Chapter 5

Role of Proline in modulation of Aluminum-induced stress responsive proteins: Relationship between Adaptation and Proline accumulation
5.1 Introduction

There is a sizeable volume of literature available regarding Al toxicity to higher plants and several hypotheses for mechanisms explaining Al-phytotoxicity have been postulated, including phosphorous starvation (Pettersson et al., 1985a), interference with mineral nutrition (Rufty et al., 1995), inhibition of ATPase activity (Woolhouse, 1969) and interference with signal transduction (Kochian, 1995). It is known that Al toxicity is an important growth-limiting factor for plants in acid soils below pH 5.0 though it can occur at higher pH levels also (Alam and Adams, 1979). On the other hand though Al is not regarded as an essential nutrient, but low concentrations can sometimes increase plant growth or induce other desirable effects. There are reports indicating that Al has a beneficial effect on plant growth and this seems to be especially true for native plant species that are adapted to acid soils (Watanabe et al., 2005).

Some plants have the ability to accumulate enormous amounts of Al without any evidence of injury or toxicity clearly indicating the phenomenon of Al tolerance. Although there are several reports demonstrating Al tolerance nevertheless the exact mechanism underlying Al-tolerance is least understood (Kochian, 1995). The findings of some researchers state that some species have developed mechanisms to cope with Al toxicity both internally and externally (Kochian et al., 2004), which helps them to grow on acid soils. The tolerance level varies with organism and also with species and among cultivars within species in case of plants and strains in case of microbes. Al toxicity and differential Al tolerance in various plant groups were reported in some studies (Anderson et al., 1988, Foy, 1988, Giannakoula et al.,
Understanding the nature of these tolerance mechanisms has been the focus of ongoing research in the area of stress physiology.

Acclimation of cyanobacteria to metals is the result of many different physiological and biochemical mechanisms, including a series of integrated events from stress signal perception, transduction to regulation of gene expression, which lead to the adaptive changes in growth, antioxidant defenses and many other changes at the molecular level (Kozlowski and Pallardy 2002, Zhang et al., 2005, Yin et al., 2005, Lei et al., 2006). Several researchers have reported metal-tolerance or metallo-adaptive response (Rieger, et al., 1990; Subhadra et al., 1994; Crawford and Davies, 1994; Bhargava et al., 2008). Cells or organisms pre-exposed to a low non- or sub-toxic dose of a metal are able to adapt to small fluctuations in their environment, or even resistant to and can survive dramatic changes by developing inherent adaptive mechanisms enabling themselves to lessen the damaging effects when exposed subsequently to a toxic dose of similar toxin (Patra et al., 2000).

As acclimation is a long-term process, and protein synthesis dependant, it is therefore imperative to study how proteins play functional roles in stress acclimation strategies and how these may be induced by some signaling molecules like proline which is considered as a signal/regulatory molecule able to activate multiple physiological or molecular responses (Taylor 1996, Oztork and Demir 2002). Khedr et al., (2003) demonstrated that proline induces the expression of salt-stress-responsive proteins which may improve the adaptation of
Pancratium maritimum L. to salt-stress. So far, Al has not been tested for induction of adaptive response in cyanobacteria.

Apart from the antioxidants which play a pivotal role in conferring physiological tolerance to organisms against different types of stresses, various low molecular weight antioxidants also contribute to metal tolerance. One of the potentially important mechanism of metal stress tolerance is osmotic adjustment which is mainly disturbed by dehydration stress causing metals like Al can be achieved from the accumulation of compatible solutes like proline under certain metal toxicity is the one of the major responses of microalgae and plants, which is possibly associated with the protection of plant cells against oxidative damage and with signal transduction (Choudhary et al., 2006). Although in the past, most attention has been concerned with the role of proline as a compatible osmolyte (Slama et al., 2006) and osmoprotectant (Serrano and Gaxiola, 1994), its accumulation under heavy metals (Tripathi and Gaur, 2004), high salinity (Miller et al., 2005; Chris et al., 2006) and light-induced stress (Abraham et al., 2003) nevertheless its mechanism and further roles in stress tolerance have received far less attention.

Further, exogenous proline has been reported to protect plants under stress. It improved the tolerance of somatic embryos of celery (Apium graveolens L. cv. SB 12) to partial dehydration (Saranga et al., 1992). Okuma et al., (2000) found that exogenous proline improved the growth of salt stressed tobacco cell cultures and the improvement was attributed to the role of proline as an osmoprotectant for enzymes and membranes against salt inhibition rather than as a compatible solute.
So far, we have noticed a unique pattern of results with Al exposure in *N. muscorum* showing less toxicity or protection as compared to Cu or Cd which actually have resulted relatively more damaging effects on various macromolecules. The fact that proline may confer a positive role in the protection of *N. muscorum* under Al stress along with the induction of various dehydrins-like proteins commonly known to get induced in higher plants under drought or dehydration stress prompted me to explore the proline accumulation in the Al acclimated *N. muscorum*. Further, the effect of another non-redox metal, Cd, on the proline contents was evaluated to see whether Al-induced proline really protects the *N. muscorum* from deleterious effects of Cd.
5.2 Material and Methods

5.2.1 Effect of exogenous proline on Cd toxicity in *N. muscorum*

1000 μM stock solution of proline was freshly prepared and the solution was further sterilized by passing through Millipore membrane filter (0.22 μm). From the stock solution, required concentrations of 1 and 10 μM of proline were freshly prepared in the BG-11(-ve) medium. To study the role of exogenous proline in ameliorating the effect of toxicity of Cd, logarithmic phase culture of *N.muscorum* was pre-incubated with various concentrations (1 and 10 μM) of proline for 24 h followed by removal of media. The proline pre-treated *N. muscorum* was exposed to 8 μM of Cd for 4 days. The dose of Cd was selected from the studies in the chapter 3 as this produced moderate toxicity. The intracellular proline content, chlorophyll a, total peroxides and lipid peroxidation were determined as described in the chapters 2 and 3.

5.2.2 Acclimation of *N.muscorum* to Al

Briefly, the stock solution of AlCl$_3$.6H$_2$O (1000 μM) was prepared in glass-distilled water and sterilized by passing through the Millipore membrane filter (0.22 μm) as described in sec 2.2.3. *N. muscorum* never exposed to Al has been used and is henceforth referred to as the control strain. For the acclimation of the *N. muscorum*, cells were initially subjected to very low dose (0.1 μM) of Al and subsequently transferred every 15 days to the higher concentrations (1, 10, 20, 30 and 40 μM) with regular growth study at each step of cells transfer to the higher concentration. Physiological acclimation of the control strain was obtained by successive sub-cultivation at increasing doses of Al up to 40 μM (hereafter
referred to as the acclimated strain) as described in Rai et al. (1991). The protein contents were measured by the Lowry et al (1951) at each successive treatment with higher dose of Al to see the proper growth of *N. muscorum*.

### 5.2.3 Effect of Cd on the Al-acclimated *N. muscorum*

The Al-acclimated *N. muscorum* was further treated with the Cd (8 µM) for 4 days to see the modulation of Cd toxicity in these acclimated strain. The dose of Cd and incubation time was selected on the basis of its moderate toxicity at this dose described in the earlier chapters. Selected parameters such as chlorophyll a, total peroxides, MDA and proline were measured in the control, Al-acclimated, Cd alone and Al-acclimated Cd treated *N. muscorum* as per the methods described earlier in the chapters 2 and 3.

### 5.2.4 Statistical analysis.

All experiments were performed using exponentially growing cultures and repeated three times to ascertain the reproducibility of the result. Values are represented as mean ± SE (n = 3).
5.3 Results

5.3.1 Ameliorative effect of exogenous proline on chlorophyll a, total peroxides and MDA content of N. muscorum exposed to Cd.

To verify whether exogenous proline modifies the internal amino acid content, the intracellular proline content of N. muscorum was estimated (Table 5.1). The level of internal proline increased by 11 and 33 % at 1 and 10 µM proline supplementation which indicates that proline was taken up by the cyanobacterium. Cd (8µM) alone treatment increased intracellular proline by 30% compared to the control. However, proline-treated (1 and 10 µM proline) N. muscorum exposed to Cd resulted in 8 and 16 % increase in proline contents as compared to Cd (8µM) alone treatment. While a decrease was seen in the intracellular proline content in N.muscorum exposed to higher concentration (16 µM) of Cd (see Fig 3.2 in Chapter 3). This may suggest a role of proline in neutralizing the toxic effects.

Further, the level of chlorophyll a (chl a) increased by 16 and 25% at 1 and 10µM proline supplementation which indicates that proline is a growth promoter as chl a content is growth linked (Table 5.1). Cd treated N.muscorum exhibited 30% decrease in chl a content, however, when proline-treated N.muscorum was exposed to Cd, the decrease was reduced to 25% and 15% at 1 and 10 µM proline, respectively. Further, the level of total peroxides and MDA was found to be lowered in the proline-treated Cd group as compared to direct exposure to Cd. Cd treatment enhanced the peroxide content by over 2-fold after four days of exposure. Contrary to this, proline pre-treated N. muscorum showed a decrease of
7 and 29% in the level of total peroxides by 1 and 10 µM proline, respectively (Table 5.1). One of the very important stress markers, lipid peroxidation measured in terms of MDA content also showed a similar pattern of alteration. Proline pre-treatment at 1 and 10 µM proline reduced the MDA content by 5 and 29% in Cd-treated group. These results reasonably indicate the ameliorative role of proline in protecting the cyanobacterial cells exposed to metal stress.
Table 5.1 Ameliorative effect of exogenous proline on chlorophyll a, total peroxides, MDA and intracellular proline contents of *N. muscorum* exposed to Cd.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Chl a</th>
<th>Total Peroxide</th>
<th>MDA</th>
<th>Intracellular Proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.20 ± 0.02</td>
<td>668 ± 8</td>
<td>771 ± 8</td>
<td>13.5 ± 0.6</td>
</tr>
<tr>
<td>Cd (8 µM)</td>
<td>0.84 ± 0.01</td>
<td>1403 ± 16</td>
<td>1542 ± 12</td>
<td>17.6 ± 1.0</td>
</tr>
<tr>
<td>Proline (1 µM)</td>
<td>1.40 ± 0.04</td>
<td>672 ± 8 ns</td>
<td>780 ± 8 ns</td>
<td>15.0 ± 1.0 ns</td>
</tr>
<tr>
<td>Proline (10 µM)</td>
<td>1.50 ± 0.04</td>
<td>675 ± 7 ns</td>
<td>774 ± 8 ns</td>
<td>18.2 ± 1.0</td>
</tr>
<tr>
<td>Proline (1µM)+Cd(8µM)</td>
<td>0.90 ± 0.02</td>
<td>1303 ± 14</td>
<td>1465 ± 12</td>
<td>19.0 ± 1.2</td>
</tr>
<tr>
<td>Proline (10 µM)+Cd(8µM)</td>
<td>1.02 ± 0.02</td>
<td>1002 ± 11</td>
<td>1095 ± 11</td>
<td>20.5 ± 1.5</td>
</tr>
</tbody>
</table>

The values represent Means ± SE (n=3). All the treatments are significantly different (P < 0.01) from control (Student’s t-test). ns= not significant. Units for Chl a (µg/ml); Total peroxide (µmol / 10^2 g dry weight); MDA (nmol/g dry weight); proline (µg/g dry weight)
5.3.2 Al acclimation of *N. muscorum*

The gradual increase in one of the growth parameter, protein contents after each acclimation starting from 1 to 40 µM concentration of Al showed that the acclimation with increasing concentrations was successful and the *N. muscorum* adapted and tolerated (Figure 5.1). This was further taken to see the effect of Cd on the Al-acclimated *N. muscorum* as described below.

**Fig 5.1** Protein content in the Al-acclimated *N. muscorum* measured after every fifteen days before transfer to the higher concentrations. Values are represented as mean ± SE (n = 3)
5.3.3 Al adaptation mediated cross protection to Cd stress

Further in order to prove our hypothesis that Al adaptation would confer cross protection to Cd, the Al acclimated cells and control cells not acclimated to Al were exposed to 8 µM Cd and parameters relating to growth and ROS generation were measured. Cd at 8 µM concentration reduced the amount of chl a by 20% as compared to the control and Al acclimated cells. However, the Al acclimated Cd-treated cells of *N. muscorum* showed marginal reduction when compared to Cd treatment alone (Figure 5.2) clearly suggesting the role of Al adaptation in minimizing Cd toxicity.

![Graph showing chlorophyll a content (% Control) vs Metal Treatment (µM)](image)

**Fig. 5.2** Effect of Cd (8µM) on the chlorophyll a content of Al acclimated *N. muscorum*. Chl a content in untreated control was 1.2 µg mL⁻¹. Values are means ± SE of three replicates. The values are significant at (P<0.01) compared to the control (Student’s t-test). ns = not significant
The levels of total peroxide in the cells of cyanobacterium was analysed and the results are presented in Figure 5.3. Cellular level of peroxides was increased by about 2-folds following the exposure of 8 μM Cd. In contrast to this, the Al acclimated Cd-exposed cells showed a reduction of 50% in the level of total peroxides when compared to that of Cd alone.

**Fig. 5.3** Effect of Cd (8μM) on total peroxide content of Al acclimated *N. muscorum*. The total peroxide in untreated control was 668 ± 8 μM (g dry weight)$^{-1}$ x 10$^2$. Values are means ± SE of three replicates. The values are significant at (P<0.01) compared to the control (Student’s t-test). ns = not significant

Data pertaining to lipid peroxidation measured in terms of MDA content in control, Cd-treated, Al-acclimated Cd-treated *N. muscorum* are presented in Figure 5.4. Increase in MDA level was noticed in both the Al-acclimated and control
cells of *N. muscorum* following the exposure of Cd (8 µM). The Cd alone exposure resulted in approximately 2-fold increase in the MDA level while its level was reduced by 75% in the Al-acclimated Cd-treated cells as compared to the Cd alone.

![Graph](image_url)

**Fig. 5.4** Effect of Cd (8µM) on the MDA content of Al acclimated *N.muscorum*. The MDA content in untreated control was 771± 8 nmol (g dry weight)⁻¹. Values are means ± SE of three replicates. The values are significant at (P<0.01) compared to the control (Student’s t-test). ns = not significant.

Acclimation of *N.muscorum* to 40 µM of Al caused an enhancement of 50% proline as compared to non-acclimated *N.muscorum*. The Cd alone treatment induced the intracellular proline by approximately 30% when compared to the control. However, when the Al-acclimated *N.muscorum* was exposed to 8µM of
Cd there was an additional increase of ~25% in the proline content as compared to the Cd alone (Figure 5.5)

Fig. 5.5 Effect of Cd (8µM) on the Proline content of Al acclimated *N.muscorum*. The amount of proline in untreated control was 13.5 ± 0.6µg (g dry weight)⁻¹. Values are means ± SE of three replicates. The values are significant at (P<0.01) compared to the control (Student’s t-test).
5.4 Discussion

5.4.1 Ameliorative effect of exogenous proline on chlorophyll a, total peroxides and MDA content of N. muscorum exposed to Cd.

We studied the effect of metals on the antioxidants, active oxygen species and lipid peroxidation and described previously in chapter 3. The aim of the present chapter was to explore in length the role of proline in mitigating metal stress. Here, we focused the role of proline in the protection of cyanobacterium N. muscorum from Cd-induced stress.

Proline pre-treatment of the cyanobacterium N. muscorum resulted in a decline of the parameters like total peroxides and MDA after exposure to toxic metal Cd. This result finds support from the findings of Chris et al., (2006) who demonstrated the role of exogenous proline in detoxification of harmful ROS generated under UV stress. Such a possibility is not ruled out in the cyanobacterium under metal stress. We have already shown enhancement in proline content in metal treated N. muscorum in chap 3. Zhang, et al. (2008) reported the Cu induced proline accumulation in the cells of Chlamydomonas reinhardtii. Transgenic algae expressing the P5CS gene show more free proline when treated with Cd conferring plant tolerance to Cd toxicity (Siripornadulsil et al. 2002). Choudhary et al. (2006) reported proline accumulation in the cells of Spirulina platensis-S5 treated with Pb, Cu and Zn.

Proline accumulation was maximum in Al treated N. muscorum (sec 3.3.7.2) and it showed maximum survival under Al stress (sec.2.3.1) clearly demonstrating that accumulation of proline has a role in Al tolerance. Nevertheless, the proline
accumulation seems to provide protection against metal stress by acting as signals to activate additional defense machinery against the stress.

5.3.2 Al adaptation mediated cross protection to Cd stress

Treatment of cyanobacteria to successively increasing concentration of metals confers physiological tolerance to that metal which can be termed as acclimation. Bhargava et al., (2008) showed that the toxic effects of Cu on the Cu-acclimated strain was much less as compared to its effect on the non-acclimated Anabeanan doliohum.

Similarly in the present study Al adaptation reduced the severity of Cd stress to a significant level. The observed decline in the chl a content with concomitant increase in total peroxides and MDA levels in Cd-treated cells (Figures 5.2 and 5.3) depicted the general response of the cyanobacterium under stress. Interestingly, the cells of N. muscorum acclimated with Al improved the tolerance of N. muscorum to Cd as evident from the less inhibitory growth or improvement of growth than the directly exposed cyanobacterial cells to Cd. The tolerance of the N. muscorum acclimated with the Al might be due to proline accumulation as exogenous proline pre-treated cells also showed the same pattern of tolerance (Table 5.1). Further, the Al-acclimated N. muscorum, as shown in fig 5.4, also showed significant enhancement in the proline content. Thus, the functional significance of Al-induced proline accumulation in the Al-acclimated cells possibly lies in its contribution towards maintenance of water balance, as Al stress is known to disrupt this balance (Barcelo and Poschenrieder, 1990). Protective role of proline may be due to its capacity to bind water molecules to itself leading
to conformational changes in enzyme molecules through proper thermodynamic interactions between solute-water and protein/amino acids system which in turn would help in maintaining proper hydration inside the cells (Mishra and Dubey, 2006). Thereby proline-mediated alleviation of water deficit and reduction of free-radical levels, may substantially contribute to the adaptation of organisms to Al stress conditions.

Apart from their role in osmotic adjustment, compatible solutes have also osmoprotective functions. Due to their specific hydrophilic structure, they are capable of replacing water on the surfaces of proteins, protein complexes or membranes, thus preserving their biological functions (Hasegawa et al., 2000). Gunse et al. (1997) demonstrated that Al decreased the hydraulic conductivity and cell wall extensibility in an Al-sensitive maize variety, but increased the hydraulic conductivity in an Al resistant variety.

Most compatible solutes also seem to play an important role in hydroxyl radical scavenging, thus defending plants against oxidative damage, which is a common consequence of many abiotic stresses (Smirnoff, 1998; Sharma and Dietz, 2006).

In the present study significant Proline accumulation is observed in Al acclimated N.muscorum which further increased when it was exposed to Cd clearly emphasizing both its osmoprotectant and antioxidant behavior.

5.3.3 The Relationship between Al acclimation and Proline accumulation

The correlation between Al acclimation and proline can be further established by comparing the results of the different studies performed on Al acclimation and proline supplemented N.muscorum. In both the cases, the level of metal stress markers like peroxides and lipid peroxidation reduced significantly on being
treated with Cd when compared with the non-acclimated Cd alone and non-proline pre-treated *N. muscorum*. A simple conclusion can be drawn from these similarities that proline accumulation may be the pivotal mechanism in mediating adaptive response during Al acclimation. Two proteins associated with proline synthesis against the stress responses in plants were induced. Arginine tRNA ligase, an enzyme that participates in arginine and proline metabolism was induced by 1.96 fold while glutamate dehydrogenase was increased by 1.66 fold in Al-treated tissues. Glutamate dehydrogenase was found to be activated by ROS in order to detoxify ammonia and to produce glutamate for the synthesis of proline (Zhou *et al*., 2009).

Osmotic stress leads to the induction stress responsive proteins like dehydrins. Based on the results of the protein profile of Al stressed *N. muscorum* (Fig 4.1) that unequivocally pointed to the up-regulation of some polypeptides which coincided with those of dehydrin like proteins, we can say that their induction could be mediated by proline to combat the Al stress. Further, based on the reports by Khedr *et al*., (2003) who demonstrated that proline induces the expression of salt-stress-responsive proteins which improved the adaptation of *Pancratium maritimum* L. to salt-stress it can be suggested that proline is the active inducer of stress responsive proteins like dehydrins. Garcia *et al*., (1997) also found that the salt gene of rice is induced by proline and it was found previously that this gene is induced by drought (Claes *et al*., 1990). However, Iyer and Caplan (1998) found that pyrroline-5-carboxylate, which is a product of proline catabolism, but not proline itself was able to induce dehydrins in rice. From these results it can be comfortably concluded that proline could act as a
component of signal transduction pathways that regulate stress responsive genes in addition to its previously described osmoprotective roles, thereby improving the tolerance to Al stress.

**Conclusions**

In conclusion, Al-induced adaptive response demonstrated in the present study to Cd could therefore possibly be attributed to the involvement of antioxidant defense especially proline and proline-mediated expression of dehydrin like proteins. The precise mechanism underlying the Al-adaptive response particularly to Cd, however, remains obscure at this moment warranting further research.

The overall, conclusion of the whole thesis is that Al-induced stress in *N.muscorum* is probably dealt with a different mechanism(s) as compared to the Cu and Cd. Largely unknown nature and role of these Al-induced proteins in *N.muscorum* certainly added sufficient curiosity to further isolate, identify and characterize their physical, chemical and biological properties which will likely to enhance our understanding of Al-induced tolerance in *N.muscorum*. 