CHAPTER 7

EFFECT OF ARSENIC ON PHYTOSIDEROPHORES AND MINERAL NUTRITION OF BARLEY SEEDLINGS GROWN IN IRON-DEPLETED MEDIUM

ABSTRACT

A hydroponic experiment with barley seedlings (*Hordeum vulgare* L. cv. Minorimugi) grown in Fe-depleted medium in presence of added arsenic (As) at the rates of 0, 0.67, 6.7 and 67 μM (equivalent to 0, 0.05, 0.5 and 5 mg L^{-1} As, respectively) showed that increasing As concentrations in the medium caused lowering phytosiderophores (PS) release and concentration in roots. This Fe-depleted experiment was conducted to observe the relationship among As, physiological response, and mineral nutrients. Chlorophyll index increased at 67 μM As treatment as compared to the others. This result indicated that As may show internal toxicity in plants without showing visible toxicity symptom in shoot. Arsenic at 67 μM level increased Fe concentration in shoots. Increased As concentration in shoot might also be responsible for lowering the release and concentration of PS in roots. Arsenic lowered the concentrations of P, K, Ca and Mg in shoot at 67 μM level. Higher Fe concentration and higher ratio of Fe/P in shoot may be the factors responsible for the greening of the leaves in 67 μM As treatment. It was found that total amounts of Mn, Zn and Cu were reduced by the high As. There was a concomitant increase in the As contents in shoots with higher As levels in the growth medium. A negative relationship between P and As, or P and Fe in shoots were observed. It appeared that higher As played a role to modulate the mobility of root Fe in barley tissues grown in Fe-depleted medium.

Abbreviations: CDL (critical deficient levels); DAT (days after treatments); DW (dry weight); PS (phytosiderophores)

7.1 INTRODUCTION

In our previous papers (Shaibur et al. 2006; Shaibur et al. 2008abc), we reported that As may induce Fe-chlorosis in plants grown in Fe-containing medium probably because of the formation of Fe-precipitation on the surface of roots (Yamane 1989). On the other hand, PS produced and released by grasses is known to solubilize and mobilize Fe. Typical phenomenon of PS release is associated with Fe-deficient grasses e.g. barley (Kawai et al. 1988b) and wheat (Ma et al. 1995). It should be investigated whether formation and release of PS may be affected by the presence of As. In order to investigate the relationship between As and PS, physiological response of grasses grown in Fe-depleted medium at high As concentrations needs to be

investigated. At present, no information about it is available. Concentration and release of PS in root could be affected and the Fe nutrition in plants might also be affected by As. Barley is known to produce and release the highest amounts of PS among the grasses (Kawai et al. 1988b; Kawai et al. 1994). It is known that PS are released by Fe-sufficient and Fe-deficient barley (Yoshida et al. 2004). These considerations promoted us to take up this physiological study.

7.2 MATERIALS AND METHODS

7.2.1 Seed Germination and Plant Culture

Seedling of barley (*Hordeum vulgare* L. cv. Minorimugi) were grown in usual way as described in **CHAPTER 2** and Shaibur et al. (2008b) Arsenic concentrations applied were 0, 0.67, 6.7 and 67 µM (0, 0.05, 0.5 and 5 mg L^{-1} As, respectively). Seedlings were allowed to grow in As treated medium for 28 days. The pH was maintained at 6.5 (March-April 2004). The Fe-depleted medium did not contain EDTA-Fe^{3+} and 0.5 mM NaH_2PO_4 substituted for 0.5 mM $NH_4H_2PO_4$.

7.2.2 Chlorophyll Index (SPAD value)

Measurement of chlorophyll index as a measure of SPAD value in the fully expanded young third leaves was taken using a SPAD-502 chlorophyll meter at 28 DAT as described in section 2.6 of **CHAPTER 2.**

7.2.3 Analysis of Plant Samples

Determination of mineral elements on 28 DAT has been described in **CHAPTER 2** and **CHAPTER 3.** A hydride generation atomic absorption spectrophotometric (HGAAS) technique (Hitachi HFS-3) was used for As determination.

7.2.4 Measurement of Phytosiderophores (PS)

Three bunches of plants from each replicate were collected just before the onset of the light time on 14, 21 and 28 DAT to assay PS concentrations in roots. Shoots and roots were washed with deionized water, put into plastic packet and were stored in a refrigerator at -20°C

for 7 days. Root washings were collected by soaking roots in beakers containing 500 mL deionized water (1 bunch per beaker) for 3 h starting at the onset of light time on 14, 21 and 28 DAT as described in section 2.5 of **CHAPTER 2**.

7.2.5 Calculations for the Parameters

The PS concentrations and released are expressed in g^{-1} root DW. Concentration of an element is defined as the amount of the element g^{-1} DW (mg or µg element g^{-1} DW), while accumulation refers to the total amount of element $plant^{-1}$ shoot or $plant^{-1}$ root (mg or µg of element $plant^{-1}$).

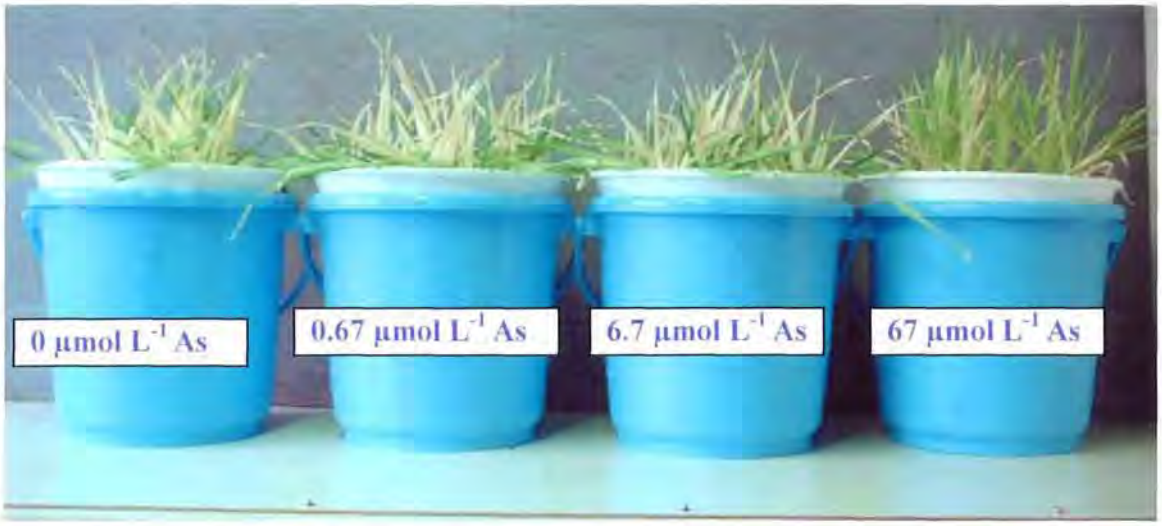

Figure 7.1 Physiological response of barley shoots at different concentrations of As in the growth medium. This photograph was taken 28 days of As exposure.

7.3 RESULTS

7.3.1 Visible Symptoms

The first and second leaves were green in all the treatments, because the plants were grown in Fe-containing medium up to its second leaf before transferring to As treatments in Fe-depleted medium. The young third leaves were green at 67 µM As treatment. However, the leaves were chlorotic at 0, 0.67 and 6.7 µM As treatments. Visible symptoms in shoots were presented in **Fig. 7.1**. Necrosis was observed in the older leaves of 67 µM As treated plants.

The reddish color of the roots increased and the roots came to be slippery with increasing As. Formation of lateral roots was low at 67 μM As treatment.

7.3.2 Dry Weight (DW), Shoot Height, Root Length and Width of Leaf Blade

Shoot DW, shoot height and width of leaf blade were similar in all of the As treatments (**Figs. 7.2a,b & 7.3a**). Root lengths decreased in all of the As treatments (**Fig. 7.2b**) comparing to control, and the root DW decreased only in 67 μM As treatment (**Fig. 7.2a**).

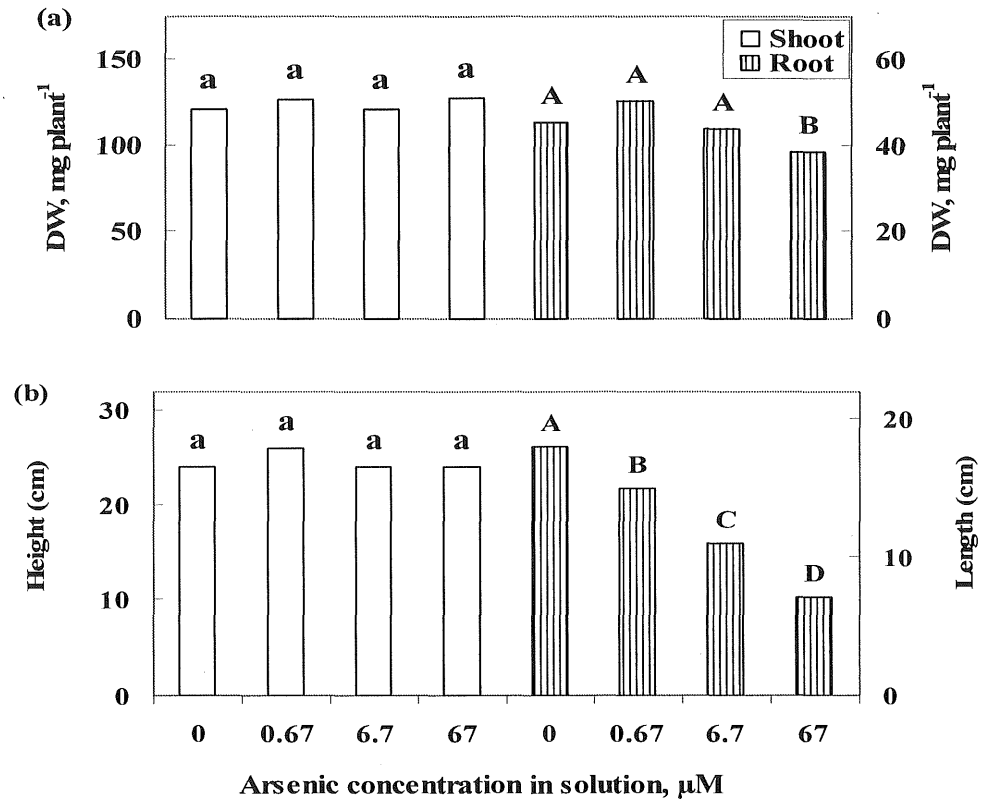

Figure 7.2 (a) Dry weight (DW) and (b) shoot height and root length of As-stressed barley seedlings grown in Fe-depleted medium. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

7.3.3 Chlorophyll Index (SPAD value)

Chlorophyll index measured as SPAD value was not detected in the fully developed young third leaves of 0, 0.67 and 6.7 μM As levels. However, high SPAD value was found in the 67 μM As level (**Fig. 7.3b**). The detectable SPAD value was not found in the 0, 0.67 and 6.7 μM As levels in the Fe-depleted medium.

7.3.4 Phytosiderophores

Concentrations of PS was similar in the 0, 0.67 and 6.7 μM As treated plants on 14 and 21 DAT but decreased in the 67 μM As treatment (**Fig. 7.4a**). Release of PS decreased with the increase of As concentration (**Fig. 7.4b**).

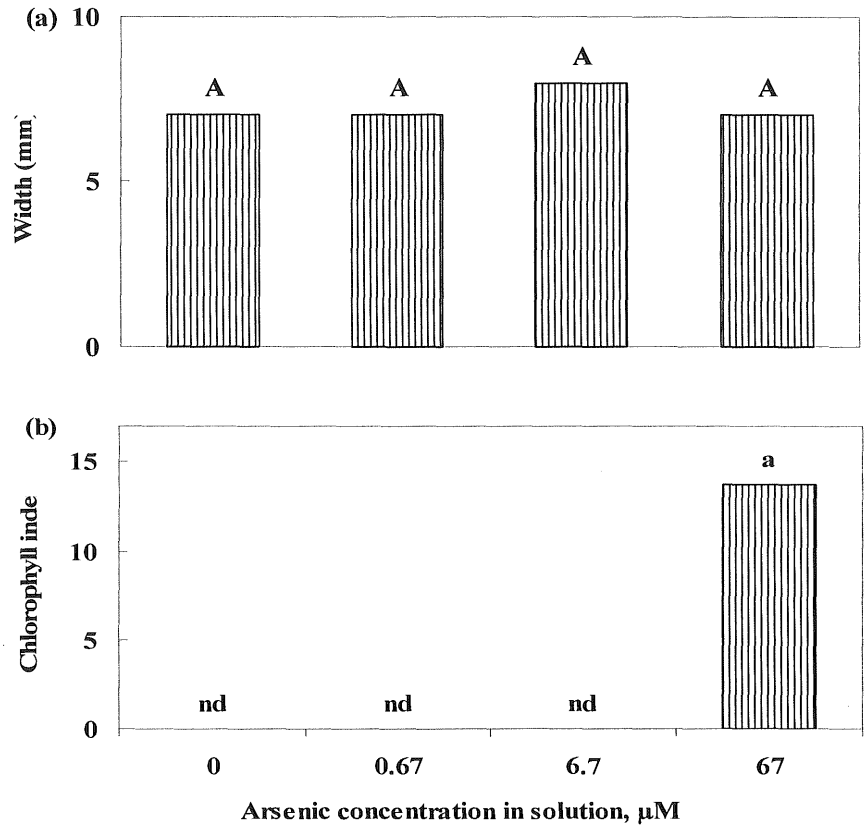

Figure 7.3 (a) Width of leaf blade and (b) chlorophyll index of fully developed young third leaves of As-stressed barley seedlings grown in Fe-depleted medium with different levels of As. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test. nd = not detected.

7.3.5 Macro and Micronutrients

Phosphorus concentration in shoots was increased in plants treated with 0.67 μM As but decreased with the higher As in the medium (**Table 7.1**). Similar trends were also noticed for P accumulation in shoots (**Table 7.2**). Phosphorus concentration and accumulation in roots showed a decreasing trend with the increase of As concentrations (**Tables 7.1 & 7.2**). Potassium concentration and accumulation were reduced both in shoots and roots at 67 μM As

treatment (**Tables 7.1 & 7.2**). Although Ca concentration decreased in shoots at 67 µM As treatment (**Table 7.1**) only, Ca accumulation decreased both at 6.7 and 67 µM As treatments (**Table 7.2**). Magnesium concentration and accumulation decreased both in shoots and roots at either 6.7 or 67 µM As treatments (**Tables 7.1 & 7.2**).

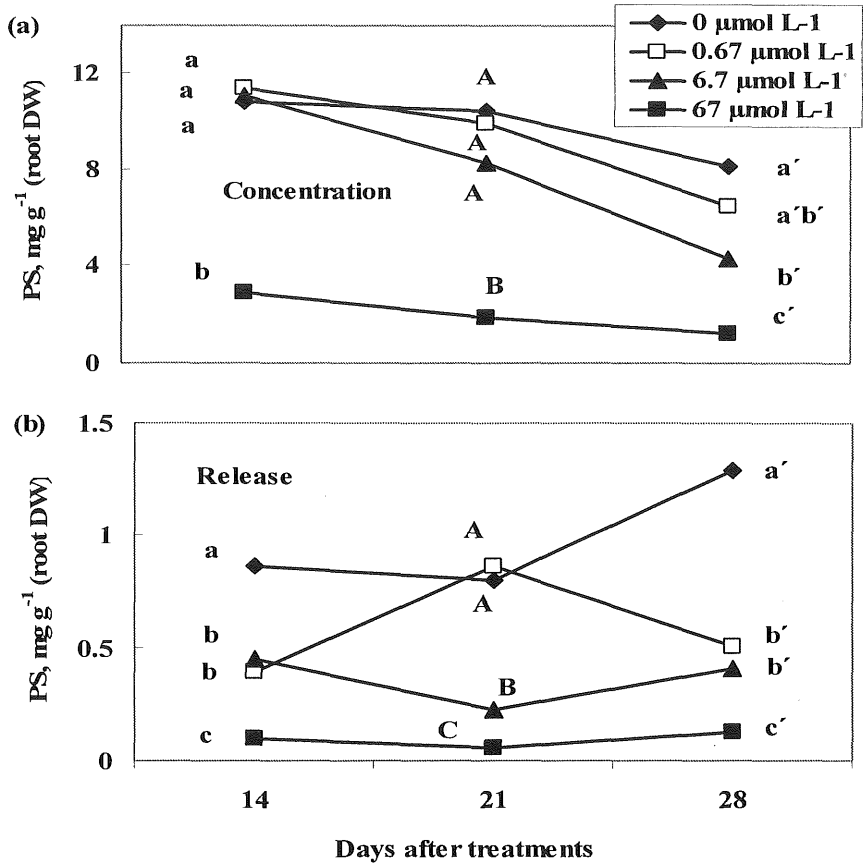

Figure 7.4 Effect of As on the PS (a) concentration in root and (b) release from barley roots grown in Fe-depleted medium. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test. PS = phytosiderophores. DW = dry weight.

Both concentration (**Table 7.1**) and accumulation (**Table 7.2**) of Fe in shoots increased in the 67 µM As treatment. However, the concentration and accumulation of Fe in roots were reduced for the same. Manganese concentration and accumulation were decreased in shoots due to the 6.7 and 67 µM As treatments (**Tables 7.1 & 7.2**). Arsenic was found to have a negative influence on the Zn concentration and accumulation in shoots only at the 67 µM level, though the Zn concentration in the roots was not much affected (**Tables 7.1 & 7.2**). The concentration

and accumulation of Cu were reduced in both of the organs due to As treatments (**Tables 7.1 & 7.2**).

Table 7.1 Concentrations of elements in shoots and roots of barley seedlings grown in Fe-depleted medium with different levels of As.

	----------mg g^{-1} DW ----------				----------µg g^{-1} DW ----------				
Treatment	P	K	Ca	Mg	Fe	Mn	Zn	Cu	As
(µM As)	Concentrations in shoots								
0	21.7 b	30.6 a	8.40 a	3.62 a	32.3 c	95.5 a	89.1 a	14.01a	nd
0.67	25.5 a	33.3 a	8.49 a	3.61 a	35.4 bc	96.4 a	100.4 a	14.1 a	2.57 c
6.7	15.7 c	29.8 a	6.08 a	2.89 b	37.2 b	79.0 b	91.2 a	7.64 b	6.85 b
67	5.21 d	11.6 b	2.66 b	1.23 c	56.2 a	9.61 c	20.2 b	1.67 c	43.7 a
	Concentrations in roots								
0	18.3 A	64.4 A	2.67 A	3.03 A	176 A	64.1 B	47.1 B	104.9 A	nd
0.67	16.5 B	63.2 A	2.64 A	3.05 A	166 A	54.8 C	50.3 B	129.1 A	43.5 C
6.7	15.3 B	58.8 A	2.36 A	2.52 B	158 A	92.7 A	74.0 A	108.5 A	975 B
67	4.62 C	14.8 B	1.14 B	0.72 C	127 B	14.8 D	49.1 B	26.0 B	1204 A

Means followed by different letters in each column are significantly different (p= 0.05) according to a Ryan-Einot-Gabriel-Welsch multiple range test. DW = dry weight, nd = not detected.

7.3.6 Arsenic

Arsenic concentration and accumulation showed increasing trend with the increase of As in the medium (**Tables 7.1 & 7.2**). However, the magnitudes of the concentration and accumulation were much greater in roots than in shoots. The As concentrations in roots were almost 17, 142 and 28 times more than those of shoots in the 0.67, 6.7 and 67 µM As levels, respectively, while the accumulations were almost 7, 52 and 8 times higher.

7.3.7 Relationship between P and As; Fe and As; and Fe and P Content in Barley Tissues

The polynomial two order relationships between P and As concentration in shoots and roots tissues showed that with increasing As concentration in plant tissue P concentration decreased (**Figs. 7.5a,b**), indicating that As repressed P concentrations.

The polynomial two order relationship between Fe and As concentrations in shoot tissues showed a positive relationship ($R^2 = 0.8848$), but the correlation co-officient between them was not high for roots (**Figs. 7.6a,b**). This was a further proof that As plays a vital role in the uptake and translocation of Fe in the barley plants when grown in Fe-depleted conditions.

Table 7.2 Accumulation of elements in shoots and roots of barley seedlings grown in Fe-depleted medium with different levels of As.

	----------mg plant^{-1} (DW)---------				----------µg plant^{-1} (DW)----------				
Treatment	P	K	Ca	Mg	Fe	Mn	Zn	Cu	As
(µM As)	Accumulation in shoot								
0	2.61 b	3.67 a	1.01 a	0.437 a	3.87 b	11.38 a	10.8 a	1.70 a	nd
0.67	3.23 a	4.16 a	1.08 a	0.456 a	4.48 b	12.20 a	12.7 a	1.77 a	0.33 c
6.7	1.89 c	3.60 a	0.734 b	0.349 b	4.48 b	9.54 b	11.0 a	0.92 b	0.82 b
67	0.66 d	1.47 b	0.339 c	0.156 c	7.14 a	1.22 c	2.56 b	0.21 c	5.6 a
	Accumulation in root								
0	0.827 A	2.92 A	0.120 A	0.138 A	7.98 A	2.83 B	2.09 B	4.75 B	nd
0.67	0.817 A	3.16 A	0.132 A	0.158 A	8.23 A	2.75 B	2.51 B	6.45 A	2.17 B
6.7	0.667 B	2.57 B	0.103 B	0.110 B	6.89 B	4.05 A	3.24 A	4.75 B	42.7 A
67	0.179 C	0.563 C	0.044 C	0.028 C	4.84 C	0.57 C	1.88 C	0.99 C	46.3 A

Means followed by different letters in each column are significantly different (p= 0.05) according to a Ryan-Einot-Gabriel-Welsch multiple range test. DW = dry weight, nd = not detected.

The polynomial two order relationships between P and Fe concentration in plant tissues showed that P concentration decreased in shoots with the increasing Fe concentration ($R^2 = 0.7267$) (**Fig. 7.7a**). Antagonistic relationship between P and Fe was observed in shoots in this experiment. On the other hand, P concentration had positive correlation with Fe concentration in the roots where R^2 value was 0.8488 (**Fig. 7.7b**).

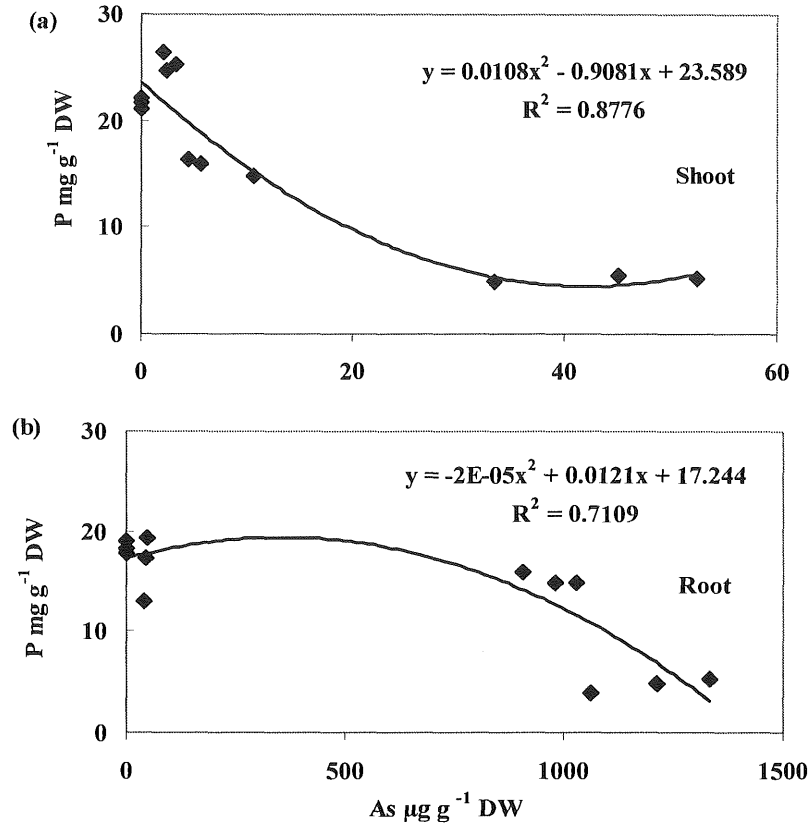

Figure 7.5 Polynomial two order relationship between P and As concentrations in (a) shoot and (b) root tissues of As-stressed barley seedlings grown in Fe-depleted medium. DW = dry weight.

7.4 DISCUSSION

Greening of the young third leaves at 67 µM As treatment could probably be due to an increase of the Fe concentration in the shoots (**Table 7.1**). In this Fe-depleted experiment, As ameliorated chlorosis in 67 µM As level. On the contrary, in our previous work with Fe-containing medium, As-induced whitish chlorosis symptom was observed in barley at 33.5 and 67 µM levels by reducing Fe translocation (Shaibur et al. 2008b). It was found that chlorophyll indices were different in Fe-depleted and Fe-containing medium at 67 µM As level. It seemed that As and Fe in plant tissue had intimate physiological relationship and affected the formation of chlorophyll. In the current experiment, Fe translocation, among the heavy metals, was specifically elevated under 67 µM As level. These opposite results in Fe concentration in shoots were obtained in Fe-depleted and Fe-containing conditions where As was supplied. Our

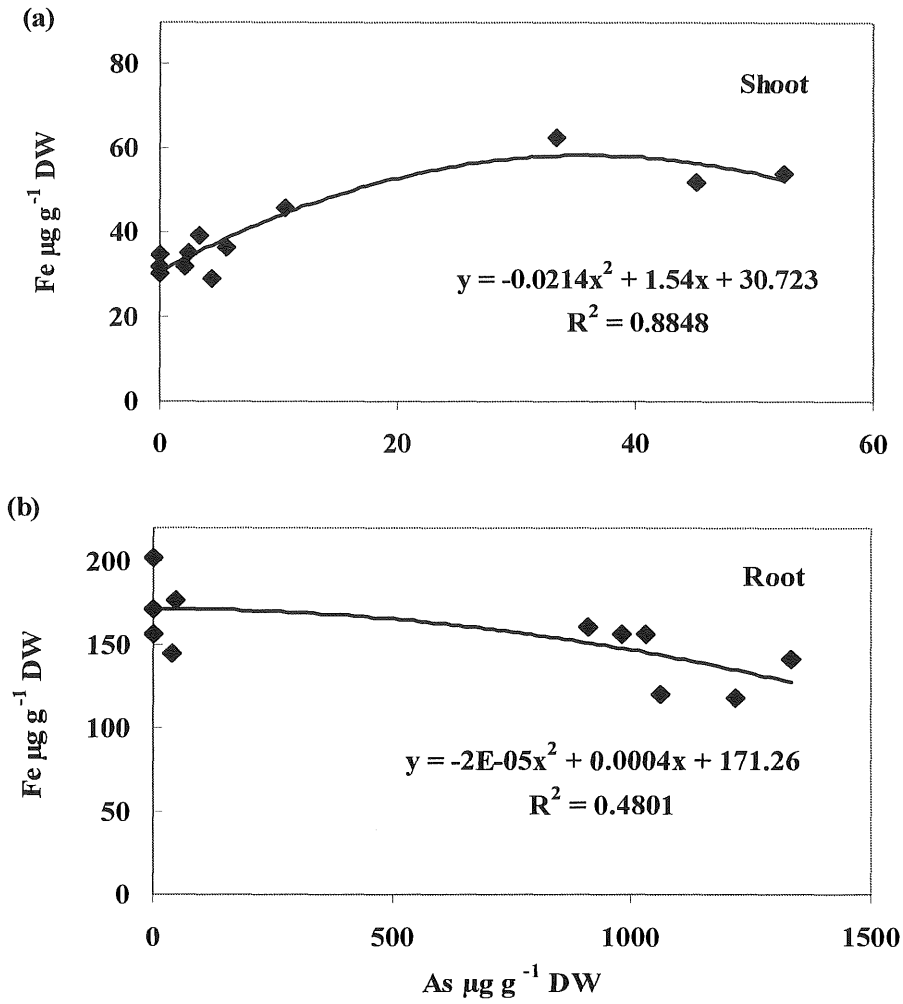

Figure 7.6 Polynomial two order relationship between Fe and As concentrations in (a) shoot and (b) root tissues of As-stressed barley seedlings grown in Fe-depleted medium. DW = dry weight

speculations for the reason of the phenomenon are- (1) higher As in Fe-containing medium: Fe may be immobilized by As in roots as inorganic precipitation, resulting in low Fe translocation and higher Fe content in roots (2) higher As in Fe-depleted medium: newly absorbed Fe does not occur and Fe absorbed during preculture had been immobilized as bound forms with organic compounds in root cells or root apoplast. When higher concentration of As (67 µM As) was supplied to the roots, the compounds for immobilizing Fe might be repressed and

decreased, resulting in gradual liberation of Fe. Liberated Fe from the compounds might be conveyed to xylem tubes by PS in the roots without precipitation with As, though PS content was lower in 67 μM As. These phenomena might cause higher Fe translocation, low Fe concentration in roots, and greening of the leaves. However, the mechanism for Fe mobilization and immobilization in plants has not been characterized. Therefore, explanation with evidences is not possible.

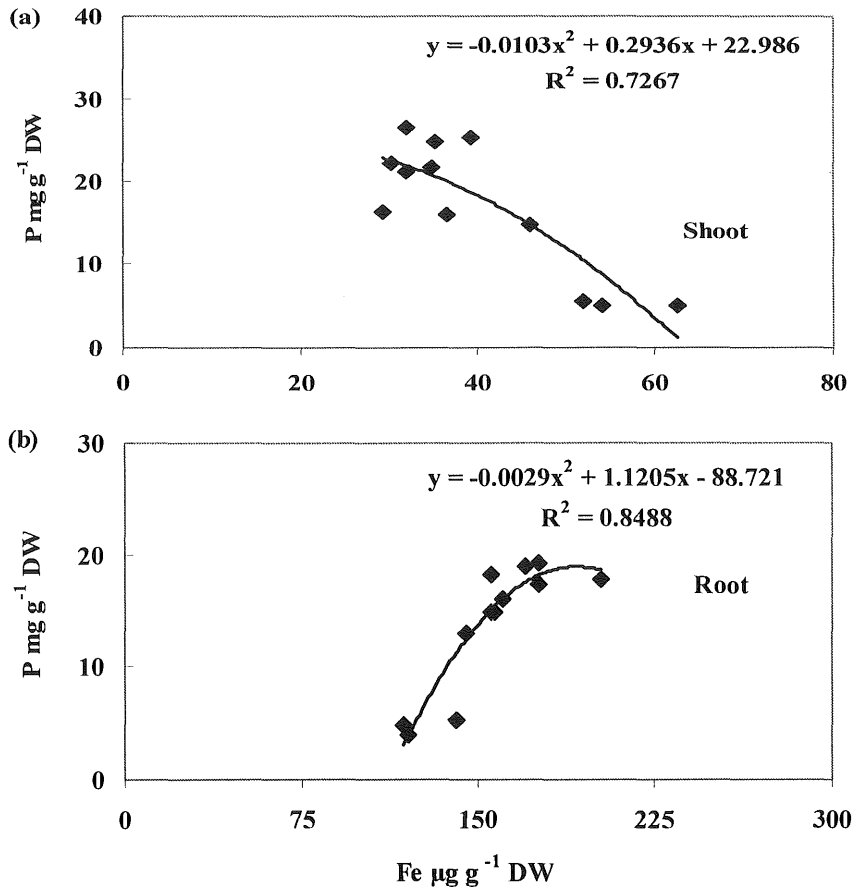

Figure 7.7 Polynomial two order relationship between P and Fe concentrations in (a) shoot and (b) root tissues of As-stressed barley seedlings grown in Fe-depleted medium. DW = dry weight.

The greening of the leaves at the 67 μM As level is commensurate with our recent findings of Japanese Mustard spinach. The plant was green in spite of containing 19.7 to 90.3 μg As g^{-1} DW in leaf tissues as well as high As in the growth medium (Shaibur et al. 2007).

However, whitish chlorosis in the young leaves of rice is also the symptom of As-toxicity (Shaibur et al. 2008c). Formation of Fe-plaque has been described earlier (Armstrong 1967; Chen et al. 1980; Batty and Younger 2003; Liu et al. 2005; Shaibur et al. 2006; 2008a). In this experiment, seedlings were grown in Fe containing medium for 16 days and afterwards the As treatments were started together with depletion of Fe. Reddish color on the root surface may be derived from the Fe-As compounds formed from applied Fe in preculture and As. Otte et al. (1991) reported that in arsenite-treated plants arsenite might be converted to arsenate after oxidation in the rhizosphere and co-precipitate with Fe^{3+} in or at the root surface.

Shoot DW, shoot height and the width of leaf blade did not change much in response to As treatments. Internal As-toxicity might be prevailed in these plants without decreasing DW. Root DW was similar in 0, 0.67 and 6.7 μM treatments. However, root length decreased in 0.67, 6.7 and 67 μM As treatments as compared to control (**Figs. 7.2ab**). The only significant reduction in root DW was in the 67 μM As treatment as compared to the others (**Fig. 7.2a**). In As treated plants, the thickness of roots increased but the length decreased, resulting in almost similar DW of roots in presence of 0.67 and 6.7 μM As. Reduction of root length by the lower concentrations of As was most probably due to the fact that the elongating hormones might be more sensitive to As.

Present result indicated that roots were more sensitive to As than shoots when grown in Fe-depleted media. Arsenic might deform the root structure, resulting in a decrease of root length. We reported that shoot height of rice decreased even at 6.7 μM As level, and width of leaf blade and root length decreased at 13.4 μM As level when grown in Fe-sufficient media (Shaibur et al. 2006).

At the time of As treatment initiation in Fe-depleted condition, the seedlings had only two leaves. Plants showed Fe-chlorosis in the third and remaining leaves of 0, 0.67 and 6.7 μM As levels (**Fig. 7.3b**). However, the Fe-chlorosis in the third and remaining leaves was not developed in the plants of 67 μM As level. It seemed that As at 67 μM level ameliorated chlorosis in barley grown in Fe-depleted condition. Total Fe concentrations in the shoots of the plants in 0, 0.67 and 6.7 μM As treatments were within the range of CDL of Fe, 30-50 μg g^{-1} DW (Bergmann 1988), below which plants showed Fe-chlorosis in the young leaf. We measured Fe concentration from the whole shoots (old first and second leaves together with young third and remaining chlorotic leaves). In this experiment, the higher SPAD value in the

third leaf of 67 μM As level may be induced by the higher Fe-concentration in shoots (Table 1), especially in the young leaves. It is not considered that As had positive effect on chlorophyll synthesis. In contrast, As at 67 μM level reduced the concentration and accumulation of Fe in roots and increased those of Fe in shoot of barley grown in Fe-depleted medium (**Tables 7.1 & 7.2**). A part of Fe in roots must be translocated to shoots in Fe-depleted medium at 67 μM As level. We believe that Fe concentration was one of the most responsible factors for the enhancement of SPAD value at 67 μM As treatment. Higher Fe/P ratio or active Fe in shoot tissues might also be involved in this case. The ratios (%) of Fe:P (concentrations) were 0.149, 0.139, 0.237 and 1.08 for the 0, 0.67, 6.7 and 67 μmol L^{-1} As treatments, respectively. The higher value of Fe/P ratio may be considered as a factor regulating the chlorophyll formation in plants. It was reported that chlorophyll increased with the increase of Fe:P ratios in barley (Ladouceur et al. 2006).

Our result indicated that 67 μM As repressed PS synthesis (**Fig. 7.4a**). Arsenic at 0, 0.67 and 6.7 μM levels did not reduce PS concentrations much at 14 and 21 DAT but decreased at 28 DAT, indicating that longer exposure to lower As concentrations induced the similar effect as short exposure to higher concentration, 67 μM. However, this phenomenon needs to be further assessed in soil culture to determine the critical As concentration and/or the critical exposure time. The PS are synthesized in the apical root zones in Fe-deficient condition (Römheld and Marschner 1986) and are released from the root apex (Marschner et al. 1987). Yoshida et al. (2004) reported that the PS was mainly released from the apical zones of the primary root and whole root surface area in some secondary roots of barley. It is well established that arsenite reacts with sulfhydryl groups of proteins of roots (Speer 1973) causing disruption of the root function (Isensee et al. 1971; Orwick et al. 1976) and even cellular death. We supplied As as $NaAsO_2$ and found that the lengths of roots decreased with the increase of As (**Fig. 7.2b**) and that the formation of new roots was also decreased by As. Therefore, PS production might be repressed by the As-toxicity to SH group in root proteins. Speer (1973) reported inactivation of SH group by As. It was reported that toxic metal cadmium (Cd) also decreased PS release in barley grown in Fe-depleted medium at 0.05, 0.5 and 5 μM levels (Kudo et al. 2007).

Reduction of P concentrations in the 6.7 and 67 μM As levels (**Table 7.1**) was most probably due to competition between P and As anions. Because the P concentrations decreased

more at the 67 μM As than the others, P concentration may be involved in the induction of green leaves. In this Fe-depleted experiment, higher Fe concentration may also be a vital factor for greening of the young leaves of 67 μM As treatment.

It is well known that arsenate is absorbed by the phosphate uptake system in barley (Asher and Reay 1979). We used aeration; therefore, supplied arsenite might partially be converted to arsenate and competed with P absorption, resulting in a decrease of the concentration and accumulation of P both in shoots and roots in the higher As concentrations. However, the fact that increasing the phosphate concentration of nutrient solutions could decrease As-uptake in plants was also in report (Hurd-Karrer 1936). Poynton et al. (2004) reported that phosphate inhibited arsenate influx in a directly competitive manner in hyperaccumulator *Pteris species* and hypothesized that As^{5+} entered into roots through Pi-transporters, similarly to that for non-accumulating plant species.

Reduction of K concentration and accumulation both in shoots and roots in the higher As concentration was most probably due to toxic effect of As on roots. Up to now, we did not see any direct antagonistic relationship between K and As during absorption. Reduction of Ca concentration might be linked to the reduced P concentration as roots absorb Ca together with negative charged phosphate (Caldwell and Haug 1982). Transpiration has a decisive influence on the transport of Ca upon its absorption (Mengel and Kirkby 2001). Reduction of transpiration would cause a reduction in the upward transport of Ca (Carbonell-Barrachina et al. 1997). Reduction of Mg concentration and accumulation might also be due to toxic effect of As on roots. This result was consistent with our previous findings in hydroponic rice (Shaibur et al. 2006). Uptake and translocation of Mg^{2+} can be greatly depressed by an excess of other cations, especially of K^+, Ca^{2+} and NH_4^+ (Mengel and Kirkby 2001). However, the reduction of Mg concentration and accumulation was not related to competition among cations in plant tissues in this experiment.

Iron concentration was enhanced in shoots and decreased in roots in the higher As treatment **(Table 7.1)**. Higher Fe concentrations in shoots might be responsible for higher chlorophyll content in the highest As concentration **(Fig. 7.3b)**. In the 67 μM As plants, Fe concentration in shoots **(Table 7.1)** was higher than CDL (30-50 μg Fe g^{-1} DW) (Bergmann 1988), but was still lower than 72 μg Fe g^{-1} DW (Smith et al. 1984) and the leaves were green. Increased Fe concentrations in shoots might be responsible for the reduction of PS synthesis

and its release **(Figs. 7.4a,b)**. It is known that the response of roots to Fe-deficiency is regulated by the Fe-deficiency symptom in shoots (Landsberg 1984). The results of this experiment indicated that total Fe in plants (shoots plus roots) were similar among all As levels **(Table 7.2)**, but As changed the allocation of Fe in roots and shoots. Accumulation of Fe also showed a similar trend like that for its concentration in the two organs **(Table 7.2)**. It could be postulated that Fe was more mobile in the roots of 67 μM As treated plants as compared to the other treatments in Fe-depleted condition.

It was clearly shown that Mn concentration was more affected in shoots than in roots in 67 μM As treatment **(Table 7.1)**, which was also true for Zn and Cu. It was sure that As interfered absorption of these heavy metals. It is known that accumulation of Mn is metabolically regulated in a similar way to Ca^{2+} and Mg^{2+} (Marschner 1998). Photosynthesis might be reduced by the shortage of Mn in plants at 67 μM As treatment.

Reduction of Zn concentration and accumulation might be due to toxic effect of As on Zn. Interaction between P and Zn is well known (Marschner and Schropp 1977). Phosphorus and As are chemically similar. Therefore, As may substitute P in metabolic process in plant tissue (Lepp 1981). It could explain the fact that As at higher concentration may cause Zn deficiency **(Tables 7.1 & 7.2)**. For most plant species, the CDL of Zn concentrations in leaves are 10 to 15 μg g^{-1} DW; concentrations in the range of 20-100 μg Zn g^{-1} DW are sufficient (Boehle and Lindsay 1969) and concentrations of 150 to 200 μg Zn g^{-1} DW of plant tissue are generally considered as toxic (Sauerbeck 1982). It was considered that Zn concentration of the barley of current experiment was in the range of normal.

Reports showed interactions between P and Cu regarding availability and uptake (Marschner 1998). Concentration and accumulation of Cu might be depressed in plant tissues like the reduction of Zn by As. Copper concentrations in the rage of 1.67 to 14.1 μg g^{-1} DW in shoots of experimental plants indicated normal concentration, though there was a decreasing tendency with the As treatments.

Arsenic concentration in roots was tremendously high, almost similar level as macronutrients in the plants treated with 67 μM As. This is because arsenite shares the same highly efficient pathway as silicon and concentrated the high content of As (Ma et al. 2008). It was considered that As was concentrated on the surface of the roots. The negative charged ions are strongly adsorbed to the root membrane; therefore, As anions (arsenite or arsenate) could

adsorb to the root surface, resulting in enhancement of As concentrations in or on the roots (Wauchope 1983). The Fe-plaque in roots might also be involved for the increase of As concentration in the roots.

7.5 CONCLUSIONS

The present work revealed that As-toxicity lowered PS concentration in roots and its subsequent release. It was shown that more Fe was mobilized in the roots and translocated to the shoots in plants grown under 67 μM As in Fe-depleted medium. Plants did not show Fe-chlorosis and PS release and concentration in roots was lowered under Fe-depleted and 67 μM As condition. The fact suggested that Fe-deficiency was alleviated and resulted in greening of the leaves. Higher ratio of Fe/P also seemed to be responsible for the greening of the leaves. Treatment with higher As concentration reduced accumulation of macroelements, such as P, Ca, Mg in shoots and total amount of heavy metals, such as Mn, Zn and Cu. We should pay attention to the fact that grasses grown under the condition of low available Fe may not show Fe-chlorosis in spite of the presence of high concentration of As in environment. It means that we may overlook the presence of As-toxicity in crops in nature.

CHAPTER 8

EFFECT OF PHYTOSIDEROPHORES ON THE ABSORPTION AND TRANSLOCATION OF ^{59}Fe IN ARSENIC TREATED BARLEY

ABSTRACT

A short term experiment with barley (*Hordeum vulgare* L. cv. Minorimugi), arsenic (As), phytosiderophores (PS) and ^{59}Fe labeled iron (Fe) was conducted. Arsenic was used as sodium meta-arsenite ($NaAsO_2$) and Fe was used as EDTA-Fe^{3+} in the medium. Seedlings were grown in the phytotron at pH 6.5. The experiment was conducted to observe the effect of PS on the absorption and translocation of ^{59}Fe in barley treated with As for 14 days. The treatments were 0 μM As + 10 μM EDTA-Fe^{3+} (control) and 33.5 μM As + 10 μM EDTA-Fe^{3+} (As-treated). It was found that As-induced conspicuous whitish chlorosis in the fully developed young leaves. Chlorophyll index was lower in As-treated plants as compared to the control plants. Control and As-treated plants were fed with 10 μM labeled ^{59}Fe in absence or in presence of 10 μM PS for 4 h starting from 10.30 a.m. to 2.30 p.m. Absorption and translocation of ^{59}Fe increased in control plants fed with PS as compared to those without PS treated plants, indicating that PS effectively played a role in ^{59}Fe absorption and translocation. In As-treated seedlings, PS also increased the absorption of ^{59}Fe in roots as compared to those without fed PS and increased ^{59}Fe translocation to shoots, indicating that ^{59}Fe could be translocated to shoots by PS in 33.5 μM As-treated seedlings. Our result suggested that As-induced chlorosis in young leaves of barley might be due to Fe absorption problem in roots.

Abbreviations: DAT (days after treatment); DW (dry weight); PS (phytosiderophores).

8.1 INTRODUCTION

We reported that arsenic (As) effectively induced whitish chlorosis in the fully developed young leaves of hydroponic barley (Shaibur et al. 2008b). We also found that Fe translocation in plants were the most decreased among the other elements when the barley seedlings were grown in Fe supplied medium (Shaibur et al. 2008b). On the contrary, As at 67 μM level ameliorated chlorosis by increasing Fe concentration in shoots of barley grown in Fe-depleted medium **(CHAPTER 7;** Shaibur et al. 2009a). It meant that As played a role in different ways depending on the presence of Fe in the growth medium. Enhancement of Fe translocation in plants grown in Fe-depleted condition implies that the translocation site of Fe in barley was not damaged completely at 67 μM level **(CHAPTER 7).** The long term effect of As (21 days) decreased the Fe translocation in barley grown in Fe-containing medium (Shaibur et al. 2008b), however, As enhanced Fe translocation in barley grown in Fe-depleted medium

(CHAPTER 7; Shaibur et al. 2009a). The present experiment was conducted to evaluate the long term effect of As on Fe absorption and translocation in barley with the short term experiment by taking ^{59}Fe labeled Fe and the Fe chelating substance PS.

8.2 MATERIALS AND METHODS

8.2.1 Seed Germination and Plant Culture

Seedlings of barley were grown for 14 days in As treatments in the phytotron as described in the section 2.3.2 of **CHAPTER 2.**

8.2.2 Applied Treatments

The treatments were 0 μM As + 10 μM EDTA-Fe^{3+} (control) and 33.5 μM As + 10 μM EDTA-Fe^{3+} (As-treated). These treatments were chosen on the basis of our previous report (Shaibur et al. 2008b). The seedlings were fed with 10 μM $FeCl_3$ labeled with ^{59}Fe in presence or absence of PS.

8.2.3 Chlorophyll Index (SPAD value)

Chlorophyll index was measured from third leaves as described in the section 2.6 of **CHAPTER 2.**

8.2.4 Radio Isotope (RI) Experiment with PS

Barley seedlings were transferred to RI laboratory from phytotron on 11 DAT (days after treatment) for getting adjustment with the phytotron environment for 3 days (day/night time 14/10 h; temperature 25/20°C, respectively; light intensity 280 μmol m^{-2} s^{-1}). On 14 DAT, roots of control and As-treated plants were washed with deionized water, then transferred to freshly prepared 100 mL half-strength nutrient solution containing 10 μM ^{59}Fe as $^{59}FeCl_3$, whose pH was 6.5 with or without PS (10 μM) to examine the long-term effect of As on ^{59}Fe absorption and translocation with this short-term experiment. Radioactivity of individual beaker was 37 kBq of ^{59}Fe. Each beaker contained 1 bunch of seedlings (3 plants). Beakers were wrapped with aluminium foil. We compared the results between without PS and with 10 μM PS treatments for individual treatment. Feeding time was 4 h starting at 2 p.m. (6 h after

the onset of light), when the release of PS does not occur. After 4 h of absorption, the extracellular (apoplastic) ^{59}Fe of roots was removed (Bienfait et al. 1985). After removing the apoplastic ^{59}Fe, seedlings were washed with tap water. Shoots were separated from the roots, air dried for 24 h and then oven dried at 70 ± 2°C for 24 h and weighed. After that the radioactivity was measured by the method described in **CHAPTER 4**.

8.2.5 Source of PS and Radioactive ^{59}Fe

Source of PS and radioactive ^{59}Fe was described in **CHAPTER 4.**

8.2.6 Terminologies Used

Total absorption of ^{59}Fe refers to the sum of the total amount (shoots plus roots accumulation) of ^{59}Fe. Translocation ^{59}Fe refers to the total amount of ^{59}Fe in the shoots only. Translocation activity refers to the content of ^{59}Fe in shoot per gram DW. Absorption activity of roots was calculated by dividing the total amount of ^{59}Fe (shoots plus roots) with the root DW.

8.3 RESULTS

8.3.1 Visible Symptoms

Plants grown under 33.5 µM As produced leaf tip necrosis (the older leaves were turned yellow, fell off and finally died). The youngest leaves failed to unfold, leaf size (both length and width) were reduced drastically in presence of 33.5 µM As. Whitish chlorosis was observed in the fully developed young leaves at 33.5 µM As treatment (**Fig. 8.1**). Wilting of younger leaves was a typical symptom of As-toxicity. The most common symptom of As-toxicity, however, was shoot height reduction. Arsenic-toxicity also resulted in poor root length, roots were discolored (reddish) and felt 'slippery' to the touch.

8.3.2 Chlorophyll Index (SPAD value)

Chlorophyll index of fully developed young third leaves decreased significantly at 33.5 µM As treatment as compared to the control plants **(Fig. 8.2)**. Chlorosis in the fully developed youngest leaves at 33.5 µM As level suggested that As hindered chlorophyll formation. We reported that reduction of chlorophyll index was most probably due to the reduction of Fe

concentration in the shoots (Shaibur et al. 2008b). Arsenic at 33.5 µM level decreased the chlorophyll index by 69% in this experiment.

8.3.3 Dry Weight (DW), Shoot Height and Root Length

Shoot DW decreased in 33.5 µM As level as compared to control, however, root DW was not much affected for the same **(Fig. 8.3a)**. Arsenic at 33.5 µM level was accountable almost 55% shoot DW reduction and almost 10% root DW reduction, indicating that barley shoots were more sensitive to As-toxicity than that of roots. Similarly, shoot height decreased by 38% and root length decreased by 4% with 33.5 µM As (from **Fig. 8.3b)**.

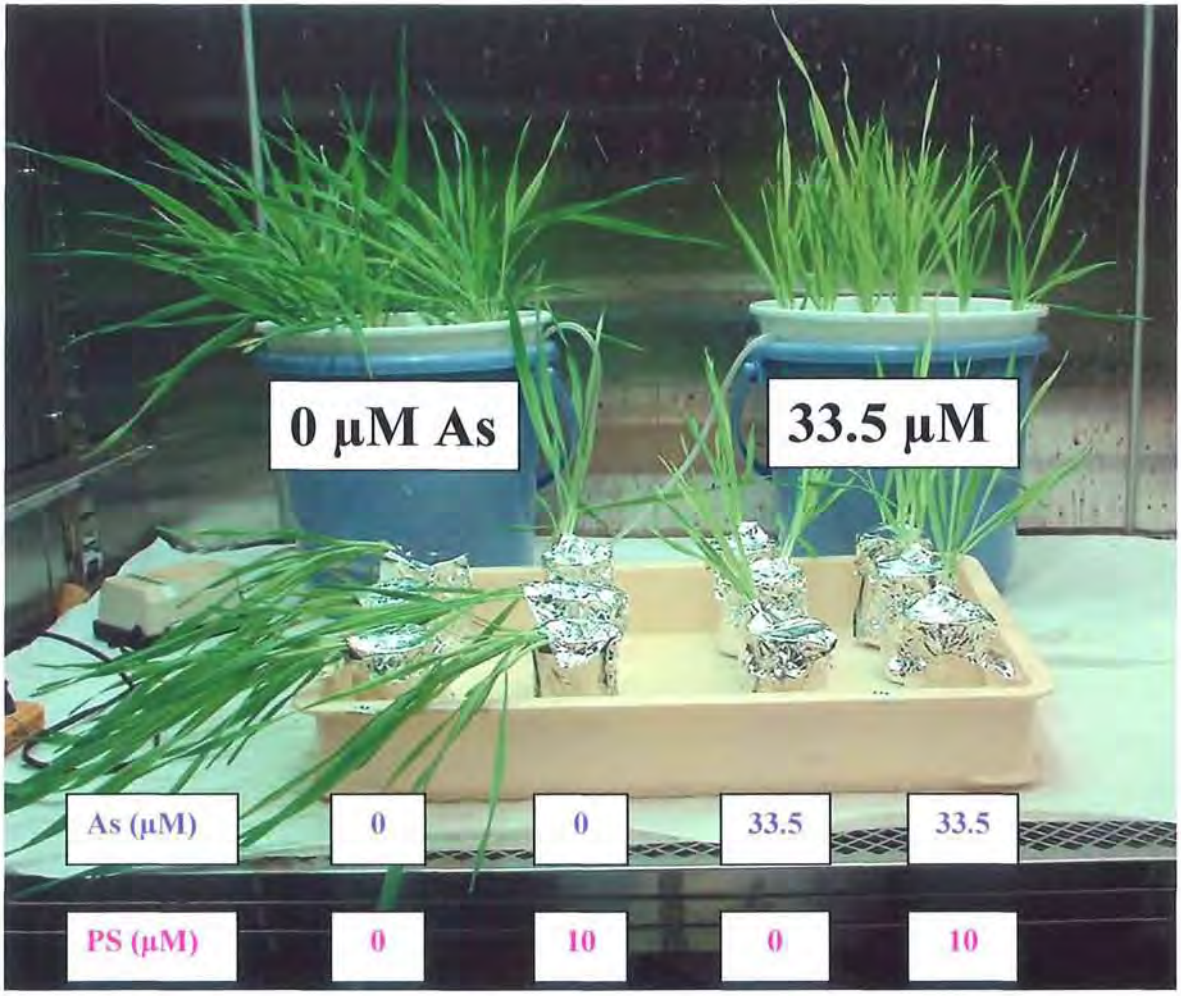

Figure 8.1 Photograph of barley seedlings at elevated concentration of As. Seedlings were fed with 10 µM $FeCl_3$ labeled with ^{59}Fe in presence or absence of PS for 4 h. Feeding experiment was conducted on 14 DAT (days after treatments).

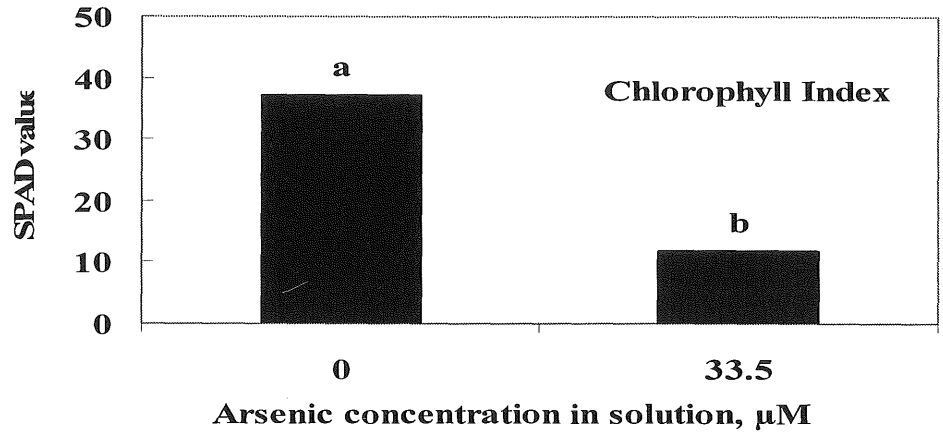

Figure 8.2 Chlorophyll index of barley in two levels of As. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

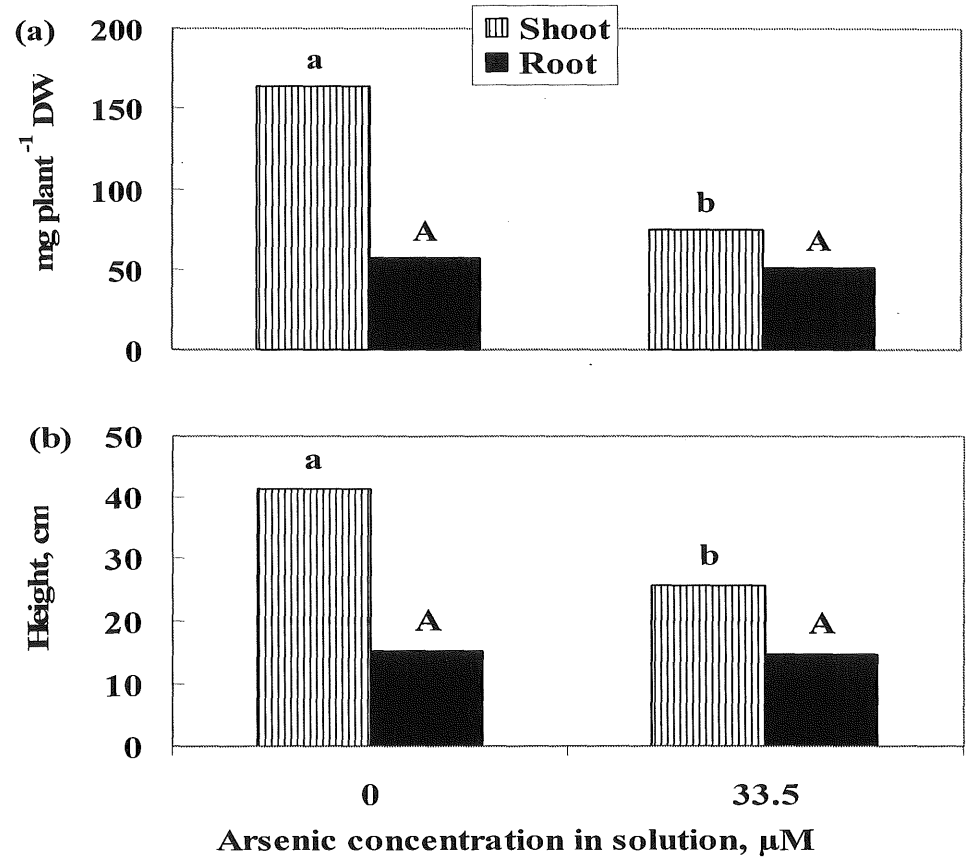

Figure 8.3 (a) Dry weight, DW (b) shoot height and root length of barley in two levels of As. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

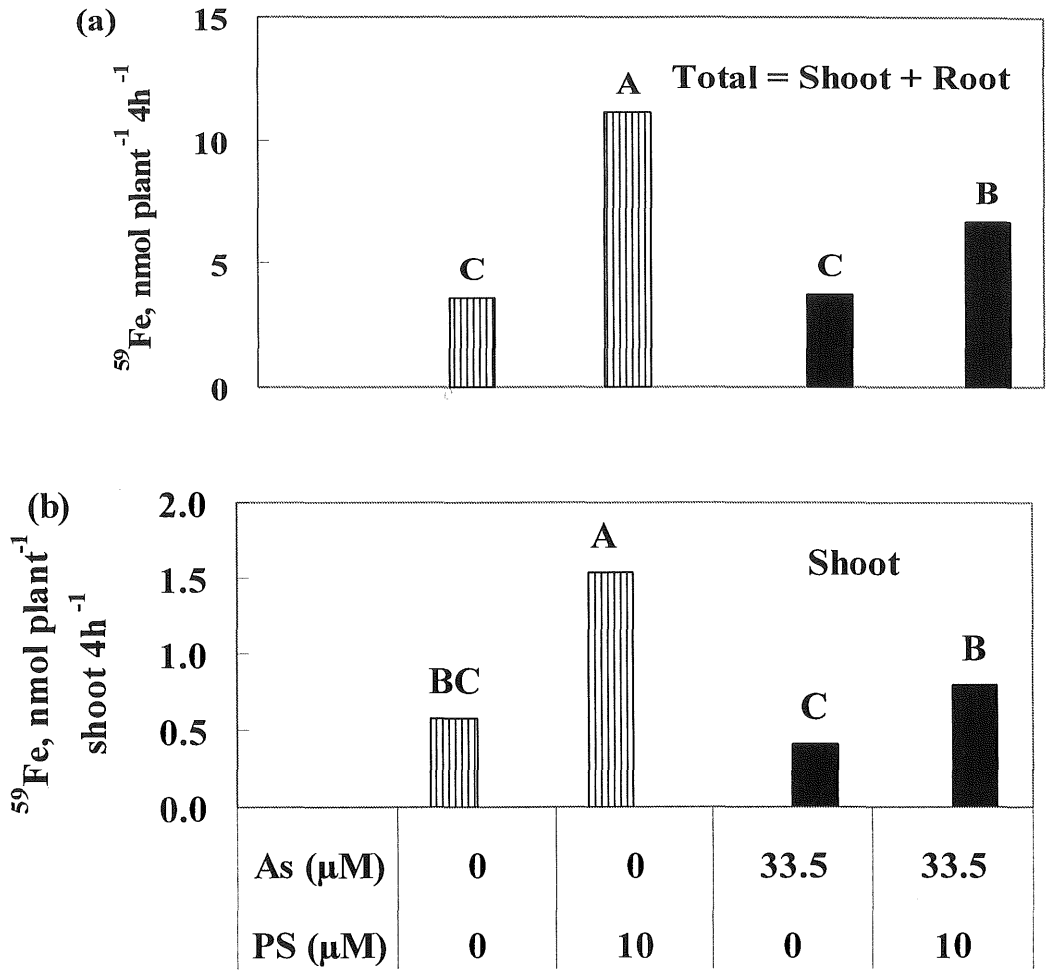

Figure 8.4 (a) Total absorption (shoot + root) and (b) translocation of ^{59}Fe to shoots of barley seedlings with two levels of As with or without PS. Seedlings were fed with 10 μM $FeCl_3$ labeled with ^{59}Fe in presence or absence of PS for 4 h.

8.3.4 Total Absorption (Shoot plus Root) and Translocation of ^{59}Fe

Total absorption of ^{59}Fe was found to be the higher in PS treated control plants as compared to without PS treated plants **(Fig. 8.4a)**. Similarly, PS also enhanced total ^{59}Fe absorption in As-treated plants **(Fig. 8.4a)**. Translocation **(Fig. 8.4b)** data showed that in presence of PS, ^{59}Fe translocation was enhanced almost 2.65 times in control plants. Similar to the control plants, PS also enhanced 1.95 times higher translocation of ^{59}Fe in As-treated plants **(Fig. 8.4b)**.

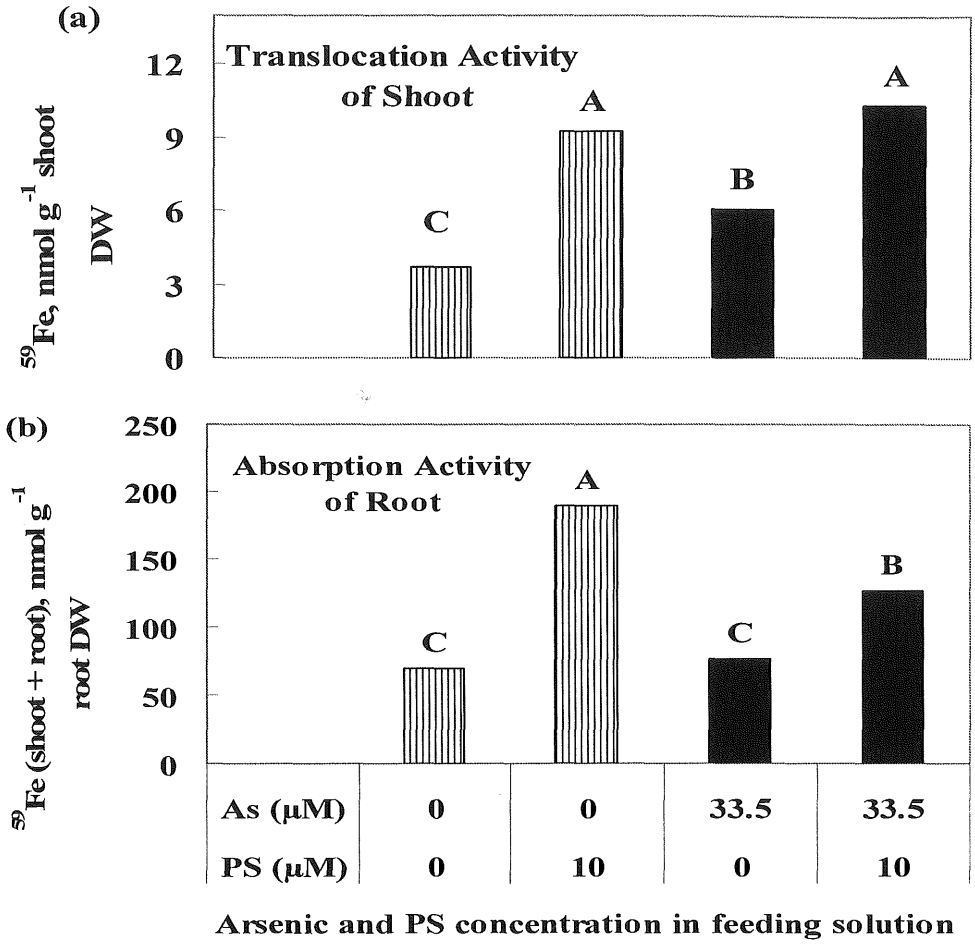

Figure 8.5 (a) Translocation activity of shoots (nmol g $^{-1}$ shoot DW) and (b) absorption activity of roots; total absorption (shoots + roots) per gram root DW to ^{59}Fe in barley seedlings as affected by two levels of As with or without PS. Seedlings were fed with 10 μM $FeCl_3$ labeled with ^{59}Fe in presence or absence of PS.

8.3.5 Translocation Activity of Shoots and Absorption Activity of Roots

Phytosiderophores enhanced translocation activity by 2.49 times in control and 1.72 times higher in As-treated plants, respectively **(Fig. 8.5a)**. Plants treated with As (but without PS) enhanced translocation activity by 1.63 times as compared to control (without PS). The reason was not clearly understood. Therefore, more research is needed by increasing the As concentration in the nutrient solution.

Absorption activity of roots was the highest in PS treated control plants as compared to the others **(Fig. 8.5b)**. It was clear that As-treated plants showed a lower absorption activity

compared to plants grown in absence of As **(Fig. 8.5b).** This was most probably due to the fact that in As-treated plants, root DW decreased. Absorption activity in PS treated control plants was 2.74 times higher compared to without PS treated plants; however, it was 1.65 times in As-treated plants. It meant that the magnitude of absorption activity was lower in As-treated plants as compared to control plants.

8.4 DISCUSSION

Previously, we reported that As at 33.5 and 67 μM levels produced whitish chlorosis in barley and suggested that Fe translocation might be the responsible factor for the induction of Fe-chlorosis (Shaibur et al. 2008b). In this current experiment, whitish chlorosis was also found at 33.5 μM As treatment **(Fig. 8.1)**. Our result showed that PS effectively enhanced total absorption, translocation, translocation activity and absorption activity of ^{59}Fe both in control and As-treated plants **(Figs. 8.4ab & 8.5ab)**. However, the magnitude of those enhancements was lower in As-treated plants. This might be due to the fact that As reduced the shoot growth by 55% and root growth by 10%. Present result showed that absorption activity of roots **(Fig. 8.5b)** was lower in plants fed with PS and As together as compared to PS and without As, but translocation activity in shoots **(Fig. 8.5a)** was not decreased in plants treated with PS and As as compared to PS treated without As. Therefore, induction of whitish chlorosis in barley at 33.5 μM As level might not be due to the translocation problem of Fe. However, this is only short term experiment and more research is needed. The main problem might be associated with the reduction of Fe absorption by roots. Recently we found that Fe translocation was enhanced in barley at 67 μM As level grown in Fe-depleted medium **(CHAPTER 7**; Shaibur et al. 2009a). Our present result is different from the result of rice (**CHAPTER 4**). Physiological response regarding induction of whitish chlorosis was similar in rice and barley; however, short term ^{59}Fe translocation was not similar. Reason behind this demands further study.

8.5 CONCLUSIONS

Finally, it could be concluded that translocation activity of barley shoots was not decreased in As treated plants. Reduction of Fe absorption might be the most responsible for the induction of whitish chlorosis in the young leaves of barley. Transporter of Fe seemed to be not affected much in barley.

CHAPTER 9

EFFECT OF PHOSPHORUS CONCENTRATION ON ARSENIC TOXICITY IN BARLEY GROWN HYDROPONICALLY

ABSTRACT

An experiment with barley (*Hordeum vulgare* L. cv. Minorimugi) grown hydroponically was conducted to observe the effects of different phosphorus (P) levels on the physiological and mineralogical response in presence of elevated arsenic (As). Plants were treated with 10 μM As in presence or absence of P. A treatment was also used without As and P. Phosphorus was used as ammonium phosphate ($NH_4H_2PO_4$) and As was used as sodium meta-arsenite ($NaAsO_2$). The half-strength nutrient solution was used at pH 5.5. Treatments were 10 μM As + 500 μM P, 10 μM As + 250 μM P, 10 μM As + 50 μM P, 10 μM As + 0 μM P and 0 μM As + 0 μM P for 14 days. Intensity of reddish color in roots increased with decreasing P concentration. Iron (Fe)-plaque with reddish color was clearly visible in roots of plants grown in As-treated and P-depleted condition (10 μM As + 0 μM P treatment). However, reddish color was not found in absence of As and in absence of P (0 μM As + 0 μM P treatment), indicating that As played a vital role in the formation of Fe-plaque in roots. Shoots and roots dry weight (DW) decreased with decreasing P in presence of As in the medium, suggesting that As-toxicity was very much dependent on the P concentration in the growth medium. The most severe effect was in As-treated and P-depleted condition (10 μM As + 0 μM P treatment). Phosphorus and magnesium (Mg) concentration was decreased both in shoots and roots by As with decreasing P concentration in the medium. Iron concentration decreased in shoots with decreasing P concentration in the medium and the lowest value was in P-depleted condition. Iron and As were mostly concentrated in roots. Our result suggested that low P might enhance the formation of As-Fe complex (Fe-plaque) in or on the roots.

Abbreviations: DW (dry weight); DAT (days after treatments); DMAA (dimethylarsinic acid).

9.1 INTRODUCTION

Phosphate can decrease or increase the uptake of arsenic (As) by plants depending on the species of As or plants and on the composition of the rooting medium (Tsutsumi 1980; Otte et al. 1990). Phosphorus (P) and As are the elements of same group in the periodic table and have the similar electric configuration. Phosphate and arsenate are analogue of each other and therefore competing for the same sorption sites in root apoplast and for the same uptake system in the root plasmalemma (Asher and Reay 1979; Meharg and Macnair 1992; Meharg and

Hartley-Whitaker 2002). Arsenate uptake in duckweed (*Spirodella polyrhiza* L.) occurred through the phosphate uptake system (Rahman et al. 2008). Reports have shown that arsenate may be removed partially from the soil colloids using inorganic phosphate, however, complete removal or desorption is not possible even after using high contents of P (Smith et al. 1998; Frankenberger 2002; Violante and Pigna 2002). Arsenic uptake has been shown to be increased after application of P in pot soil (Jiang and Singh 1994) and in agricultural field (Small and McCants 1962). Application of P fertilizer to soil increased (Peryea 1998) or decreased (Hanada et al. 1975) the phytoavailability or bioavailability of As in soils.

Arsenite is more mobile compared to arsenate (Meharg 2004) and arsenite is more toxic than arsenate. Most of the experiments have been done with additional P in presence of arsenite or arsenate in hydroponic or soil culture experiments. However, physiological data related to the response of plants at higher to low P condition in hydroponic culture is hardly available. Physiological response of As-toxic plants may vary with decreasing P levels, including in P-depleted condition. Therefore, the present experiment was conducted by taking different P levels. In this experiment, sodium meta-arsenite ($NaAsO_2$) was used in aerated condition, because we considered that some arsenite could be converted to arsenate with aeration. Moreover, underground irrigation water contains arsenite or arsenate. In paddy field conditions, inorganic As species is inter converted between arsenite and arsenate (Meharg 2004). In this study, barley is chosen, because barley is a fast growing plant, easy to set up experiment in the greenhouse. Moreover barley is widely distributed in the globe. The main objectives of this experiment were to observe physiological and mineralogical response of barley to As-toxicity in high to low P levels, especial focus has been given on visible symptom, Fe and As concentrations both in shoots and in roots.

9.2 MATERIALS AND METHODS

9.2.1 Seed Germination and Plant Culture

Barley (*Hordeum vulgare* L. cv. Minorimugi) seeds were grown in usual way (September-October, 2006) in the greenhouse. In this experiment, each bucket (5 L) contained 8 bunches (3 seedlings/bunch) of seedlings. Treatments were 10 μM As + 500 μM P, 10 μM As + 250 μM P, 10 μM As + 50 μM P, 10 μM As + 0 μM P and 0 μM As + 0 μM P for 14 days.

Arsenic concentration at 10 μM was used on the basis of our primary experiments, because 10 μM As did not produce acute toxicity symptoms in barley when the nutrient solution contained 10 μM EDTA-Fe^{3+}. Phosphorus was added as ammonium phosphate ($NH_4H_2PO_4$; Kanto Chemical Company, Tokyo, Japan) and As was used as $NaAsO_2$.

9.2.2 Chlorophyll Index (SPAD value)

Chlorophyll index (SPAD value) of fully developed third leaves was measured as described in section 2.6 of **CHAPTER 2**.

9.2.3 Analysis of Plant Samples

Sample preparation has been described in section 2.7 of **CHAPTER 2**. A hydride generation atomic absorption spectrophotometric (HGAAS) technique (Hitachi HFS-3) was used for As determination.

9.3 RESULTS

9.3.1 Visible Symptoms

Shoots of 10 μM As + 500 μM P, 10 μM As + 250 μM P and 0 μM As + 0 μM P treatments were almost similar in color **(Fig. 9.1)**. However, interveinal chlorosis was observed in 10 μM As + 50 μM P and 10 μM As + 0 μM P plants, in which the severe response was found in 10 μM As + 0 μM P treatment **(Fig. 9.1)**. Not only chlorosis but also necrotic symptom was found at 10 μM As + 0 μM P treatment **(Fig. 9.1)**. Visible growth, wilting in leaves and turgidity decreased in 10 μM As + 0 μM P treatment as compared to others.

Reddish color intensity increased in roots with decreasing P in As containing condition. Conspicuous reddish color (Fe-plaque) appeared at 10 μM As + 0 μM P treatment. However, it was not present in 0 μM As + 0 μM P treatment. Roots of 0 μM As + 0 μM P were very lengthy and thin but not highly branched.

9.3.2 Chlorophyll Index (SPAD value)

In P-supplied condition, chlorophyll index decreased with decreasing P in the medium **(Table 9.1)**. In this experiment, the lowest value of chlorophyll index was in absence of P

(Table 9.1). Chlorophyll index was higher in the plants grown in without P and As (0 μM As + 0 μM P) treatment as compared to plants grown in without P and As-containing (10 μM As + 0 μM P) condition.

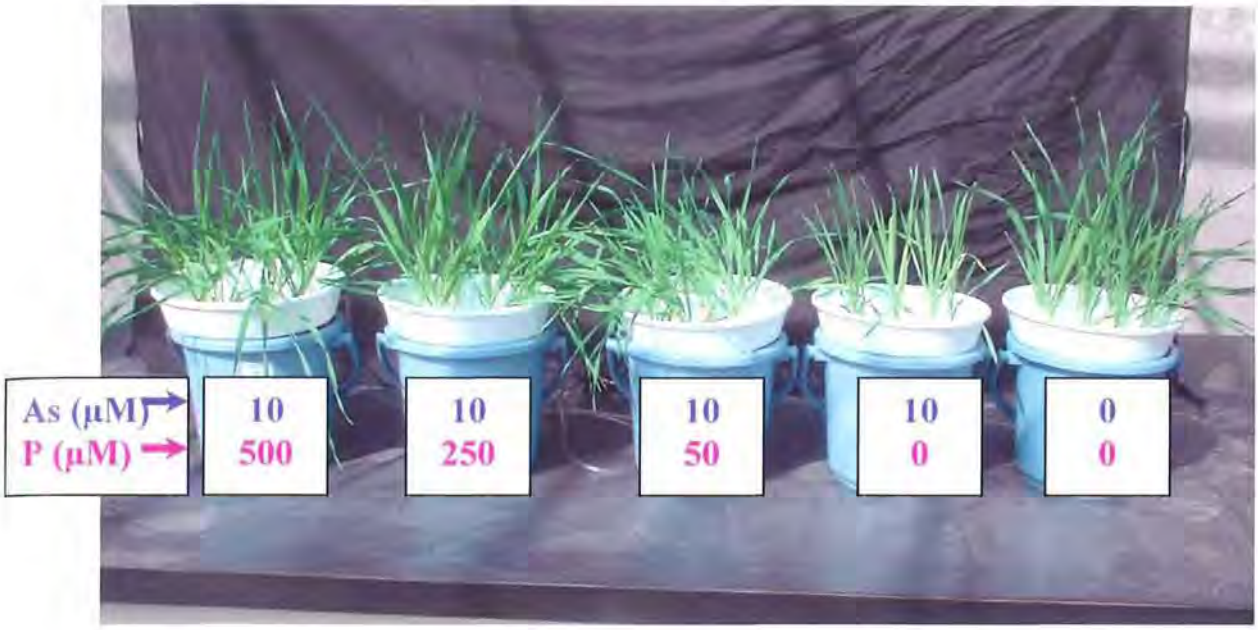

Figure 9.1 Photograph of barley seedlings grown at elevated concentration of As together with different P levels in nutrient solution. This picture was taken after 14 days of As exposures.

Table 9.1 Agronomic parameters of barley seedlings grown in different levels of P and As.

Treatments (μM)		DW (mg plant $^{-1}$)		Height (cm)		Leaf		Chlorophyll index	Tiller No. bunch^{-1}
		Shoot	Root	Shoot	Root	Width, mm	No. plant $^{-1}$		
As	P								
10	500	156.8a	61.5a	37.7a	13.0b	9.33a	4.78a	38.6a	3a
10	250	170.5a	63.3a	35.2a	15.0b	9.00a	4.44a	38.4a	3a
10	50	128.5b	57.5a	38.3a	14.3b	8.67a	4.00ab	33.3b	3a
10	0	67.0d	20.3b	24.7b	10.0c	5.00b	3.00b	14.2c	3a
0	0	106.1c	62.5a	36.7a	35.3a	9.00a	3.56b	33.6b	3a

Means followed by the different letters in each column are significantly different (p= 0.05) according to Ryan-Einot-Gabriel-Welsch multiple range test. DW = dry weight.

9.3.3 Leaf Number, Width of Leaf Blade and Tiller Number

Leaf number and width of leaf blade also decreased with decreasing P concentration in the medium **(Table 9.1)**. However, tiller number was not affected at all. At transplantation,

each bunch contained 3 tillers (3 seedlings) and it was similar at the harvest on 14 DAT in every treatment.

9.3.4 Dry Weight (DW), Shoot Height and Root Length

In As-containing condition, shoot and root DW decreased with decreasing P in the medium **(Table 9.1)**. The lowest DW was at 10 μM As + 0 μM P treatment. However, DW was higher when the plants were grown in absence of As and in P (0 μM As + 0 μM P) treatment as compared to 10 μM As + 0 μM P treatment **(Table 9.1)**. Shoot height did not seem to be affected much with the treatments except for 10 μM As + 0 μM P treatment. Root length was the lowest at 10 μM As + 0 μM P treatment as compared to the others. Interestingly, the highest root length was found in 0 μM As + 0 μM P treatment **(Table 9.1)**.

9.3.5 Macro and Micronutrients

In P-supplied condition, P concentration was lower both in shoots and in roots of low P condition as compared to that of high P condition **(Table 9.2)**. The lowest P concentration was found in shoots and in roots of 0 μM As + 0 μM P treatment. Similar trend was also found in case of accumulation. In case of K and Mg, the lowest concentration as well as accumulation was found at 10 μM As + 0 μM P treatment **(Table 9.2)**. Translocation of K and Mg was higher in 10 μM As + 0 μM P treatment as compared to others.

In P-supplied condition, Fe concentration decreased in shoots with decreasing P in the medium **(Table 9.2)**. Iron concentration was higher in shoots of P-supplied plants as compared to plants grown in P-depleted condition **(Table 9.2)**. In roots, Fe concentration was not much affected in P-supplied condition. However, Fe concentration increased in roots abruptly in plants grown in P-depleted condition **(Table 9.2)**. This is the most interesting result of this experiment. Iron translocation decreased with decreasing P concentration in the medium. In this experiment, almost 81% Fe was translocated when the plants were grown in absence of P and As **(Table 9.2)**.

In P-supplied condition, Mn concentration was not much affected in shoots by As, but it decreased in shoots of plants grown in P-depleted condition **(Table 9.2)**. Most of the cases, the concentration of Zn and Cu decreased in shoots by As in P-depleted condition **(Table 9.2)**.

Zinc translocation decreased with decreasing P in the medium. However, Cu translocation seemed to be not much affected with the treatments.

Table 9.2 Concentrations, accumulations and translocations of elements in shoots and in roots of barley seedlings grown in different levels of P and As.

Treatments (μM)		P	K	Ca	Mg	Fe	Mn	Zn	Cu
		----------mg g^{-1} DW ----------				----------μg g^{-1} DW ----------			
As	P	Concentrations in shoots							
10	500	4.86a	82.0a	5.69ab	1.12b	82.4a	18.5b	16.7a	7.78b
10	250	4.44a	82.7a	5.69ab	1.17b	79.3a	22.6b	14.8a	7.78b
10	50	2.86b	73.6a	4.70b	1.06b	59.4b	22.9b	7.21b	9.96a
10	0	0.79c	54.5b	5.87ab	0.83c	26.3c	6.58c	5.79c	5.91c
0	0	0.46d	80.0a	6.58a	1.53a	63.8b	29.6a	7.72b	9.03a
		Concentrations in roots							
10	500	5.64a	75.1a	1.41a	1.75b	129.1b	15.7b	22.0b	23.6b
10	250	4.91a	72.2a	1.63a	1.66b	119.0b	19.7b	19.2c	32.2a
10	50	1.67b	46.0b	1.51a	1.33c	113.8b	30.3a	17.5c	16.2c
10	0	1.80b	20.5c	1.29a	0.36d	262.6a	3.26c	23.8b	28.1ab
0	0	0.47c	53.8b	1.49a	2.58a	24.3c	34.5a	40.1a	25.4b
		----------mg $plant^{-1}$ ----------				----------μg $plant^{-1}$----------			
		Accumulation in shoots							
10	500	0.768a	12.8a	0.895a	0.177ab	12.9a	2.93b	2.64a	1.21a
10	250	0.754a	14.1a	0.970a	0.199a	13.5a	3.85a	2.51a	1.33a
10	50	0.372b	9.44b	0.604b	0.137c	7.81b	2.99b	0.91b	1.28a
10	0	0.053c	3.63c	0.379c	0.056d	1.71c	0.43c	0.38c	0.40c
0	0	0.049c	8.47b	0.689b	0.161b	6.82b	3.15ab	0.81b	0.96b
		Accumulation in roots							
10	500	0.349a	4.60a	0.087a	0.107b	8.00a	0.95d	1.38b	1.40b
10	250	0.312a	4.57a	0.103a	0.106b	7.54a	1.25c	1.21b	2.04a
10	50	0.096b	2.65c	0.087a	0.077c	6.59ab	1.77b	1.00c	0.94c
10	0	0.036c	0.41d	0.026c	0.007d	5.33b	0.06e	0.48d	0.57d
0	0	0.029d	3.36b	0.094b	0.161a	1.52c	2.16a	2.50a	1.59b
		Translocation (%)							
10	500	68.8ab	73.6b	91.2a	62.3b	62.1b	75.2b	66.2a	46.3b
10	250	70.6ab	75.5b	90.4a	65.3b	64.2b	75.5b	67.4a	39.4bc
10	50	79.2a	78.1b	87.4a	64.0b	53.4c	63.3c	47.7b	57.8a
10	0	59.0b	89.8a	93.4a	88.1a	24.3d	86.9a	44.3b	40.9bc
0	0	62.1b	71.5b	88.0a	50.0c	81.4a	59.2c	24.4c	37.5c

Means followed by the different letters in each column are significantly different (p= 0.05) according to Ryan-Einot-Gabriel-Welsch multiple range test. Translocation refers to the ratio of accumulation of element in shoot to the total accumulation (shoot + root). The translocation was expressed in %. DW = dry weight.

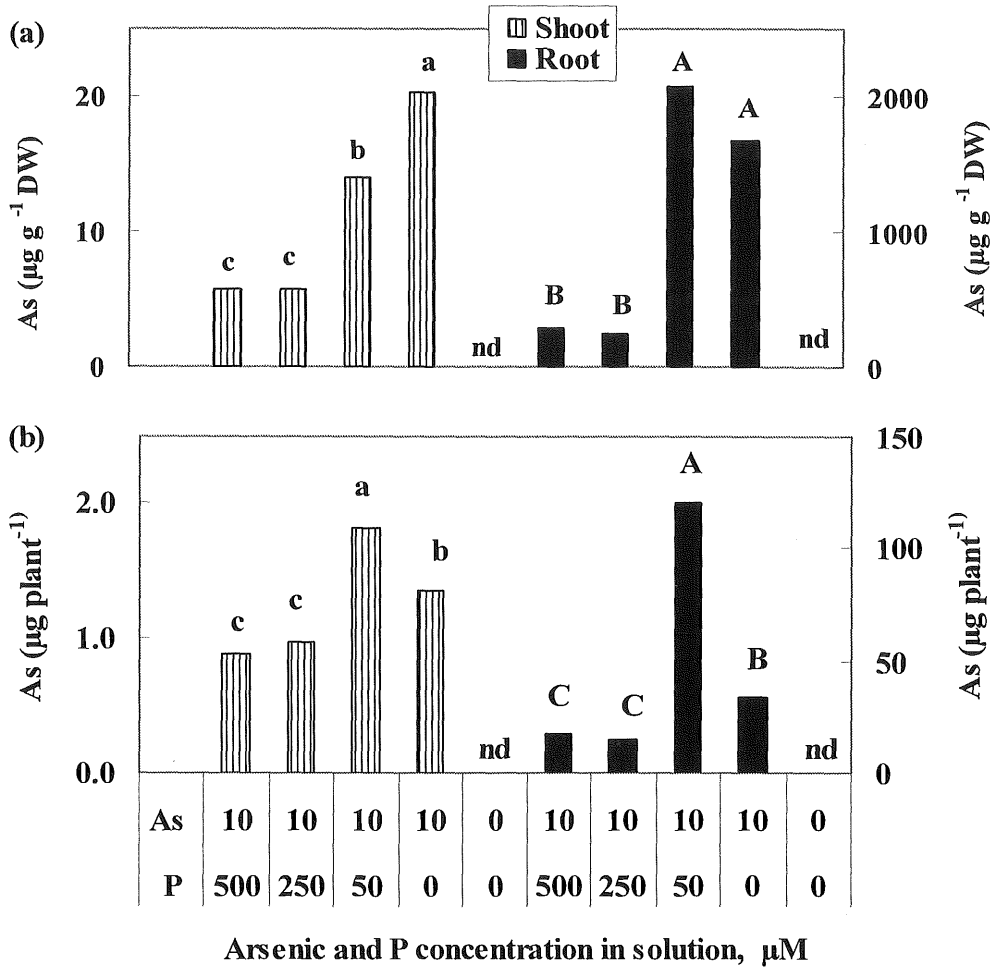

Figure 9.2 Effect of As on the (a) concentration and (b) accumulation of As in shoots and roots of barley seedlings at elevated concentration of As together with different P levels in nutrient solution. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

9.3.6 Arsenic

Arsenic concentration increased both in shoots and in roots with decreasing P concentration in the medium **(Fig. 9.2a)**. In shoots, the highest As concentration was found in absence of P. Similar to concentration, accumulation seemed to be also higher in low P containing solution **(Figs. 9.2ab)**. The highest accumulation was for the 10 µM As + 50 µM P treatment both in shoots and in roots. Arsenic translocation was not much affected in high P levels. However, As translocation was reduced in low P level in the medium **(Fig. 9.3)**.

9.4 DISCUSSION

Plants showed chlorosis symptom at 10 μM As + 0 μM P treatment, which was most probably due to the formation of Fe-plaque with reddish color at this treatment. Rice exhibit Fe-deficiency due to the formation of plaque on roots (Meharg 2004). Plants were green at 0 μM As + 0 μM P treatment **(Fig. 9.1)**. This was most probably due to P-deficiency. It is now well established that plants are green in P-deficient condition (Hopkins 1995; Ladouceur et al. 2006). In this case the ratios (%) of Fe:P (concentration) in shoot tissues might be the vital point. In P-deficient plants, the ratios of Fe:P in shoots are generally higher and shows greening in the leaves (Ladouceur et al. 2006).

In roots, reddish color appeared in 10 μM As + 0 μM P treatment which was most probably due to the formation of Fe-plaque on the root surface. The explanation regarding Fe-plaque has bee describe extensively in **CHAPTER 3**. Root length was the highest at 0 μM As + 0 μM P treatment as compared to others **(Table 9.1)**. Generally, root length is higher in P-deficient condition (Anuradha and Narayana 1991).

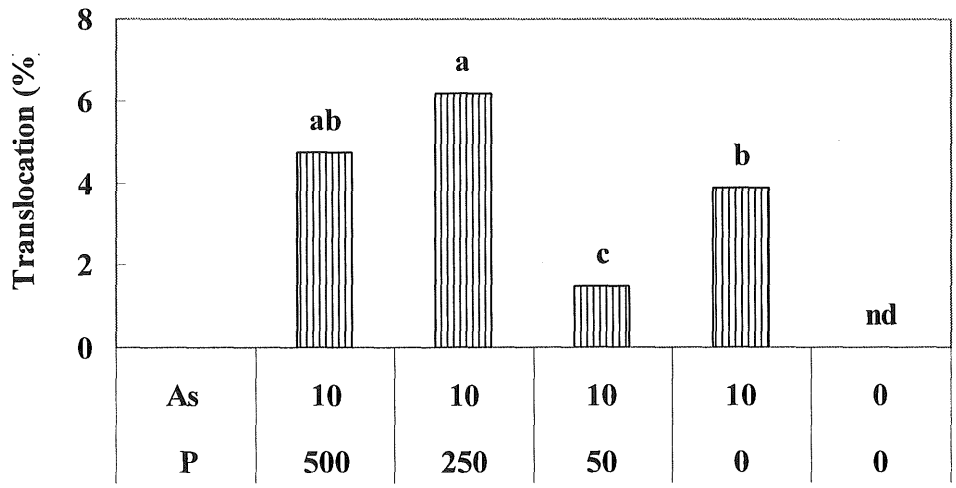

Figure 9.3 Effect of As on the translocation (%) of As from roots to shoots of barley seedlings at elevated concentration of As together with different P levels in nutrient solution. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

Reduction of DW, shoot height, root length, width of leaf blade, leaf number and chlorophyll index at 10 μM As + 0 μM P treatment as compared to the +P containing treatments implied that As-toxicity was higher in low or P-depleted condition. Our result demonstrated that if same concentration of As is present in different P levels, the plants of low P level will be affected severely. Oppositely, it may be noted that As-toxicity is lower in comparatively high P levels. Therefore, it was suggested that application of P might be partially effective to reduce As-toxicity. Application of inorganic P fertilizer prevents As-toxicity in wheat grown in pot soil (Pigna et al. 2009).

Phosphorus concentration and accumulation decreased with decreasing P concentration in the medium. In our experiment, plant growth decreased with decreasing P in the medium, resulting in lower accumulation. We found that As concentration increased both in shoots and roots. The increase of As concentration might also be responsible for the reduction of P concentrations in the plant parts. We used aeration; therefore, some arsenite might be converted to arsenate and competition might be occurred between them. Arsenate uptake was negatively correlated with phosphate uptake in duckweed (*Spirodella polyrhiza* L.; Rahman et al. 2008). Mkandawire and Dude (2005) suggested that arsenate uptake in *Lemna gibba* L. might occur through the phosphate uptake system.

In roots, Fe concentration in 0 μM As + 0 μM P treatment was 24.3 μg g^{-1} DW **(Table 9.2)** but in 10 μM As + 0 μM P treatment the value was 262.6 μg g^{-1} DW for the same, indicating that the activity of As was much higher in absence of P to concentrate Fe in or on the roots. In presence of higher P, Fe concentration was much lower in roots, suggesting that higher P in the medium reduced the activity of As to concentrate Fe in roots. Arsenic significantly decreased the concentrations of Fe in roots and in shoots of rice when the solution contained 50 μM EDTA-Fe^{2+} in presence of arsenate (Liu et al. 2004). However, in our experiment, we used 10 μM EDTA-Fe^{3+} in presence of 10 μM As (arsenite). The differences were most probably due to the source of Fe and As together with the species of plants. Decreases of Mn, Zn and Cu concentration in P-depleted condition were also most probably due to toxic effect of As on plants nutritional status.

In our experiment, As concentrations were significantly higher in arsenite-treated barley shoots and roots grown in low P or P-depleted medium than in high P-supplied medium **(Fig. 9.2a)**, suggesting that Fe-plaque might not sequestrate much arsenite. As a result sufficient

amount of As was concentrated or accumulated in the shoots of low or P-depleted condition. The reason, why higher amount of As was present in or on the roots of low P or P-depleted condition needs to be clarified. The reason behind this demands further study. In this experiment, As absorption increased in plants with decreasing P concentration in the medium. It is well known that arsenite has low affinity for the Fe-plaque compared to arsenate. This may be the reason why Fe-plaque did not reduce Fe-translocation much in this experiment at 10 μM As + 0 μM P treatment **(Fig. 9.3)**. During absorption, a competition between P and As might be occurred, because they are chemically similar. At low P or P-depleted condition high amount of As absorption might be possible, resulting in the higher concentration and accumulation of As both in shoots and roots in this experiment.

It is reported that As concentrations were significantly lower in arsenate-treated rice shoots grown in P-depleted medium than in P-containing medium (Liu et al. 2004). Iron-plaque might sequestrate As and consequently reduced the translocation of As from roots to shoots (Liu et al. 2004). Rice varieties sequester more As in plaque and translocate less As to aboveground tissues (Liu et al. 2004).

Our result showed that As and Fe were positively correlated in roots but negatively in shoots. Arsenate concentration was positively correlated with Fe concentration in roots of duckweed (*Spirodella polyrhiza* L.; Rahman et al. 2008).

9.5 CONCLUSIONS

Finally, it could be concluded that As-toxicity was very much depended on the concentration of P in the growth medium. The lower was the P concentration in the medium, the higher was the As toxicity. The severest toxicity symptom was observed in absence of P in the growth medium. Most of the cases the accumulation was the lowest in the plants grown in P-depleted condition. Formation of reddish color Fe-plaque was very much depended on the P concentration in the medium in As containing condition. Intensity of reddish color increased with decreasing P in the medium.

CHAPTER 10

EFFECT OF PHOSPHORUS ON IRON-PLAQUE FORMATION IN ARSENIC TREATED HYDROPONIC BARLEY

ABSTRACT

An experiment with arsenic (As) and barley (*Hordeum vulgare* L. cv. Minorimugi) grown hydroponically was conducted to observe the effects of phosphorus (P) status on the formation of iron (Fe)-plaque. Plants were grown for 16 days treated with 10 μM As in presence or in absence of P. A set of treatment also was without As and P. Phosphorus was used as ammonium phosphate ($NH_4H_2PO_4$) and As was used as sodium meta-arsenite ($NaAsO_2$). The half-strength nutrient solution was used at pH 5.5. The treatments were- 10 μM As + 500 μM P, 10 μM As + 250 μM P, 10 μM As + 50 μM P, 10 μM As + 0 μM P and 0 μM As + 0 μM P for 16 days. Iron-plaque with root reddish color was clearly visible on barley roots grown in As-treated and P-depleted condition. However, Fe-plaque with reddish color was not found in P-depleted and without As containing medium, suggesting that As played a vital role in the formation of Fe-plaque in P-depleted condition. Intensity of reddish color increased with decreasing P in the medium. Apoplastic-Fe together with other elements in the apoplast were extracted by the method of Bienfait et al. (1985). Contents of elements in Fe^{3+}-complex or in apoplast and in roots were determined. Particular emphasis was given on the concentration of P, Fe and As. Detectable amount of P was not found with apoplastic-Fe in all treatments, indicating that P might not be complexed with apoplastic-Fe. Phosphorus may be present inside of roots as organic or inorganic phosphate. Iron was mostly concentrated in apoplast. However, As was mostly concentrated in roots and a little amount was found to be complexed with Fe^{3+} in the apoplast. Our result suggested that P might repress the formation of As-Fe complex in the apoplast, somehow.

Abbreviations: CBE (citrate-bicarbonate-ethylenediaminetetraacetic acid); DCB (dithionite-citrate-bicarbonate); DAT (days after treatments); DW (dry weight)

10.1 INTRODUCTION

A little concentration of As may hamper the plant growth and nutritional quality of plants. Plants show As-toxicity response by changing some physiological responses in shoots and in roots. One of the toxicity responses of As in roots is the formation of reddish color. Formation of reddish color in root surface of aquatic plants is due to Fe-plaque in As-treated condition. Iron-plaque, coating of Fe hydroxides/oxides is commonly formed on the root

surface of aquatic plants such as rice (*Oryza sativa* L.). It is the consequence of oxidation of roots by release of oxygen and oxidants into the rhizosphere (Armstrong 1967; Chen et al. 1980). Iron-plaque has been reported to consist of a mixture of amorphous or crystalline Fe (Bacha and Hossner 1977; Chen et al. 1980).

Iron (hydro-) oxide has a high affinity for arsenate in soil or solution (Meng et al. 2002; Belzile and Tessier 1990; Jain et al. 1999). Arsenate reacts with Fe^{3+} on the rice root surface to give the highly insoluble Fe arsenate (Meharg 2004). Arsenic concentrations in Fe-plaque on rice roots were significantly higher in plants grown in arsenate treated condition as compared to those grown in arsenite treated condition. Therefore, it was suggested that Fe-plaque of rice roots had a higher affinity to arsenate than to arsenite (Liu et al. 2005). Plaque formation was governed by P concentration in the growth medium (Liu et al. 2004). Under low P condition, plaque formation was generally higher in arsenate treated rice (Liu et al. 2004). Rice treated with 6.7 μM As (arsenate) showed reddish color in roots after 24 h grown in the medium without P (Liu et al. 2004).

Depending on the species of As, species of plants and the composition of growth medium, P can decrease or increase the uptake of As by plants (Tsutsumi 1980; Otte et al. 1990). Phosphorus is chemically similar to As and, therefore has antagonistic relationship between them. Arsenate has been reported to be taken up by phosphate transporter (Asher and Reay 1979) and arsenite has been shown to be transported into rice roots via silicon transporter (Ma et al. 2008) and aquaporins (Meharg and Hartley-Whitaker 2002). In flooding condition, arsenate and arsenite co-exist in the soil solution (Smith et al. 1998; Abedin et al. 2002b). We used arsenite in aerated condition, because barley is an upland crop.

Apoplast is located between plasma membranes and the rhizosphere and is in contact with soil solution (Strasser et al. 1999). We think that apoplast is the space outside of root membrane and inside of the cell wall where elements can move freely. Apoplast is thought to be the main place for free Fe in roots. Zribi et al. (2002) reported that almost 75% Fe was concentrate as extraplasmic Fe and 25% was present as root Fe in pea grown hydroponically. This consideration prompted us to take up this physiological study to extract apoplastic-Fe together with other elements in the apoplast by the method of Bienfait et al. (1985). Moreover, we thought that Fe might be precipitated with As in apoplast, Fe might also be precipitated with P in apoplast or adsorbed on phosphate group of root membranes. In order to clarify these

considerations, relationship among P, Fe and As in apoplast needs to be examined. The main objectives of this experiment were to observe the appearance of Fe-plaque with reddish color with decreasing P in the medium and to determine the contents of elements in Fe-plaque (apoplast) and in barley roots treated with arsenite.

10.2 MATERIALS AND METHODS

10.2.1 Seed Germination and Plant Culture

Seedlings of barley were grown in usual way (September-October, 2006) in the greenhouse of Iwate University as described in section 2.3.2 of **CHAPTER 2**. In this experiment, each bucket (5 L) contained 8 bunches seedlings (3 plants/bunch). The half-strength modified nutrient solution at pH 5.5 was used. The treatments were 10 μM As + 500 μM P, 10 μM As + 250 μM P, 10 μM As + 50 μM P, 10 μM As + 0 μM P and 0 μM As + 0 μM P for 16 days. Arsenic concentration at 10 μM was used on the basis of our primary experiments, because 10 μM As did not produce acute toxicity symptom in barley when the nutrient solution contained 10 μM EDTA-Fe^{3+}.

10.2.2 Solubilization of Apoplastic-Fe and Sample Preparation

Apoplastic-Fe and other elements in apoplast were solubilized by the method of Bienfait et al. (1985). The Mes buffer solution at pH 5.5 (a solution of 10 mM Mes, calcium nitrate, 0.5 mM $Ca(NO_3)_2$ and 1.5 mM 2,2′ Biphydril) was used. Firstly, plants were washed with deionized water **(Fig. 10.1; Step 1)** before transferring to the step wise procedure. After washing, roots were shocked in 100 mL calcium sulfate solution (0.5 mM $CaSO_4$) for 10 min **(Fig. 10.1; Step 2)**, then shocked in 100 mL Mes buffer solution for 5 min **(Fig. 10.2; Step 3)**. After that, 5 mL sodium dithionite solution (250 mM $Na_2S_2O_4$) was added with the Mes buffer solution for another 10 min more **(it is also in Step 3)**. Direct contact of roots with $Na_2S_2O_4$ solution was avoided. After adding $Na_2S_2O_4$ solution, plants were shaken gently for 10 min. Plants were retransferred to $CaSO_4$ solution for another 10 min more. After that plants were collected, washed with deionized water and roots were separated from the shoots. Root samples were dried at 60 ± 5°C for 48 h and digested with a nitric acid perchloric acid mixture (HNO_3-

$HClO_4$). Extract of Mes solution was collected and was evaporated by digesting it with a HNO_3-$HClO_4$ mixture.

Figure 10.1 Solubilization of apoplastic-Fe (Bienfait et al. 1985) together with other elements in apoplast. Step 1: washing with deionized water and Step 2: is the washing step with calcium sulfate solution (0.5 mM $CaSO_4$) for 10 min.

Figure 10.2 Solubilization of apoplastic-Fe (Bienfait et al. 1985) together with other elements in apoplast. Step 3 is the solubilization of apoplastic-Fe with Mes buffer solution.

Digested samples (roots and Mes extract) were condensed to 5 mL. The final volume was made to 50 mL with MQ water. Digested samples were analyzed with a Hydride Generation Atomic Absorption Flame Emission Spectrophotometer (AA-6200; Shimadzu Corporation, Kyoto, Japan) for all elements except P. Phosphorus was determined colorimetrically using a UV-visible Spectrophotometer (model UV mini 1240, Shimadzu Corporation, Kyoto, Japan) at 420 nm wavelengths (Imamul-Huq and Alam 2005).

10.3 RESULTS

10.3.1 Visible Symptoms

Reddish color intensity increased with decreasing P in the medium. Roots appeared conspicuous reddish color on 16 days after treatments at 10 μM As + 0 μM P **(Fig. 10.3)**. Reddish color indicates the presence of Fe-plaque (Armstrong 1967; Chen et al. 1980; Liu et al. 2004; 2005). Roots of 0 μM As + 0 μM P was very lengthy and thin as compared to others **(Fig. 10.3)** and was not highly branched.

Figure 10.3 Photograph of barley seedlings grown at elevated concentration of As (μM) together with different P (μM) levels in nutrient solution. This picture was taken after 16 days of As exposure. Reddish color intensity increased with decreasing P in the medium and conspicuous reddish color Fe-plaque was formed in presence of As but in absence of P.

10.3.2 Root Dry Weight (DW)

Root DW decreased with decreasing P in the medium and the lowest value was in As-treated plants grown in P-depleted condition **(Fig. 10.4)**. Root DW was significantly higher in absence of P and As compared to As-treated plants grown in P-depleted condition.

10.3.3 Macro and Micronutrients

Phosphorus concentration and accumulation decreased in roots with decreasing P **(Table 10.1)**. Solubilized P in apoplast was not detected at all. Potassium concentration decreased in roots with decreasing P (without 10 μM As + 0 μM P treatment). Potassium concentration increased suddenly in roots in 10 μM As + 0 μM P treatment **(Table 10.1)**. Very little amount of solubilized K in apoplast was detected but this content seemed to be within the range of error. Potassium accumulation decreased in As-treated roots with decreasing P in the medium. Calcium and Mg concentration decreased in As-treated roots in absence of P. Most of the Ca was concentrated and accumulated in the apoplast **(Table 10.1)**.

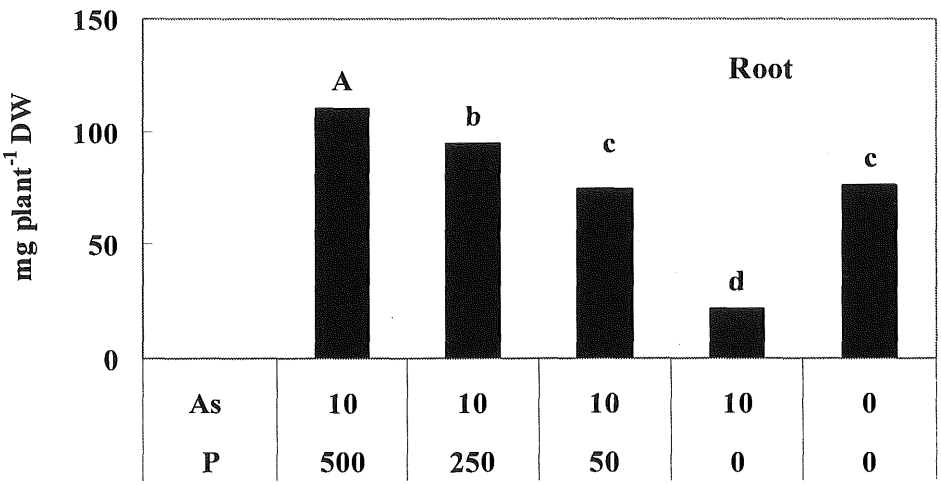

Figure 10.4 Root dry weight (DW) of barley seedlings at elevated concentration of As together with different P levels in solution. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

In P-supplied condition, Fe concentration in apoplast was almost similar, but in P-depleted condition, Fe concentration in apoplast was much higher **(Table 10.1)**. Major portion

of Fe was concentrated in apoplast as compared to roots. Formation of apoplastic-Fe was low in absence of As and P (0 μM As + 0 μM P treatment; **Table 10.1**).

Table 10.1 Concentrations and accumulations of elements in roots and in apoplast of barley seedlings grown in different treatments of P and As.

Treatments (μM)		P	K	Ca	Mg	Fe	Mn	Zn	Cu
		----------mg g^{-1} DW ----------				----------μg g^{-1} DW ----------			
As	P	Concentrations in roots							
10	500	4.64a	92.7b	1.79ab	2.89a	118.0a	9.02c	34.8b	12.7b
10	250	3.40b	88.7b	2.11a	2.57b	148.2a	14.4b	27.6c	15.7b
10	50	1.35c	72.3c	2.13a	1.36c	118.3a	25.0a	29.0bc	9.57c
10	0	0.88d	132.3a	1.39b	0.28d	116.6a	14.1b	23.1d	19.6a
0	0	0.84d	86.2b	1.89a	2.71ab	60.0b	25.3a	41.1a	13.6b
		Concentrations in apoplast							
10	500	nd	3.84c	5.87c	0.095b	720c	2.21d	50.6c	1.36d
10	250	nd	4.63c	7.15bc	0.099b	714c	3.76c	110.0b	3.68c
10	50	nd	4.38c	6.61c	0.098b	827b	7.12b	129.9b	4.45c
10	0	nd	10.5a	24.2a	0.078c	2631a	22.9a	684.7a	41.3a
0	0	nd	6.16b	7.91b	0.126a	114d	9.67b	117.2b	9.81b
		----------mg $plant^{-1}$ ----------				----------μg $plant^{-1}$----------			
		Accumulation in roots							
10	500	0.51a	10.2a	0.197a	0.318a	13.0a	1.00c	3.84a	1.39a
10	250	0.32b	8.28a	0.199a	0.247b	13.9a	1.35b	2.62b	1.44a
10	50	0.09c	5.24b	0.158b	0.103c	9.01b	1.82a	2.17b	0.70c
10	0	0.02d	2.84c	0.030c	0.006d	2.56d	0.30d	0.50c	0.41d
0	0	0.07c	6.52b	0.143b	0.206b	4.02c	1.94a	3.13a	1.03b
		Accumulation in apoplast							
10	500	nd	0.42a	0.65a	0.010a	79.4a	0.24d	5.51c	0.15d
10	250	nd	0.43a	0.64a	0.009a	65.8b	0.33c	8.91b	0.32c
10	50	nd	0.31b	0.47c	0.007b	60.9b	0.50b	9.03b	0.32c
10	0	nd	0.23c	0.51b	0.002c	58.0b	0.49b	15.5a	0.95a
0	0	nd	0.46a	0.60ab	0.010a	8.57c	0.72a	9.36b	0.74b

Means followed by the different letters in each column of individual group are significantly different (p= 0.05) according to Ryan-Einot-Gabriel-Welsch multiple range test. DW = dry weight. nd = not detected.

Manganese concentration was higher both in roots and in apoplast in low P condition. High content of Mn was present in roots compared to adsorb in apoplast. Zinc concentration in roots was lower in low P condition but was higher in absence of As and P. In general, Zn concentrations in apoplast were higher as compared to roots. However, Cu concentration was higher in roots compared to apoplast. Copper concentration in apoplast was higher in low P condition compared to high P condition **(Table 10.1)**.

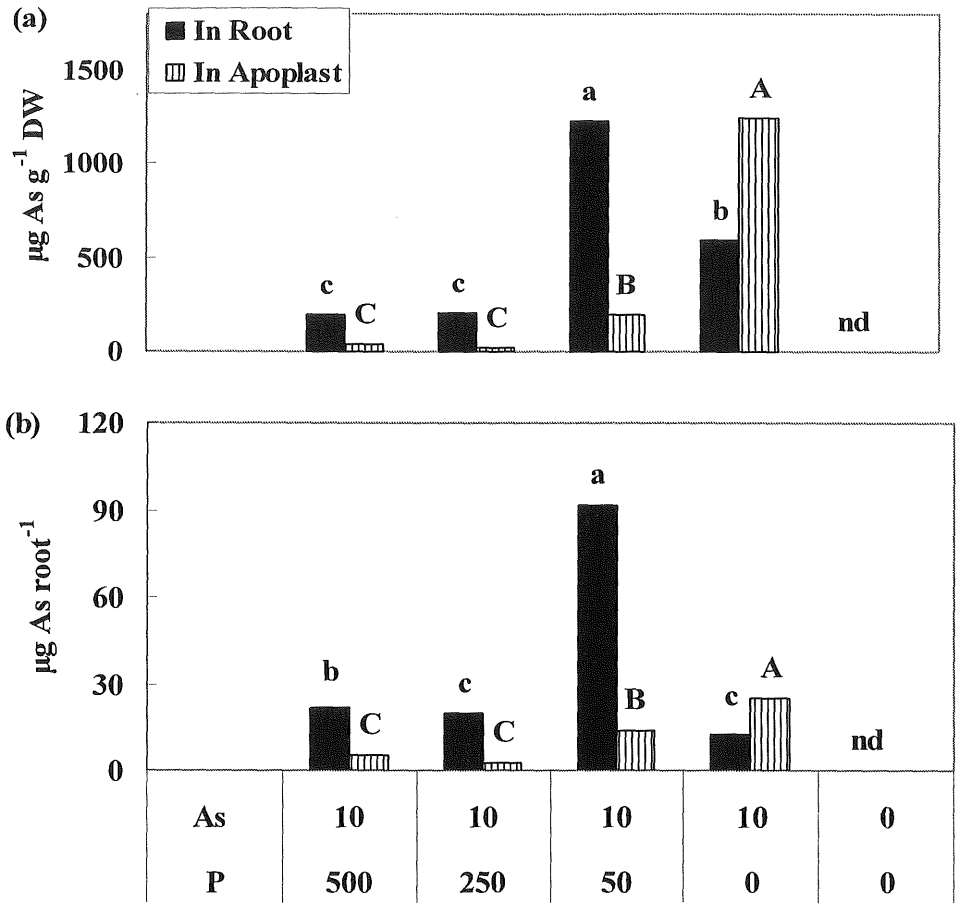

Figure 10.5 Effect of As on the (a) concentration and (b) accumulation of As in roots and in apoplast of barley seedlings at elevated concentration of As together with different P levels in nutrient solution. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test. nd = not detected.

10.3.4 Arsenic

In P-supplied condition, As concentrations in roots and in apoplast increased with decreasing P in the medium and the highest content was at 10 µM As + 50 µM P treatment **(Fig. 10.5a)**. Arsenic concentration was much lower in the roots of 10 µM As + 0 µM P treatment compared to 10 µM As + 50 µM P treatment **(Fig. 10.5a)**. In P-supplied condition, As concentrations in apoplast were much lower as compared with that of roots. However, in P-depleted condition, As concentration in apoplast was much higher as compared with that of

roots. In every case, similar results were obtained in accumulation. It meant that P might interfere the formation of As-Fe complex in apoplast. In P-depleted condition, formation of As-Fe complex might not be interfered by P, resulting in higher As in apoplast.

10.4 DISCUSSION

Conspicuous reddish color appeared in barley roots grown in P-depleted but As-containing treatment **(Fig. 10.3)**. Intensity of reddish color increased with decreasing P in As containing condition, indicating that As intensified the reddish color on the root surface in low or P-depleted condition. The result suggested that the activity of As to form Fe-plaque increased with decreasing P. In other word, formation of Fe-plaque might be very much dependent on the P concentration in the medium **(Fig. 10.3)**. In our case, Fe-plaque was visible as reddish coating on the root surface of barley. Recently, it was suggested that formation of Fe-plaque in rice roots might be governed by P nutritional status in the medium (Liu et al. 2004).

In this experiment, P in apoplast or adsorbed with apoplastic-Fe was not detected, suggesting that P might not be precipitated with Fe in apoplast. Phosphorus was mainly found in the roots, suggesting that P might mostly be present as inorganic or organic (phospholipids) P in roots. This supposition demands further study. Mes buffer solution dissolving apoplastic-Fe contained no P, suggesting that the root membrane may not be broken by this reducing agent used in the current experiment. It was mentioned that Fe-plaque locked up phosphate (Meharg 2004). However, our result suggested that Fe-plaque might not lock up P in barley roots, because we did not see any detectable amount of P adsorbed with apoplastic-Fe **(Table 10.1)**.

In presence of As, Fe in apoplast increased with decreasing P **(Table 10.1)**. In P-supplied condition, Fe concentration in apoplast was almost similar, but in P-depleted condition Fe concentration in apoplast was much higher as compared to P-supplied condition **(Table 10.1)**. It was suggested that P hindered the formation of apoplastic-Fe in presence of As. Apoplast contained almost 6, 5, 7, 23 and 2 times higher Fe than the roots in 10 μM As + 500 μM P, 10 μM As + 250 μM P, 10 μM As + 50 μM P, 10 μM As + 0 μM P and 0 μM As + 0 μM P treatments, respectively, indicating that apoplast was the sink of Fe in roots. The highest apoplastic-Fe was in the plants treated with As in P-depleted condition **(Table 10.1)**. However, the formation of apoplastic-Fe was low in absence of As and P.

Roots contained almost 5.37, 9.01 and 6.47 times higher As as compared to adsorbed As with apoplastic-Fe in 10 μM As + 500 μM P, 10 μM As + 250 μM P and 10 μM As + 50 μM P treatments, respectively, indicating that roots are the main sink of As in arsenite treated and P-supplied condition. However, in absence of P, As concentration in apoplast was 2.09 times higher compared to roots. It was suggested that absorption of As was partially regulated by P nutritional status in the medium. Recently, it was reported that most of the As was concentrated in roots of rice when the plants were grown in arsenite treated and P-supplied medium (Liu et al. 2005). However, it was also reported that As was mainly concentrated in DCB extracts or on the root surface, when arsenate treated rice was grown in P-depleted medium. It was reported that As concentrations in Fe-plaque were significantly higher (up to 1180 mg kg^{-1}) in rice roots grown in arsenate treated and P-depleted medium compared to plants grown in arsenate treated and P-sufficient solution (Liu et al. 2004). About 56% of the total As was concentrated in the root tissues and 44% was adsorbed on Fe-plaque (CBE-extract) when duckweed (*Spirodella polyrhiza* L.) were grown in arsenate treated solution (Rahman et al. 2008). It was suggested that the concentration of As in apoplast or in roots is mostly dependent on the species of As (arsenite or arsenate), plant species and also the presence and or absence of P in the growth medium.

10.5 CONCLUSIONS

Formation of Fe-plaque was very much dependent on the P concentration in the nutrient solution. The lower was the P concentration; the higher was the Fe-plaque intensity. Phosphorus might not be precipitated with Fe in the apoplast and may be present as organic or inorganic form in the roots. In low P condition, Fe was mostly concentrated in apoplast compared to the roots. In arsenite treated seedlings, most of the As was concentrated or accumulated in roots compared to apoplast. A portion of Fe might be precipitated with arsenite in apoplast. Phosphorus may hinder the formation of Fe-As complex in the apoplast.

CHAPTER 11

QUANTITATIVE ANALYSIS OF PHOSPHORUS, IRON AND ARSENIC IN ROOTS AND IN APOPLAST OF RICE: INTERACTION WITH ARSENIC AND IRON

ABSTRACT

Previously we found that iron (Fe) and arsenic (As) were mostly concentrated in the roots of As-treated rice (*Oryza sativa* L. cv. Akitakomachi). Outer space (apoplast) and inner space (in root) are the two major portions of roots. Contents of the elements may be varied in these two spaces. This report consists of two experiments. The first experiment was conducted to observe the effect of As on phosphorus (P), Fe and As concentration in roots and in apoplast. The second experiment was conducted to observe the effect of additional EDTA-Fe^{3+} on the P, Fe and As concentration in roots and in apoplast in As containing condition. Apoplastic-Fe together with elements in apoplast were extracted by the method of Bienfait et al.. Phosphorus, Fe and As were measured with PIXE (Particle-Induced X-ray Emission). In the first experiment, the treatments were 0 μM As + 10 μM EDTA-Fe^{3+}, 6.7 μM As + 10 μM EDTA-Fe^{3+}, 13.4 μM As + 10 μM EDTA-Fe^{3+} and 26.8 μM As + 10 μM EDTA-Fe^{3+} for 14 days. In the second experiment, the treatments were 0 μM As + 10 μM EDTA-Fe^{3+} (control), 13.4 μM As + 10 μM EDTA-Fe^{3+} (As-treated), 13.4 μM As + 25 μM EDTA-Fe^{3+} (medium-Fe^{3+}) and 13.4 μM As + 50 μM EDTA-Fe^{3+} (high-Fe^{3+}) for 14 days. In the first experiment, Fe-plaque representing as root reddish color was clearly visible in the roots of As-treated plants. Intensity of reddish color increased along with roots with increasing As in the medium, suggesting that As played a vital role in the formation of Fe-plaque. Detectable amount of P in apoplast or adsorbed with apoplastic-Fe was not found in all the treatments, suggesting that P might not form complex with apoplastic-Fe. Iron was mostly concentrated in apoplast as compared to roots. However, As was mostly concentrated in roots and a little amount was in apoplast. Additional-Fe^{3+} in the medium decreased P concentration in roots. Additional-Fe^{3+} in the medium increased Fe concentration both in roots and in apoplast. However, the magnitude was 1.86 and 2.63 times higher in apoplast as compared to roots in 13.4 μM As + 25 μM EDTA-Fe^{3+} and 13.4 μM As + 50 μM EDTA-Fe^{3+} treatments, respectively. Additional-Fe^{3+} decreased As concentration in roots.

Abbreviations: DW (dry weight); DAT (days after treatments)

11.1 INTRODUCTION

Root has outer (apoplast) and inner space (in root). Apoplast is the space in roots for free movements of ions especially iron (Fe) (Bienfait et al. 1985) and most of the Fe is present in the free spaces in roots (Bienfait et al. 1985; Zribi and Gharsalli 2002). Iron has very high affinity to form complex with phosphorus (P) and arsenic (As). Relationship among free space of root, P, Fe and As leaded us to take up this physiological study to find out the Fe content in root or in free space of As-treated rice seedlings.

Inorganic As in the medium sharply governs the As-toxicity and the higher is the concentration, the higher is the toxicity (Shaibur et al. 2006). Beside As concentrations, As phytotoxicity is also depended on soil properties such as pH, concentration of phosphate, Fe, aluminium (Al), amount of organic matter and the sensitivity of the crops (Carbonell-Barrachina et al. 1995). Especially, As-Fe interaction may be a significant matter to be investigated as As induced reddish color Fe-plaque in roots of hydroponic rice. This report consists of two experiments. The main objectives of the first experiment were to observe the concentration of Fe in apoplast and in roots in arsenite-treated rice. The second experiment was conducted to observe the effect of additional-Fe^{3+} on the formation of Fe-plaque in roots of rice treated with arsenite. In this research, arsenite was used, because in anaerobic paddy field condition arsenite, the most toxic form of As is mostly prevailed.

11.2 MATERIALS AND METHODS

11.2.1 Seed Germination and Plant Culture

Seedlings of rice were grown as described in section 2.3.1 of **CHAPTER 2**. Seeds were germinated in an electric incubator for 60 h at 25 ± 2°C. Duration of these experiments was from 24 June to 6 August 2008. In the first experiment, the treatments were 0 μM As + 10 μM EDTA-Fe^{3+}, 6.7 μM As + 10 μM EDTA-Fe^{3+}, 13.4 μM As + 10 μM EDTA-Fe^{3+} and 26.8 μM As + 10 μM EDTA-Fe^{3+} for 14 days. In the second experiment, the treatments were 0 μM As + 10 μM EDTA-Fe^{3+} (control), 13.4 μM As + 10 μM EDTA-Fe^{3+} (As-treated), 13.4 μM As + 25 μM EDTA-Fe^{3+} (medium-Fe^{3+}), 13.4 μM As + 50 μM EDTA-Fe^{3+} (high-Fe^{3+}). Arsenic was used as sodium meta-arsenite ($NaAsO_2$) for 14 days.

11.2.2 Environmental Condition of the Greenhouse

Experiments were carried out in the greenhouse with ambient light (roughly 14 h day/10 h night). Temperature was around 18 to 32°C at night and day, respectively.

11.2.3 Solubilization of Apoplastic-Fe and Sample Preparation

Apoplastic-Fe together other elements in apoplast were solubilized by the method of Bienfait et al. (1985) as described in **CHAPTER 10**. After solubilization, the roots were washed with deionized water, dried and were digested with HNO_3-$HClO_4$ mixture as described in section 2.7 of **CHAPTER 2**. Similar to root samples, solubilized extract was also digested. Digested root and digested solution samples were analyzed with PIXE for P, Fe and As.

11.2.4 Calculation for the Parameter

First of all concentration was calculated in mg or μg of element g^{-1} DW and accumulation was calculated as mg or μg of element $plant^{-1}$ shoot or root (Shaibur et al. 2006). After that, concentration and accumulation values were expressed as mmol g^{-1} DW (for P) and μmol g^{-1} DW (for Fe and As). The mmol g^{-1} DW and μmol g^{-1} DW values were obtained by dividing the unit mg or μg of element g^{-1} DW with the molecular weight of the particular element.

11.3 RESULTS

11.3.1 Visible Symptoms

In the first experiment, roots of control plants were white at harvest. However, Fe-plaque with reddish color appeared in roots with 6.7 μM As + 10 μM EDTA-Fe^{3+}, 13.4 μM As + 10 μM EDTA-Fe^{3+} and 26.8 μM As + 10 μM EDTA-Fe^{3+} treatments. The intensity of reddish color in roots increased with increasing As in the medium. In the second experiment, roots of control plants were also white, but Fe-plaques were clearly visible as a reddish coating on the root surface of the plants treated with 13.4 μM As + 10 μM EDTA-Fe^{3+}, 13.4 μM As + 25 μM EDTA-Fe^{3+} and 13.4 μM As + 50 μM EDTA-Fe^{3+} treatments.

11.3.2 Dry Weight (DW)

In the first experiment, root DW decreased in 6.7 μM As + 10 μM EDTA-Fe^{3+}, 13.4 μM As + 10 μM EDTA-Fe^{3+} and 26.8 μM As + 10 μM EDTA-Fe^{3+} treatments as compared to the roots of control plants. Though the root DW reduction was not proportional to the As concentration in the nutrient solution **(Fig. 11.1a)**. Similar to the first experiment, root DW was also decreased by 13.4 μM As + 10 μM EDTA-Fe^{3+} treatment as compared to control **(Fig. 11.1b)**. Root DW did not increase in 13.4 μM As + 50 μM EDTA-Fe^{3+} as compared to 13.4 μM As + 10 μM EDTA-Fe^{3+} treatment **(Fig. 11.1b)**.

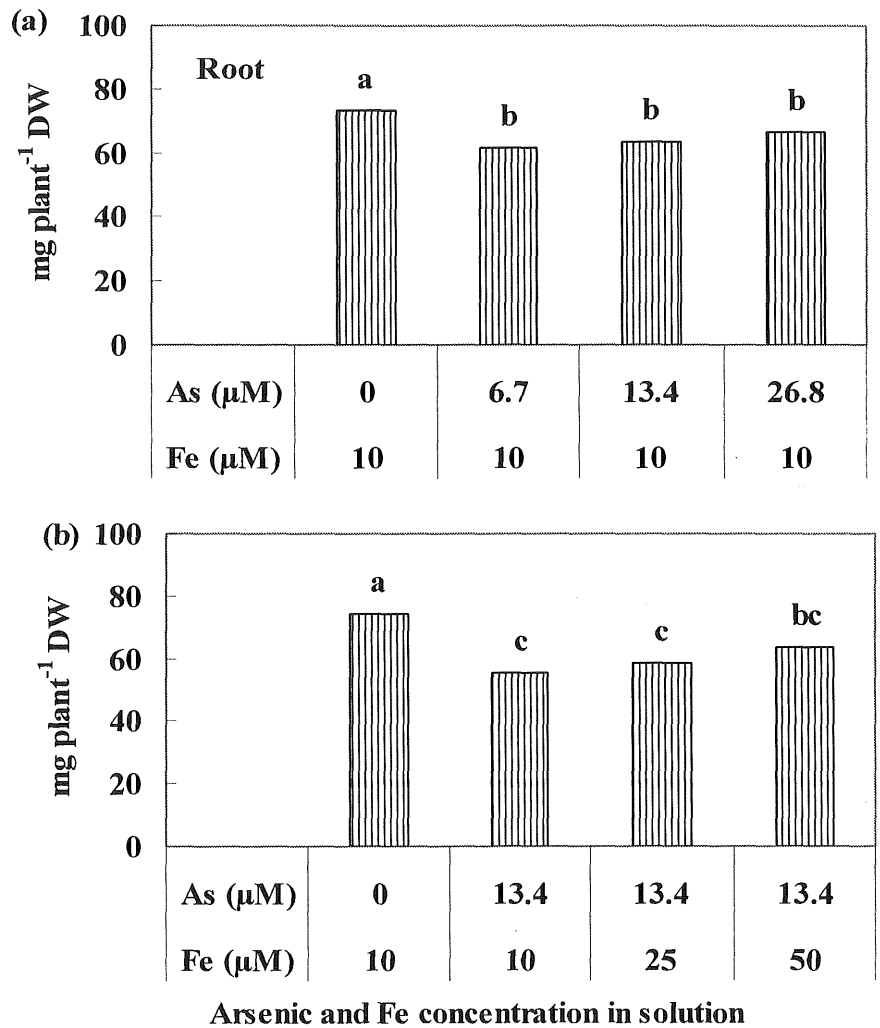

Figure 11.1 Root dry weight (DW) of rice seedlings (a) at elevated concentrations of As and or (b) in presence of additional EDTA-Fe^{3+} treatments. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

11.3.3 Macro and Micronutrients

In the first experiment, P concentration in roots was not much affected by 6.7 μM As + 10 μM EDTA-Fe^{3+} or 13.4 μM As + 10 μM EDTA-Fe^{3+} treatments but decreased at 26.8 μM As + 10 μM EDTA-Fe^{3+} treatment as compared to control **(Table 11.1)**. Similar trend was also found for accumulation **(Table 11.1)**. Detectable amount of P in apoplast or adsorbed with apoplastic-Fe was not found at all.

Table 11.1 Concentrations and accumulations of P, Fe and As in roots or in apoplast of rice seedlings grown with different treatments of As (first experiment).

Treatments (μM)		----------P----------		----------Fe----------		----------As----------	
		In root	In Apoplast	In root	In Apoplast	In root	In Apoplast
As	EDTA- Fe^{3+}	μmol g^{-1} DW		μmol g^{-1} DW		μmol g^{-1} DW	
0	10	71.9a	nd	0.81c	1.62b	nd	nd
6.7	10	87.7a	nd	0.83c	1.76b	0.93b	0.02c
13.4	10	72.0a	nd	1.36a	3.20a	2.56a	0.09a
26.8	10	55.1b	nd	1.08b	3.39a	2.64a	0.06b
		μmol plant $^{-1}$ root		μmol plant $^{-1}$ root		μmol plant $^{-1}$ root	
0	10	5.26a	nd	0.06b	0.12b	nd	nd
6.7	10	5.47a	nd	0.05b	0.11b	0.057b	0.001c
13.4	10	4.52b	nd	0.09a	0.21a	0.162a	0.006a
26.8	10	3.67c	nd	0.09a	0.23a	0.175a	0.004b

Means followed by the different letters in each column are significantly different (p= 0.05) according to Ryan-Einot-Gabriel-Welsch multiple range test. DW = dry weight. nd = not detected.

In the second experiment, P concentration in roots was not much affected by 13.4 μM As + 10 μM EDTA-Fe^{3+} or 13.4 μM As + 25 μM EDTA-Fe^{3+} treatments as compared to control **(Table 11.2)**. However, it decreased at 13.4 μM As + 50 μM EDTA-Fe^{3+} treatment as compared to the others **(Table 11.2)**. Accumulation of P decreased in roots at 13.4 μM As + 10 μM EDTA-Fe^{3+}, 13.4 μM As + 25 μM EDTA-Fe^{3+} and 13.4 μM As + 50 μM EDTA-Fe^{3+} treatments as compared to control roots **(Table 11.2)**. Similar to the first experiment, P was not detected in apoplast.

Table 11.2 Concentrations and accumulations of P, Fe and As in roots or in apoplast of rice seedlings grown in different treatments of As and EDTA-Fe^{3+} (second experiment).

Treatments (μM)		P		Fe		As	
		In root	In Apoplast	In root	In Apoplast	In root	In Apoplast
As	EDTA- Fe^{3+}	μmol g^{-1} DW		μmol g^{-1} DW		μmol g^{-1} DW	
0	10	74.3a	nd	0.88c	1.40d	nd	nd
13.4	10	77.9a	nd	1.95b	2.93c	3.44a	0.11b
13.4	25	67.0a	nd	2.22ab	4.14b	2.94b	0.44a
13.4	50	48.6b	nd	2.53a	6.66a	2.79b	0.13b
		μmol $plant^{-1}$ root		μmol $plant^{-1}$ root		μmol $plant^{-1}$ root	
0	10	5.51a	nd	0.07c	0.10d	nd	nd
13.4	10	4.32b	nd	0.11b	0.16c	0.19a	0.01b
13.4	25	3.97b	nd	0.13b	0.24b	0.17a	0.03a
13.4	50	3.09c	nd	0.16a	0.42a	0.18a	0.01b

Means followed by the different letters in each column are significantly different (p= 0.05) according to Ryan-Einot-Gabriel-Welsch multiple range test. DW = dry weight. nd = not detected.

In the first experiment, Fe concentration was higher in roots of 13.4 μM As + 10 μM EDTA-Fe^{3+} and 26.8 μM As + 10 μM EDTA-Fe^{3+} treatments as compared to the roots of control plants **(Table 11.1)**. Similarly, Fe concentration was also higher in apoplast at 13.4 μM As + 10 μM EDTA-Fe^{3+} and 26.8 μM As + 10 μM EDTA-Fe^{3+} treatments as compared to the roots of control plants **(Table 11.1)**. Our result showed that Fe concentration increased higher in apoplast as compared to roots with increasing As in the medium **(Table 11.1)**. Similar trend was also found for accumulation both in roots and in apoplast. Iron concentration in roots were 1.02, 1.68 and 1.33 times higher as compared to control in 6.7 μM As + 10 μM EDTA-Fe^{3+}, 13.4 μM As + 10 μM EDTA-Fe^{3+} and 26.8 μM As + 10 μM EDTA-Fe^{3+} treatments, respectively. In apoplast the values were 1.09, 1.98 and 2.09 times higher for the same, respectively. Our result showed that As concentrated higher Fe in apoplast as compared to roots. In case of accumulation, 1.75 and 1.92 times higher Fe was found in apoplast at 13.4 μM As + 10 μM EDTA-Fe^{3+} and 26.8 μM As + 10 μM EDTA-Fe^{3+} treatments, respectively.

In the second experiment, Fe concentration increased in roots at 13.4 μM As + 10 μM EDTA-Fe^{3+} treatment as compared to control roots **(Table 11.2).** Iron concentrations in roots and in apoplast were also increased with higher Fe treatments in As-treated condition **(Table 11.2).** Similar to the concentration, accumulations both in roots and in apoplast were also affected by the treatments **(Table 11.2).** In the second experiment, Fe concentrations were 2.22, 2.52 and 2.88 times higher in roots as compared to control in 13.4 μM As + 10 μM EDTA-Fe^{3+}, 13.4 μM As + 25 μM EDTA-Fe^{3+} and 13.4 μM As + 50 μM EDTA-Fe^{3+} treatments, respectively. In apoplast, the concentration values were 2.09, 2.96 and 4.76 times and accumulation values were 1.60, 2.40 and 4.20 times higher for the same.

11.3.4 Arsenic

In the first experiment, As concentration increased in roots with increasing As in the rooting medium. In apoplast, As concentration was higher in higher As-treated condition. Arsenic concentration was very low in apoplast as compared to roots **(Table 11.1).** Our result showed that additional-Fe^{3+} decreased As concentration in roots **(Table 11.2).** In apoplast, As concentration increased at 13.4 μM As + 25 μM EDTA-Fe^{3+} treatment as compared to 13.4 μM As + 10 μM EDTA-Fe^{3+} treatment **(Table 11.2).** The reason is not known clearly.

11.4 DISCUSSION

Intensity of reddish color increased with increasing As in the medium. Our result suggested that the formation of Fe-plaque and absorption of Fe might be very much dependent on As concentration in the medium, the higher was the As concentration, the higher was the plaque intensity. In our experiment, Fe-plaque was visible as reddish coating on the rice root surface. Similar result was also obtained in rice root treated with arsenite (Liu et al. 2005).

In this report, P was not detected in apoplast or adsorbed with apoplastic-Fe in both experiments, suggesting that P might not be precipitated with Fe in the apoplast. Phosphorus might mostly be present as organic or inorganic P in roots. This supposition, however, demands further study. Recently, it was suggested that formation of Fe-plaque in rice roots might be governed by P nutritional status in the medium (Liu et al. 2005). Under low P condition in the medium plaque formation is increased (Liu et al. 2004) and P concentration in plants (Meharg 2004). In our case, it might also be true, because in the first experiment P concentration was the

lowest at 26.8 μM As + 10 μM EDTA-Fe^{3+} treatment and at this treatment Fe-plaque was clearly visible. Root membrane might not be broken by the reducing agent $Na_2S_2O_4$ used to extract the elements in apoplast, because P was not detected in the solution which extracted elements from the apoplast. Our result suggested that Fe-plaque might not lock up P, because plaque did not contain any detectable amount of P.

In presence of As, Fe concentration increased both in roots and in apoplast but the value of apoplastic-Fe was much higher than the roots **(Table 11.1**; first experiment). Apoplast contained almost 1.59, 1.50, 1.86 and 2.63 times higher Fe than the roots **(Table 11.1**; first experiment), indicating that apoplast was the main sink of Fe, not roots. The highest apoplastic-Fe was found in the plants treated with highest As **(Table 11.1)**.

Arsenic concentration was 46.5, 28.4 and 44.0 times higher in roots as compared to apoplast at 6.7 μM As + 10 μM EDTA-Fe^{3+}, 13.4 μM As + 10 μM EDTA-Fe^{3+} and 26.8 μM As + 10 μM EDTA-Fe^{3+} treatments, respectively **(Table 10.1**; first experiment), indicating that roots are the main sink of As in rice treated with $NaAsO_2$. Similarly, As concentrations in roots were 31.3, 6.68 and 21.5 times higher as compared to apoplast at 13.4 μM As + 10 μM EDTA-Fe^{3+}, 13.4 μM As + 25 μM EDTA-Fe^{3+} and 13.4 μM As + 50 μM EDTA-Fe^{3+} treatments, respectively **(Table 11.2**; second experiment). It meant that most of As was present in roots and a little amount was in apoplastic portion. We found that major portion of Fe was concentrated in apoplast but a little content of As was found in apoplast. Our result suggested that a part of As might be precipitated with Fe in the apoplast. Increase of Fe concentration in apoplast of As-treated plants in an unknown pathway. By using an X-ray micro analyzer, it was suggested that Fe and As was accumulated on the surface of rice roots (Yamane 1989). Generally, anions are strongly adsorbed to the membrane surface of roots (Wauchope 1983). In our experiments, P and As were mostly found in roots, not in the apoplast. Very little amount of As was found in the apoplastic portion. Arsenic was mostly found in roots; and this may be the reason why arsenite As shows its toxicity compared to arsenate. Arsenate As is mostly found in the outer portion of the root cells (Liu et al. 2004).

Liu et al. (2004) suggested that As was mainly concentrated in DCB extracts; when arsenate treated rice was grown in P-depleted medium. However, most of the As was concentrated or accumulated in the roots if plants grown in P-containing medium (Liu et al. 2004). Arsenic concentrations in arsenate treated Fe-plaque were significantly higher (up to

1180 mg kg^{-1}) in rice roots grown in P-depleted condition as compared to P-supplied condition (Liu et al. 2004). In Fe-plaque treatment, the results indicated that most of the As was sequestered in roots when arsenite was supplied and most As concentrated in Fe-plaque when arsenate was supplied (Liu et al. 2005).

11.5 CONCLUSIONS

Arsenic concentration increased in roots with increasing As in the medium. Root cells are the main sink of As in arsenite treated condition. Phosphorus may not form complex with Fe in apoplast. In arsenite treated condition, Fe was mostly concentrated in the apoplast.

CHAPTER 12

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE NEEDS

In my research, I gave focus on the physiology of Fe in rice and barley though other physiological and mineralogical properties have been discussed throughout the dissertation.

In the first experiment **(CHAPTER 3)**, rice (*Oryza sativa* L. cv. Akitakomachi) seedlings were treated with 0, 6.7, 13.4 and 26.8 μM As in presence of 10 μM citrate-Fe^{3+} in the greenhouse of Iwate University, Japan to observe the physiological and mineralogical responses of rice at elevated concentrations of As in the rooting medium. Arsenic significantly decreased shoot DW at 6.7, 13.4 and 26.8 μM levels and root DW at 13.4 and 26.8 μM levels. In most of the cases, the nutrient elements like K, Ca, Mg, Fe, Mn, Zn and Cu decreased with As treatments. However, P concentration in shoots increased in As-treated rice. Iron concentrations decreased in shoots at 13.4 and 26.8 μM As treatments and were almost similar or within the CDL of Fe (30-50 μg Fe g DW), resulting in the whitish chlorosis in the fully developed young leaves. On the contrary, Fe concentration increased in roots with increasing As concentration in the medium. Among the nutrient elements, Fe translocation from roots to shoots was the most decreased, resulting in Fe-chlorosis. In roots, most of the cases Fe and As concentration increased with increasing As in the medium. Our speculation was that As concentrated Fe in or at the root surface of rice. Root has outer surface and inner tissues. Therefore, we need to investigate in which portion of roots is mostly responsible for concentrating As and Fe.

In the second experiment **(CHAPTER 4)**, we tried to prove that As-induced whitish chlorosis in the fully developed young leaves was Fe-chlorosis. In order to prove that, we added additional citrate-Fe^{3+} with the 26.8 μM As treatment. The treatments were 0 μM As + 10 μM citrate-Fe^{3+} (control), 26.8 μM As + 10 μM citrate-Fe^{3+} (As-treated) and 26.8 μM As + 50 μM citrate-Fe^{3+} (additional citrate-Fe^{3+}). Chlorosis was induced in As-treated seedlings. Additional citrate-Fe^{3+} could not recover or alleviate As-induced chlorosis. It meant that in presence of citrate-Fe^{3+}, As-toxicity was very severe that can not be alleviated with additional citrate-Fe^{3+}. Therefore, we could not conclude certainly that As-induced chlorosis was Fe-chlorosis in presence of citrate-Fe^{3+}. To find out the reason why additional citrate-Fe^{3+} failed to ameliorate As-induced chlorosis, we applied phytosiderophores (PS) and radioactive ^{59}Fe to observe if radioactive ^{59}Fe could be translocated with Fe-chelating substance PS or not in As-treated rice. We found that Fe-chelating substances PS failed to translocate radioactive ^{59}Fe from roots to shoots in As-treated rice seedlings, suggesting that in presence of citrate-Fe^{3+}, As

might inactivate Fe-transporter severely and therefore can not ameliorate the chlorosis in presence of additional citrate-Fe^{3+}.

A preliminary experiment was conducted with As at the rate of 0, 6.7, 13.4, 26.8 and 33.5 μM levels in presence of 10 μM EDTA-Fe^{3+}. In this preliminary experiment, we found that As effectively induced whitish chlorosis in the fully developed youngest leaves at 13.4 and 26.8 μM levels in presence of 10 μM EDTA-Fe^{3+}, in which the chlorosis was more pronounced at 13.4 μM As treatment. Subsequently, the experiment with additional EDTA-Fe^{3+} was conducted by taking 13.4 μM As as a standard level. An interaction experiment between As and EDTA-Fe^{3+} was conducted to evaluate the effectivity of EDTA-Fe^{3+} to ameliorate As-induced chlorosis in rice. The treatments were 0 μM As + 10 μM EDTA-Fe^{3+} (control), 13.4 μM As + 10 μM EDTA-Fe^{3+} (As-treated), 13.4 μM As + 25 μM EDTA-Fe^{3+} (medium-Fe^{3+}) and 13.4 μM As + 50 μM EDTA-Fe^{3+} (high-Fe^{3+}). In this case, whitish chlorosis was observed at 13.4 μM As + 10 μM EDTA-Fe^{3+} treatment. Chlorophyll index and Fe concentration in shoots decreased in As-treated plants as compared to control plants. Arsenic-induced chlorosis was partially alleviated with 13.4 μM As + 25 μM EDTA-Fe^{3+} treatment and was almost completely alleviated in 13.4 μM As + 50 μM EDTA-Fe^{3+} treatment, indicating that As-induced chlorosis could be alleviated with additional EDTA-Fe^{3+}. Chlorophyll index and Fe concentration increased accordingly. Our result showed that As-induced chlorosis was very much dependent on EDTA-Fe^{3+} concentration in the medium. Our result confidently proved that As-induced chlorosis in presence of EDTA-Fe^{3+} was Fe-chlorosis.

Our first experiment **(CHAPTER 3)** showed As-induced whitish chlorosis in the fully developed youngest leaves in hydroponic rice. Second experiment **(CHAPTER 4)**, showed that additional citrate-Fe^{3+} can not alleviate As-induced whitish chlorosis. However, third experiment **(CHAPTER 5)** showed that additional EDTA-Fe^{3+} could alleviate As-induced whitish chlorosis in rice. In order to make a comparison and to evaluate the effectivity of citrate-Fe^{3+} and EDTA-Fe^{3+}, we did a subsequent experiment **(CHAPTER 6)**. In **CHAPTER 6**, the treatments were 0 μM As + 10 μM citrate-Fe^{3+} (control), 13.4 μM As + 10 μM citrate-Fe^{3+} (As-treated), 13.4 μM As + 50 μM citrate-Fe^{3+} (additional citrate-Fe^{3+}) and 13.4 μM As + 10 μM citrate-Fe^{3+} + 40 μM EDTA-Fe^{3+} (additional EDTA-Fe^{3+}). Arsenic at 13.4 μM level (13.4 μM As + 10 μM citrate-Fe^{3+}) induced whitish chlorosis in the fully developed youngest leaves. Chlorophyll index and Fe concentrations were also decreased in As-treated plants. Additional

citrate-Fe^{3+} failed to ameliorate As-induced chlorosis. However, additional EDTA-Fe^{3+} alleviated As-induced chlorosis effectively. Our result showed that the effectivity of EDTA-Fe^{3+} was much higher than citrate-Fe^{3+} to alleviated As-induced chlorosis in hydroponic rice. The reason why EDTA-Fe^{3+} can ameliorate As-induced chlorosis but citrate-Fe^{3+} can not needs to be investigated. Degradation of citrate by microorganisms in the media may be the main factor.

We reported that As-induced chlorosis in barley (*Hordeum vulgare* L. cv. Minorimugi) grown in Fe-containing medium (Shaibur et al. 2008b). However, PS production and release is related with the chlorosis in the shoot tissues. Therefore, we did this experiment **(CHAPTER 7)** with barley grown in Fe-depleted medium to observe the effect of As on PS production and release together with Fe physiology. Arsenic treatments were 0, 0.67, 6.7 and 67 μM for 28 days in presence of 10 μM EDTA-Fe^{3+}. We found that As at high concentration effectively reduced the release and concentration of PS in roots. Most of the cases, the concentration of the macro and micronutrients decreased in shoots and roots. Interestingly, Fe concentration in shoots increased but in roots it was decreased in the 67 μM As treatment, resulting in greening in leaves. Our speculation was that greening of the young third leaves at 67 μM As treatment could probably be due to an increase of the Fe concentration in the shoots. In this Fe-depleted experiment, As ameliorated chlorosis at 67 μM As level. On the contrary, in our previous work (Shaibur et al. 2008b) with Fe-containing medium, As-induced whitish chlorosis was observed in barley at 33.5 and 67 μM levels by reducing Fe translocation. It was found that chlorophyll indices were different in Fe-depleted and Fe-containing medium at 67 μM As level. It seemed that As and Fe in plant tissue had intimate physiological relationship and affected the formation of chlorophyll. In this experiment **(CHAPTER 7)**, Fe translocation, among the heavy metals, was specifically elevated under 67 μM As level. These opposite results in Fe concentration in shoots were obtained in Fe-depleted or Fe-containing conditions where As was supplied. Our speculations for the reason of the phenomenon are- (1) higher As in Fe-containing medium: Fe may be immobilized by As in roots as inorganic precipitation, resulting in low Fe translocation and higher Fe content in roots (2) higher As in Fe-depleted medium: newly absorbed Fe does not occur and Fe absorbed during preculture had been immobilized as bound forms with organic compounds in root cells or root apoplast. When higher concentration of As (67 μM) was supplied to the roots, the compounds for immobilizing Fe might be repressed and

decreased, resulting in gradual liberation of Fe. Liberated Fe from the compounds might be conveyed to xylem tubes by PS in the roots without precipitation with As, though PS content was lower in 67 μM As. These phenomena might cause higher Fe translocation, low Fe concentration in roots, and greening of the leaves. However, the mechanism for Fe mobilization and immobilization in plants has not been characterized.

Arsenic induced whitish chlorosis in the young leaves of barley at 33.5 and 67 μM levels grown in Fe-containing medium for 21 days and suggested that As might hinder Fe translocation from roots to shoots (Shaibur et al. 2008b). In addition, we also reported that, in presence of 67 μM As, Fe translocation was enhanced when the plants were grown in Fe-depleted medium for 28 days **(CHAPTER 7**; Shaibur et al. 2009a). A short term experiment **(CHAPTER 8)** was conducted to observe the effect of PS on the absorption and translocation of Fe in barley grown in presence or absence of As by using ^{59}Fe. It was found that PS effectively enhanced ^{59}Fe absorption and translocation both in control and As-treated barley plants, though the absorption and translocation were lower in As treated plants as compared to control plants. Translocation activity of shoots and absorption activity of roots were also higher in PS treated plants compared to without PS treated plants (in presence or absence of As). Absorption activity of roots was lower in PS and As treated plants as compared to PS and without As treated plants. Therefore, translocation might not be the main problem of As-induced chlorosis in this As concentration (33.5 μM) in barley. This result was different from that of rice. The system for translocation of Fe-PS was week to As in rice as compared to barley. Absorption activity of roots might be a responsible factor for the induction of whitish chlorosis in barley treated with higher As, however, this is a short term experiment and therefore more research needs to be done.

An experiment with barley grown hydroponically **(CHAPTER 9)** was conducted to observe the effects of different P levels on the physiological and mineralogical response in presence of elevated As. This research was conducted taking the consideration that As and P were chemically similar. Plants were treated with 10 μM As in presence or in absence of P. The treatments were 10 μM As + 500 μM P, 10 μM As + 250 μM P, 10 μM As + 50 μM P, 10 μM As + 0 μM P and 0 μM As + 0 μM P for 14 days. Intensity of reddish color in roots increased with decreasing P concentration in the medium. Reddish color Fe-plaque was clearly visible on roots grown in As-treated and P-depleted condition (10 μM As + 0 μM P treatment). Shoot and

root DW decreased with decreasing P in presence of As in the medium, suggesting that As-toxicity was very much dependent on the P concentration in the medium. The most severe effect was in As-treated and P-depleted condition (10 μM As + 0 μM P treatment). Phosphorus and Mg concentrations were decreased both in shoots and roots by As with decreasing P concentration in the medium. Iron concentration decreased in shoots with decreasing P concentration in the medium and the lowest value was in P-depleted condition. Iron and As were mostly concentrated in roots. However, the mechanism of Fe and As increase in roots needs to be characterized.

An additional experiment with As and barley grown hydroponically **(CHAPTER 10)** was conducted to observe the effects of P status on the formation of Fe-plaque in roots. The treatments were 10 μM As + 500 μM P, 10 μM As + 250 μM P, 10 μM As + 50 μM P, 10 μM As + 0 μM P and 0 μM As + 0 μM P for 16 days. Iron-plaque representing as root reddish color was clearly visible in barley roots grown in As-treated and P-depleted condition. However, reddish color Fe-plaque was not found in P-depleted and without As medium, suggesting that As played a vital role in the formation of Fe-plaque in roots in P-depleted condition. Intensity of reddish color increased with decreasing P in the medium. Apoplastic-Fe together with other elements was extracted with a Mes buffer solution. Contents of macro and micronutrients in roots and in apoplast were determined. Particular emphasis was given on the concentration of P, Fe and As. Arsenic decreased root DW with decreasing P concentration in the medium. Detectable amount of P was not found in apoplast in all treatments, indicating that P might not form complex with apoplastic-Fe. Phosphorus may be present inside of roots as organic or in organic phosphate. Iron was mostly concentrated in apoplast compared to roots. However, As was mostly occurred inside of roots and little amount was found in the apoplast or adsorbed with apoplastic-Fe. Similar to Fe, major portion of Zn was present in the apoplast compared to roots. Our result suggested that low P might enhance the formation of As-Fe complex (Fe-plaque) in or at the roots.

Previously we reported that (Shaibur et al. 2006) Fe and As were mostly concentrated in roots of As-treated rice seedlings. Outer space (apoplast) and inner space (in root) are the two major portions of roots and elemental contents may be varied in these two spaces. In this case two subsequent experiments were conducted **(CHAPTER 11)**. Firstly, we tried to observe the effect of As on the content of P, Fe and As in roots and in apoplast of rice. Secondly, we tried

to observe the effect of additional EDTA-Fe^{3+} on the content of P, Fe and As in roots and in apoplast in As-treated condition. For both of the cases, the duration of the treatments was for 14 days in the greenhouse. Similar to **CHAPTER 10**, apoplastic-Fe was extracted with a Mes buffer solution. Contents of P, Fe and As were measured with PIXE (Particle-Induced X-ray Emission). In the first case, the treatments were 0 μM As + 10 μM EDTA-Fe^{3+}, 6.7 μM As + 10 μM EDTA-Fe^{3+}, 13.4 μM As + 10 μM EDTA-Fe^{3+} and 26.8 μM As + 10 μM EDTA-Fe^{3+}. In the second case, the treatments were 0 μM As + 10 μM EDTA-Fe^{3+} (control), 13.4 μM As + 10 μM EDTA-Fe^{3+} (As-treated), 13.4 μM As + 25 μM EDTA-Fe^{3+} (medium-Fe^{3+}) and 13.4 μM As + 50 μM EDTA-Fe^{3+} (high-Fe^{3+}). Iron-plaque representing as root reddish color was found in or at the roots of As-treated plants, intensity of reddish color increased along with roots with increasing As concentration in the medium, suggesting that As played the vital role for the formation of Fe-plaque. Detectable amount of P was not found in apoplast in all treatments, suggesting that P might not form complex with apoplastic-Fe. Iron was mostly concentrated in apoplast compared to roots. However, As was mostly concentrated in roots and little amount was detected in apoplast. High-Fe^{3+} in the medium decreased P concentration in roots. High-Fe^{3+} in the medium increased Fe concentration both in roots and in apoplast. However, the magnitude was 1.86 and 2.63 times higher in apoplast than the roots in 13.4 μM As + 25 μM EDTA-Fe^{3+} and 13.4 μM As + 50 μM EDTA-Fe^{3+} treatments, respectively. Additional-EDTA-Fe^{3+}, however, decreased As concentration in roots.

Finally, the results may be summarized as follows:

1. Sodium arsenite ($NaAsO_2$) effectively induced whitish chlorosis in the fully developed youngest leaves of rice. Arsenic-induced chlorosis was Fe-chlorosis which can be alleviated with additional EDTA-Fe^{3+}. Reduction of Fe concentration in shoots and translocation of Fe from roots to shoots were the most responsible factor for the induction of Fe-chlorosis in the young leaves of rice, though other factors might also be responsible.

2. The problem for translocation of Fe-PS complex might also be a responsible factor for the induction of Fe-chlorosis in rice. The mechanism for the absorption and

translocation of Fe-PS seemed to be affected by As. The tolerance to As-toxicity of the Fe-PS absorption or translocation seemed to be different depending on the species of gramineae.

3. In presence of As, formation reddish color Fe-plaque in barley roots was very much dependent on the P concentration in the medium. Intensity of reddish color was very high in P-depleted condition as compared to P-containing condition. In arsenite treated plants, most of the As was prevailed inside of root cells and little amount was in apoplast. On the contrary most of the Fe was found in the apoplast and little amount was found in the roots. Phosphorus might not form complex with apoplastic-Fe.

Considering the fact that Fe nutritional status is affected by As, application of Fe materials to the field contaminated by As should be investigated more. Considering the Fe physiology in gramineae, it was sure that the system for PS-Fe was damaged by As in rice. The toxicity of As to Fe status must be related with the mechanism, which should be investigated to clarify the mechanism of As-toxicity. Additionally, Fe and P containing materials could be effective to reduce As-toxicity in the As contaminated areas.

ABSTRACT

ABSTRACT

Some experiments were conducted with rice (*Oryza sativa* L. cv. Akitakomachi) and barley (*Hordeum vulgare* L. cv. Minorimugi) in hydroponic culture to evaluate the Fe physiology under As-toxic condition. Rice seedlings were treated with 0, 6.7, 13.4 and 26.8 μM As ($NaAsO_2$) in presence of 10 μM citrate-Fe^{3+} for 14 days. Considering 10% DW (dry weight) reduction, the calculated critical toxicity level (CTL) of As was 40.2 μg g^{-1} DW in shoots and 577 μg g^{-1} DW in roots. Arsenic-induced chlorosis in the fully developed young leaves of rice at 13.4 and 26.8 μM levels. Iron concentration decreased in shoots in As-treated condition. Chlorophyll index was also decreased by the 13.4 and 26.8 μM As treatments. Iron translocation was the most affected among the elements by As-toxicity. Considering Fe concentration and translocation, it was suggested that As might induce Fe-chlorosis in the fully developed youngest leaves.

In the succeeding experiment, an interaction between As ($NaAsO_2$) and citrate-Fe^{3+} was investigated in related with alleviation of As-induced chlorosis in rice. The treatments were 0 μM As + 10 μM citrate-Fe^{3+} (control), 26.8 μM As + 10 μM citrate-Fe^{3+} (As-treated) and 26.8 μM As + 50 μM citrate-Fe^{3+} (additional-Fe^{3+}) for 14 days. Chlorophyll index decreased resulting in whitish chlorosis in the As-treated plants. Chlorosis was almost similar in As-treated and additional citrate-Fe^{3+} treatments, indicating that additional citrate-Fe^{3+} could not alleviate As-induced chlorosis in rice. Iron concentration and translocation were the most affected than the other elements in As-treated plants.

Additionally, feeding experiment with ^{59}Fe was conducted to evaluate the effect of As on absorption and translocation of ^{59}Fe in rice. Treatments were 0 μM As + 10 μM citrate-Fe^{3+} (control) and 26.8 μM As + 10 μM citrate-Fe^{3+} (As-treated) for 14 days. Control and As-treated plants were fed with 10 μM labeled ^{59}Fe in presence or absence of 10 μM PS (phytosiderophores) for 4 hours. Phytosiderophores effectively enhanced ^{59}Fe translocation in control plants but failed to enhance ^{59}Fe translocation in As-treated plants, suggesting that As-might destroy Fe transporter for PS-Fe complex in roots. The result confirmed that As-induced chlorosis was due to Fe translocation problem from roots to shoots in rice.

Another preliminary experiment with rice, As ($NaAsO_2$) and EDTA-Fe^{3+} was conducted. Rice seedlings were treated with 0, 6.7, 13.4, 26.8 and 33.5 μM As in presence of 10 μM

EDTA-Fe^{3+} for 14 days. Arsenic effectively induced chlorosis in the young leaves at 13.4 and 26.8 μM levels in which chlorosis was more pronounced at 13.4 μM treatment. Therefore, we chose 13.4 μM As for further experiment. In the succeeding experiment, the treatments were 0 μM As + 10 μM EDTA-Fe^{3+} (control), 13.4 μM As + 10 μM EDTA-Fe^{3+} (As-treated), 13.4 μM As + 25 μM EDTA-Fe^{3+} (medium-Fe^{3+}) and 13.4 μM As + 50 μM EDTA-Fe^{3+} (high-Fe^{3+}). Additional EDTA-Fe^{3+} was used to observe the efficiency of EDTA-Fe^{3+} to alleviate As-induced chlorosis. Chlorosis induced in As treated plants disappeared partially by medium-Fe^{3+} treatment and almost completely by high-Fe^{3+} treatment, indicating that As-induced chlorosis was Fe-chlorosis in rice. Our result showed that EDTA-Fe^{3+} effectively ameliorated As-induced chlorosis.

An additional experiment was conducted to compare the effectivity of citrate-Fe^{3+} and EDTA-Fe^{3+} to ameliorate As-induced chlorosis. The treatments were 0 μM As + 10 μM citrate-Fe^{3+} (control), 13.4 μM As + 10 μM citrate-Fe^{3+} (As-treated), 13.4 μM As + 50 μM citrate-Fe^{3+} (additional citrate-Fe^{3+}) and 13.4 μM As + 10 μM citrate-Fe^{3+} + 40 μM EDTA-Fe^{3+} (additional EDTA-Fe^{3+}). Chlorosis was found in As-treated plants. Additional citrate-Fe^{3+} could not alleviate the chlorosis, but additional EDTA-Fe^{3+} alleviated the As-induced chlorosis almost completely, indicating that the effectivity of EDTA-Fe^{3+} was higher than citrate-Fe^{3+} to ameliorate As-induced whitish chlorosis. Iron concentration in the shoots of additional EDTA-Fe^{3+} plants was higher than additional citrate-Fe^{3+} plants, suggesting that EDTA-Fe^{3+} might be translocated easily to the shoots than citrate-Fe^{3+}.

Arsenic could also induce Fe-chlorosis in barley leaves. It is known that production and release of PS are governed by the Fe-deficiency symptom of the shoots. Barley was treated with 0, 0.67, 6.7 and 67 μM As for 28 days in Fe-depleted medium. Increasing As in the medium decreased PS release and production in roots. Chlorophyll index increased at 67 μM As treatment as compared to the other treatments. Iron concentration increased in shoots but decreased in roots at 67 μM As treatment. Arsenic concentration increased in plant parts with increasing As in the medium. Increased As concentration may be responsible for lowering the release and concentration of PS in roots. Higher Fe concentration and higher ratio of Fe/P in shoots may also be the factors responsible for greening of the leaves in 67 μM As treatment. A negative relationship between P and As, or P and Fe in shoots was observed. It appeared that

higher As played a role to modulate the mobility of Fe in barley roots grown in Fe-depleted medium.

A short term experiment with barley, As, PS and ^{59}Fe was conducted to evaluate the efficiency of PS on ^{59}Fe translocation in As treated barley seedlings. Control and As treated plants were fed with 10 μM labeled ^{59}Fe in absence or presence of 10 μM PS for 4 hours. Absorption and translocation of ^{59}Fe increased in control or As treated plants fed with PS as compared to those without PS treated plants. Translocation activity of shoots of ^{59}Fe was not affected much in barley in As treated plants but the absorption activity decreased. Therefore, it was suggested that As induced chlorosis in barley was most probably due to the reduction of Fe absorption problem.

The succeeding experiment was conducted to observe the effects of different P levels in the media on the response of barley at elevated As concentration. Plants were treated with 10 μM As in presence or in absence of P. Treatments were 10 μM As + 500 μM P, 10 μM As + 250 μM P, 10 μM As + 50 μM P, 10 μM As + 0 μM P and 0 μM As + 0 μM P. Shoot and root DW decreased with decreasing P in presence of 10 μM As, indicating that As toxicity was very much dependent on P concentration in the medium. Phosphorus and Fe concentration in shoots decreased but As concentration in shoots increased with decreasing P in the medium. Iron and As were mostly concentrated in the roots.

Another experiment was conducted to observe the effects of P on Fe-plaque formation in barley roots. Treatments were similar to the previous experiment with varied P concentration of the medium. Iron-plaque representing as reddish color was clearly visible in the roots grown in As-treated and P-depleted condition. However, reddish color formed by Fe-plaque was not found in P-depleted and without As medium, suggesting that As played a vital role in the formation of Fe-plaque in P-depleted condition. Intensity of reddish color increased with decreasing P in the medium. Apoplastic-Fe was dissolved by the method of Bienfait et al.. Phosphorus complexed with Fe^{3+} in apoplast was not detected in all treatments. Phosphorus may be present inside of roots and not complexed with Fe in the apoplast. Iron was mostly concentrated in apoplast as compared to roots. However, As was mostly concentrated in roots and a little portion occurred in the apoplast when arsenite As was used. The results suggested that high P might repress the formation of As-Fe complex in the apoplast.

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