and 4
00	32, 29, 27, 17.5, 14, 12, 7 and 4	28, 27, 25, 12, 5 and 4	28, 22, 14, 6.5 and 3

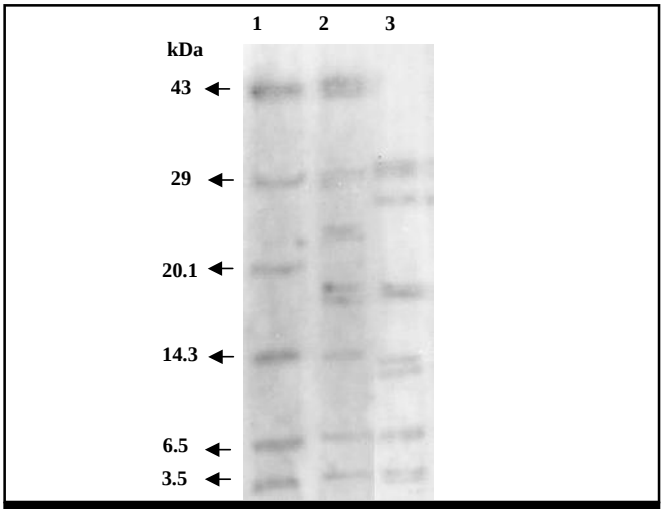

Fig. 1 : SDS- PAGE profile of cytoplasmic host plant proteins of inoculated nodules treated with (1.0mgL^{-1} : Lane 2) and without (Lane 3) zinc

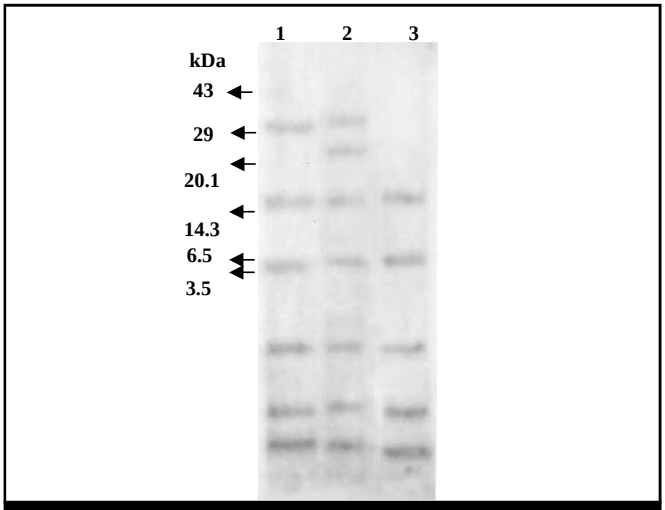

Fig. 3: SDS- PAGE profile of Bacteroid cytosol proteins treated with (1.0 mgL^{-1} : Lane 2) and without (Lane 3) zinc

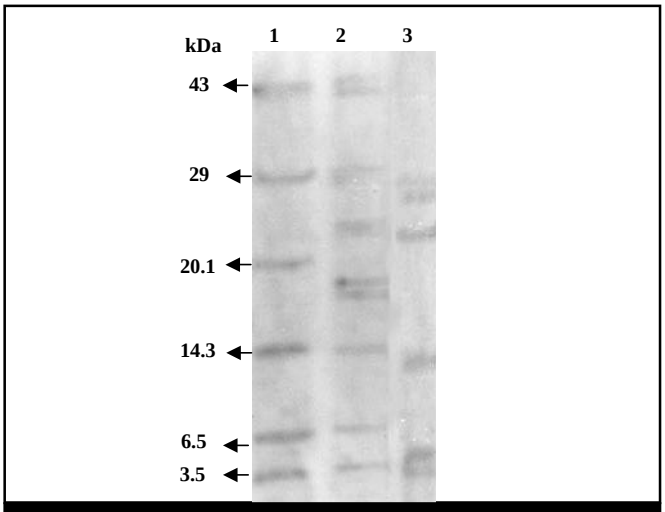

Fig. 2 : SDS- PAGE profile of Peribacteroid space (PBS) proteins of nodules treated with (1.0mgL^{-1} : Lane 2) and without (Lane 3) zinc

Fig 1, 2 and 3 show SDS – PAGE profiles and Table 3 summarize the data of molecular weights of host plant cytoplasmic, peribacteroid space and bacteroid cytosol polypeptides, respectively.

Cytoplasmic host plant proteins:

Eleven (low and high molecular weight) polypeptides were observed in nodules of plants grown at optimal level of zinc. Polypeptides of 45, 43, 25, 23 and 18 kDa were not observed in zinc deficient nodules. Instead two polypeptides of 27 and 12 kDa were observed specifically in zinc deficient nodules. This suggests a role of zinc in the synthesis of average molecular weight plant proteins in nodule.

Peribacteroid space proteins:

Nine polypeptides (45, 43, 29, 25, 18, 17.5, 14, 7 and 4.0 kDa) of plant origin were found in PBS of zinc treated nodules. However, polypeptides of 45, 43, 29, 18, 17.5, 14 and 7.0 were absent in zinc deficient nodules and may be considered as zinc dependant for their normal synthesis. Interestingly, polypeptides of 27 and 12 kDa seemed to be the essential polypeptides for sustaining nodules under zinc deficient conditions. Additional polypeptides of 28 and 5.0 kDa were also observed in PBS of zinc deficient nodules. These might be of bacterial origin.

Bacteroid cytosol proteins:

Seven polypeptides (43, 38, 29, 22, 14, 7 and 4 kDa) were observed in bacteroid cytosol of zinc treated nodules. Polypeptides of molecular weight 43 and 38 kDa were not synthesized under zinc deficient conditions. However polypeptides, of molecular weight 28, 6.5 and 3.0 kDa

appeared in zinc deficient conditions and seemed to be isoforms of molecular weight 29, 7.0 and 4.0 kDa, respectively.

This study is the initial effort in the direction of proteomics for a symbiotic organ nodule grown in with and without zinc conditions. The systematic analysis of the protein complement of an organism, organ, tissue, cell or cell fraction is termed as proteomics (Williams *et al*., 1996). It is a frequent observation, that polypeptides patterns of plants, tissues or cells change considerably in response to zinc regimes. In principle, two opposite casual interpretations of these changes are possible with respect to the physiological relevance: on the one hand zinc deficieny related proteins may be essential or atleast useful element of the plant's response in dealing with the deficiency and allowing for adaptations or development of tolerance. On the other hand, zinc deficiency may reflect changes in polypeptide composition just as a consequence of disregulation and metabolic disorder. As a third explanation which combines components of both extreme responses, maintenance of metabolic pathways in zinc deficiency may require up or down regulation of specific proteins. The results of this study must be considered in this context and may be applicable in the diagnosis of 90% phytoavailable Zn-deficient soil of Roorkee-Muzaffarnagar Indo-Gangetic plains (Singh *et al*., 2001).

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