Physiology of Iron in Rice and Barley Grown Under Arsenic Toxic Condition

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By

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I am declaring that the dissertation with the above mentioned title presented herein for the degree of Doctor of Agricultural Sciences is the result of my own experiments. All the references to other's work as sources of information are fully acknowledged.

Shaibur Rahman Molla 17.3.2009

SHAIBUR RAHMAN MOLLA

The dissertation with the above mentioned title presented herein for a degree of Doctor of Agricultural Sciences is hereby approved as to style and content by:

Shigenao Kawai 17.3.2009

(Professor Dr. Shigenao Kawai)
Major Advisory Professor

DEDICATED

To

My beloved parents

Mr. Abdul Zalil Molla and Mrs. Fatima Begum

&

My beloved son

Izyan Bin Shaibur

&

My beloved wife

Tamanna Islam

&

My beloved eldest brother

Mr. Mizanur Rahman

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Physiology of Iron in Plants

Of all the micronutrients, iron (Fe) is required by plants in the largest amounts. It is considered a macronutrient by some researchers because it is taken up by the plants in large amounts. Iron may be taken up as the ferric (Fe^{3+}) or ferrous (Fe^{2+}) ion by the plant roots (Hopkins 1995). However, it is Fe^{2+} which is taken up (Fox et al. 1996) by the plant roots through a specific channel of the plasma membrane (Fox and Guerinot 1998). Ferrous (Fe^{2+}) is more common due to its greater solubility in the soils (Hopkins 1995). The complex of Fe^{3+} and siderophores produced in the soil are transported to the roots by diffusion or mass flow and enter the root free space through which they move to the plasmalemma bound Fe^{3+}-reductase. During the course of electron transfer Fe is reversibly reduced from Fe^{3+} to the Fe^{2+} state. The importance of Fe is related to two important functions in the plants. It is the part of the catalytic group for many redox enzymes and it is required for the synthesis of chlorophyll (Hopkins 1995). Iron is a constituent of some oxidase enzymes e.g. catalase and peroxides. Iron is not a constituent of the chlorophyll molecule itself. Precise roles of Fe in chlorophyll synthesis remain somewhat of a mystery. There is no definitive evidence that any of the enzymes involved in chlorophyll synthesis are Fe-dependent. Instead, Fe requirement may be related to more general need for Fe in the synthesis of the chloroplast constituents, especially the electron transport proteins (Hopkins 1995). Iron deficiencies invariably lead to a simultaneous loss of chlorophyll and degeneration of chloroplast structure. Chlorosis appears first in the interveinal regions of the youngest leaves, because the mobility of Fe in the plant is very low and can not be withdrawn from the older leaves (Hopkins 1995). Chlorosis may progress to the veins and if the deficiency is severe enough, the leaves may actually turn white (Hopkins 1995). Brown (1961) defined Fe-chlorosis as the leaf yellowing that can be overcome by an effective Fe application.

1.2 Iron Deficiency and Iron Toxicity

Iron deficiency is a worldwide problem in crop production on calcareous soils. It is the major factor responsible for lime-induced chlorosis. Iron deficiency might limit CO_2 fixation in phytoplankton in the Pacific Ocean (Greene et al. 1992). The major symptom of Fe deficiency is inhibition of chloroplast development in leaves of all plants. The critical deficiency content of Fe in leaves is in the range of 50 ~ 150 $\mu g\ g^{-1}$ dry weight (DW; Marschner 1998). The

content refers to the total Fe in plants. In general, C_4 species require a higher Fe supply than C_3 species, but their critical deficiency contents are similar, namely about 72 μg g^{-1} DW in C_3 species and about 66 μg g^{-1} DW in C_4 species (Smith et al. 1984). In fast-growing meristematic and expanding tissues, for example, shoot apices, the critical deficiency contents are much higher, presumably in the range of 200 μg g^{-1} DW for total Fe and 60-80 μg g^{-1} DW for active Fe (Häussling et al. 1985).

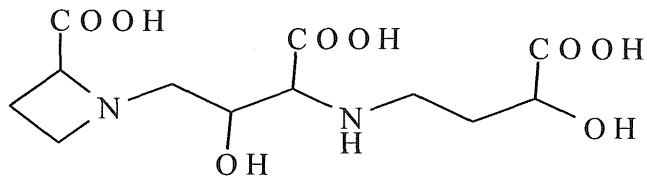
In both dicots and monocots (except graminaceous species) Fe-deficiency is associated with inhibition of root elongation, increase in the diameter of apical root zones and abundant root hair formation (Römheld and Marschner 1981a; Chaney et al. 1992b). These morphological changes are often associated with the formation of cells with a distinct wall labyrinth typical of transfer cells (Marschner 1998). These transfer cells may be induced either in rhizodermis in the hypodermis (Landsberg 1989). In graminaceous species (Strategy II) these Fe-deficiencies induced morphological and physiological changes are absent. Instead, roots of Strategy II plants release phytosiderophores (PS) as chelators Fe^{3+}.

Compare to calcareous soils, Fe is very soluble in strongly acidic soils and may appear Fe toxicity due to excess Fe uptake (Hopkins 1995). Iron-toxicity (bronzing) is a serious problem in crop production on waterlogged soils. It is the second most severe yield-limiting factor in wet land rice. The critical toxicity contents are above 500 μg g^{-1} DW in leaf, but very much dependent on other factors such as content of other mineral nutrients (Yamauchi 1989). Iron toxicity may also play a role under dry land conditions and is probably an early event of drought-induced damage in photosynthetic tissue caused by Fe-catalyzed formation of oxygen free radicals in the chloroplasts (Price and Hendry 1991).

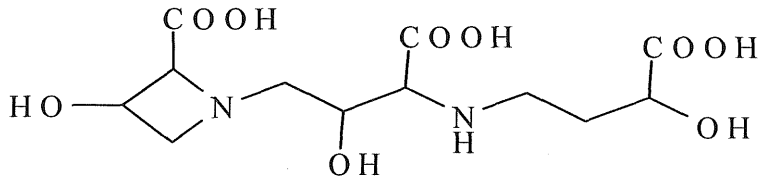
1.3 Iron Deficiency and Phytosiderophores (PS)

The study of Fe acquisition systems in graminaceous plants enters the stage of steady progress in recent years as a consequence of the discovery of PS. Phytosiderophores are non-proteinogenic secondary amino acids, which function with their amine-, hydroxyl- and carboxyl-groups as multidentate cation chelators (Sugiura et al. 1981). Six kinds of PS have already been identified (**Fig. 1.1**). Amounts and kinds of PS released vary among the graminaceous species. For example, 2'-deoxymugineic acid (DMA) is the first of the PS in the biosynthetic pathway of PS from methionine (Mori and Nishizawa 1987; Kawai et al. 1988a).

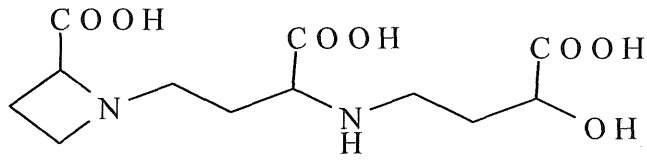
The other PS, including mugineic acid (MA), 3-hydroxymugineic acid (HMA), 3-epi-hydroxymugineig acid (epi-HMA), avenic acid (AVA) and distinchonic acid (DA) are



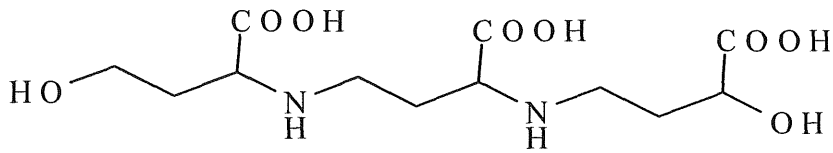
Mugineic acid (MA)



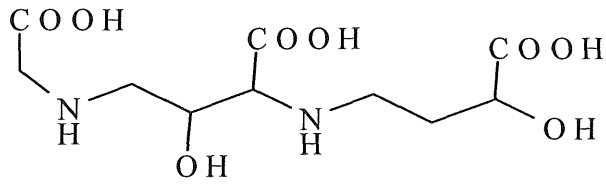
3-Hydroxymugineic acid (HMA)
3-epi-Hydroxymugineic acid (epi-HMA)



2'-Deoxymugineic acid (DMA)



Avenic acid A (AVA)



Distichonic acid (DA)

Figure 1.1 Chemical structures of different phytosiderophores (PS)

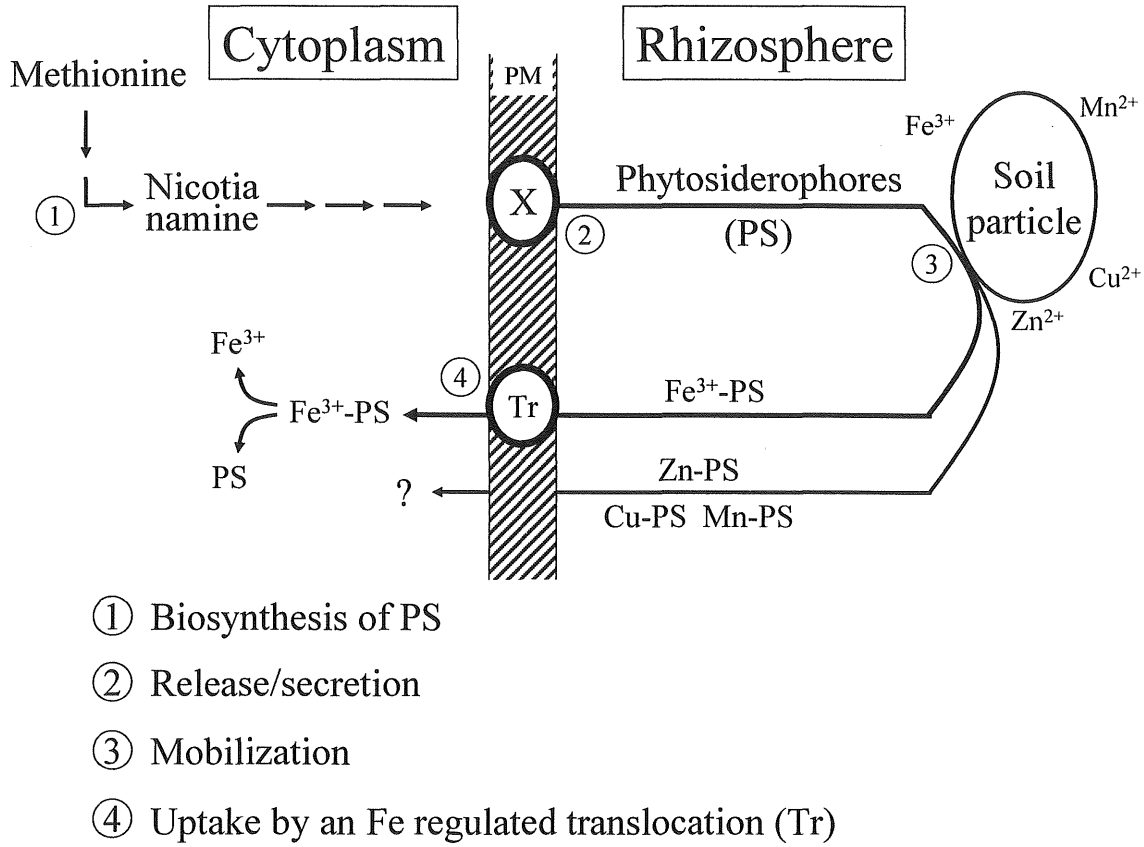


Figure 1.2 Schematic presentation of the PS system of Graminaceae (Römheld 1991).

synthesized by the subsequent hydration of DMA (Marschner 1995). The component of PS varies depending upon the plant species. For example, barley and rye can release various kinds of PS, such as DMA, MA, HMA, epi-HMA and AVA (Takagi 1993). In contrast to barley and rye, only DMA has been detected in root washings of all common varieties in wheat (Singh et al. 2000). The amounts of PS release from roots of Fe-deficient corn and sorghum have been estimated to be 1/10 and 1/100, respectively, than those release from roots of Fe-deficiency barley (Kawai et al. 1988a), which very often exceeds 10 mg day^{-1} g^{-1} root DW (Takagi et al. 1984). The release of PS from roots is roughly consistent with the ability of the plants to tolerate Fe-deficiency and generally follows the sequence: barley > wheat ≒ rye > oat > sorghum > rice (Marschner et al. 1986; Kawai et al. 1988b).

Mostly, phytosiderophore is synthesized in barley roots with the onset of Fe-deficiency (Kawai et al. 1993) and the amount synthesized often approaching up to 20 mg PS day^{-1} g^{-1} root DW (Takagi 1993). However, PS synthesis decreases immediately after the resumption of Fe supply, suggesting that the synthesis of PS is under the control of some feedback mechanism (Takagi et al. 1984). Phytosiderophores are released to the root rhizosphere for the acquisition of sparingly soluble Fe in soils. Release of PS in Fe-deficient plants is related to distinct diurnal rhythm, with maximum release rates usually 3-4 hours after the onset of light (Takagi et al. 1984). Phytosiderophores release is inhibited by the presence of potassium cyanide (KCN) and dicyclohexylcarbodiimide (DCCD), suggesting that the release is highly dependent on metabolic energy (Takagi 1990). Moreover, the PS-mediated Fe uptake is also inhibited strongly by the presence of metabolic inhibitors (Takagi et al. 1984).

Solubilization is a prerequisite for the acquisition of sparingly soluble Fe in soils by chelation with PS. It has been inferred that the chelation results in the form of 1:1 complex with PS and Fe^{3+} as in the case of PS and Cu^{2+} (Nomoto et al. 1981). It has been reported that PS form stable chelates with metal micronutrients (**Table 1.1**). Treeby et al. (1989) and Singh et al. (1992) reported that PS solubilized not only Fe, but also Mn, Cu and Zn from calcareous soils. The schematic presentation of the PS-based models for metal micronutrients is shown in **Fig. 1.2.**

1.4 Mechanisms of Iron Acquisition in Strategy I and Strategy II Plants

Available content of Fe in the soil is not sufficient. So stress of Fe is a common feature in most agricultural soil. Iron-deficiency is a common plant nutritional problem in the world, especially in calcareous soils, accounting for 1/3 of the world's land (Brown 1961). Available content of Fe varies from soil to soil and the ability to absorb Fe varies widely among plants. Based on the Fe acquisition system expressed under Fe-deficiency, plants are classified into Strategy I (dicotyledons and non-graminaceous) and Strategy II (graminaceous) plants (Römheld and Marschner 1986).

Strategy I plants respond to Fe-deficiency by 1) reducing Fe^{3+} ion at the root surface; 2) releasing H^+ from roots to the root rhizosphere; 3) releasing reduced compounds from roots; and 4) increasing the contents of citrate and other organic acids in roots (Brown 1978). In contrast, the strategy II mechanism involves the 1) synthesis of PS in apical root zones

(Römheld and Marschner 1986); 2) release of PS from the root apex (Marschner et al. 1987); 3) solubilize the sparingly soluble Fe^{3+} by chelation with PS in apical root zone; and 4) uptake of the PS-Fe^{3+}complex by roots (Takagi et al. 1984; Marschner et al. 1987).

Table 1.1 Stability constants of phytosiderophores with metal micronutrients (Murakami et al. 1989; Sugiura et al. 1981).

	Phytosiderophores		
Metal micronutrients	Epi-HMA	MA	DMA
Cu^{2+}	17.9	18.1	17.8
Fe^{3+}	-	18.1	-
Zn^{2+}	12.4	12.7	12.8
Fe^{2+}	10.0	10.1	10.5
Mn^{2+}	8.0	8.3	8.3

In Strategy II plants, PS are synthesized and released by the plants only under conditions of Fe-deficiency. The compounds have a high affinity for Fe^{3+} and very effectively scavenge Fe^{3+} from the rhizosphere. The distinctive feature of the PS system is the formation of Fe-PS complex, which is then absorbed into the roots. Once inside of the roots, the Fe^{3+} is presumably reduced to Fe^{2+} and released for use by the cell. It has been suggested that absorbed Fe is translocated as Fe^{3+}-PS complex in Strategy II plants (Takagi et al. 1984; Römheld and Marschner 1986). The fate of the PS inside the plants is not clear yet. In microorganism, it may be degraded and metabolized. The schematic presentation of PS-based models for metal micronutrients is shown in **Fig. 1.2**.

1.5 Physiology of Phosphorus in Plants

Next to nitrogen (N), phosphorus (P) has more widespread influence on both natural and agricultural ecosystems than any other essential elements. It is available in soil solution as polyprotic phosphoric acid (H_3PO_4; Hopkins 1995). At soil pH less than 6.8, the predominant form of P is the monovalent orthophosphate anion ($H_2PO_4^-$). Orthophosphate is readily absorbed by plant roots. Between pH 6.8 and pH 7.2, the predominant form is HPO_4^{2-} which is less readily absorbed by the plants. Phosphorus deficient plants are often severely stunted, since

this element takes part in the synthesis of several essential compounds upon which all plants and animals lives depend. Neither plants nor animals can grow without P. It is an essential component of the organic compound often called the energy currency of the living cell, adenosine triphosphate (ATP; Brady and Weil 2002).

Phosphorus deficiency produced several toxicity symptoms in plants. Intensification of green color in the leaves is the symptom of P-deficiency (Hopkins 1995). In severe P-deficient conditions, leaves may become malformed and exhibit necrosis spots (Hopkins 1995). In some cases, anthocyanin (Hopkins 1995; Jain et al. 2007) also accumulates giving the leaves a dark greenish-purple color (Hopkins 1995). Phosphorus-deficient plants develop a dark greenish in leaves as a purplish color of the leaves edges. Phosphorus is mobile in plants and therefore rapid senescence and death of older leaves occur first due to the translocation of P from old leaves to young leaves (Hopkins 1995).

Shorter and slender shoots and lower yield of fruits are the typical response of P-deficiency (Barry and Miller 1989). In P-deficient condition, P may re-translocate from shoots to roots (Smith et al. 1990), resulting in reduced shoot-root ratio of DW (Fredeen et al. 1989). Phosphorus deficiencies lead to decrease of leaf number (Lynch et al. 1991), reduction of leaf surface area and leaf expansion (Fredeen et al. 1989). However, chlorophyll content per unit area of the leaves is not much affected (Fredeen et al. 1989). Phosphorus concentration in P-deficient leaves may drop from 5 mM to less than 0.2 mM and ATP concentration drops to 20-30% of the original level (Theodorou and Plaxton 1993).

Root growth is progressed by retaining most of the P in roots by importation of P from shoots (Smith et al. 1990). Phosphorus deficiency triggers progressive loss of meristematic cells in the primary roots and thereby causes determinate growth (Sánchez-Calderón et al. 2005). Elongation rate of individual root cells and of the roots might be enhanced by P-deficiency (Anuradha and Narayanan 1991). In P-deficient condition, some crops multiply roots and root hairs and release organic acids to uptake sparingly soluble Pi of the medium (Mengel and Kirkby 2001). Acidification in the root rhizosphere is the response of P-deficiency. For example, in P-deficient condition net H^+ efflux increases, resulting in decreased nitrate uptake (Heuwinkel et al. 1992). Rape (Hoffland et al. 1989) and Leguminous species (Ohwaki and Hirata 1992) response to release organic acids in P-deficient condition, particularly citric acid (Mengel and Kirkby, 2001).

1.6 History of Arsenic

Arsenic (As), the king of poison has probably influenced human history more than any other element or toxic compound (**Plate 1.1**). Albertus Magnus (1193-1280) is credited with the isolation of this toxic element by heating auripigment (As_2S_3) with soap (Goessler and Kuehnelt 2002). The use of As as a deadly poison has been known since long years back. Although As has common presence in nature but it is considered as the trace element in terms of its natural abundance (Oremland et al. 2002). It is classified as a group Va element in the Periodic Table and it shares many chemical and biochemical properties in common with neighboring P and N (Oremland et al. 2002). The most oxidized (+5) oxidation state is arsenate [$HAsO_4^{2-}$ or As^{5+}]. The toxicity of arsenate is based on its action as an analogue of phosphate. The other important state of As is arsenite [$H_2AsO_3^-$ or As^{3+}]. Arsenite product binds with internal sulfhydryl groups that render it even more toxic than the original arsenate (Oremland et al. 2002).

Arsenate can be reduced to arsenite by microbes. In addition to biological reduction, arsenate can also be reduced to arsenite by strong, naturally occurring reductants, such as sulfide, although this is generally favored at low pH rather than under neutral or alkaline conditions (Newman et al. 1997; Cherry et al. 1979). Conversely, a number of naturally occurring oxidants such as Fe^{3+} and Mn^{4+} can reoxidize As^{3+} back to As^{5+} (Oscarson et al. 1981). Arsenite is formed in the presence of free sulfides at acidic or neutral pH condition and is precipitated as arsenic trisufide (As_2S_3).

1.7 Arsenic Problems in Bangladesh

Arsenic was first identified in the groundwater of West Bengal in 1983, following a medical diagnosis of As poisoning (Saha 1984; 1995; Mazumder et al. 1988). Investigations in India in the 1980s and early 1990s progressively identified the extent of pollution there (PHED [Public Health Engineering Department, Government of West Bengal] 1991; Das et al. 1994; 1996). Unfortunately, this information was effectively unknown in Bangladesh until the early 1990s. The earliest known analyses of As in groundwater in Bangladesh was reported by Dhaka Water and Sewerage Authority from three municipal supply wells in Dhaka City (DWASA 1991). All were below the analytical method detection limit (10 $\mu g\ L^{-1}$) and therefore attracted no attention. Arsenic contamination in groundwater in Bangladesh was detected in

December 1993 (Ahmed et al. 2006), by the Department of Public Health Engineering (DPHE), but the fact remained behind the screen till 1996 (DCH [Dhaka Community Hospital] 2006). However, according to Ali and Tarafdar (2003), all credits are to the Chemistry Division of the Atomic Energy Centre, Dhaka (AECD) for the first time invention of As-contamination in the groundwater in Bangladesh at the village of Barogharia, Chapainawabganj District, Rajshahi Division (Ali 1995). Between 1995 and 1998, a series of survey revealed the extent of the catastrophe in Bangladesh (NRECA 1997; Jakariya et al. 1998 and DPHE 1999). **Plate 1.2** is showing the As contaminated areas in Bangladesh.

Arsenic contaminates groundwater across much of southern, central and eastern Bangladesh. Groundwater from the Holocene alluvium of the Ganges, Brahmaputra and Meghna Rivers locally exceeds 200 times the World Health Organization (WHO) guideline value for drinking water of 10 $\mu g\ L^{-1}$ of As. Approximately 25% of wells in Bangladesh exceed the national standard 50 $\mu g\ L^{-1}$ of As affecting at least 25 million people. Arsenic has entered the groundwater by reductive dissolution of ferric oxyhydroxides, to which As was adsorbed during fluvial transport (Ravenscroft et al. 2005).

1.8 Effect of Arsenic on Human Health

Chronic exposure to As in drinking water results in skin ailments such as hyperpigmentation and Keratosis and leads progressively to cancers of the skin, to damage to internal organs, cancer and ultimately death (WHO 1993; National Academy Press 2001). Arsenic toxicity symptoms may take five to fifteen years or longer to develop. The current standard for arsenic in drinking water in both Bangladesh and India is 50 $\mu g\ L^{-1}$ (Ravenscroft et al. 2005). In 1993 the WHO recommended a provisional guideline level of 10 $\mu g\ As\ L^{-1}$ for public water supplies. Even this lower limit is not expected to be protective at the one excess cancer in 10^6 life time exposure (National Academy Press 2001). The WHO guideline has not been adopted either in Bangladesh or in India.

Several studies have shown that ingestion of inorganic As can increase the risk of skin cancer and cancer in the lunges, bladder, liver and kidney. Inhalation of inorganic As can cause increase risk of lung cancer. The International Agency for Research on Cancer (IARC) and the EPA have determined that inorganic As is carcinogenic to humans (URL: **http://www.atsdr.cdc.gov/toxfaq.html**).

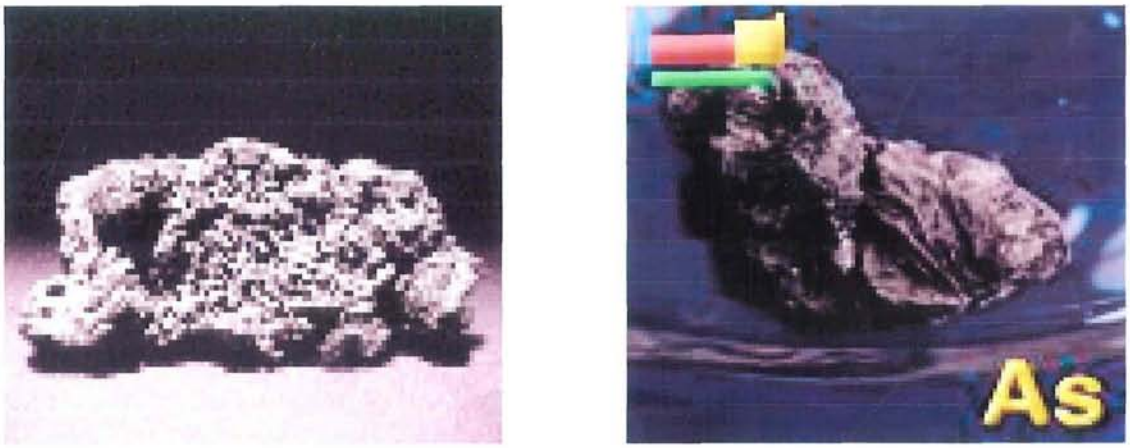


Plate 1.1 Photographs of crystal-shaped As minerals

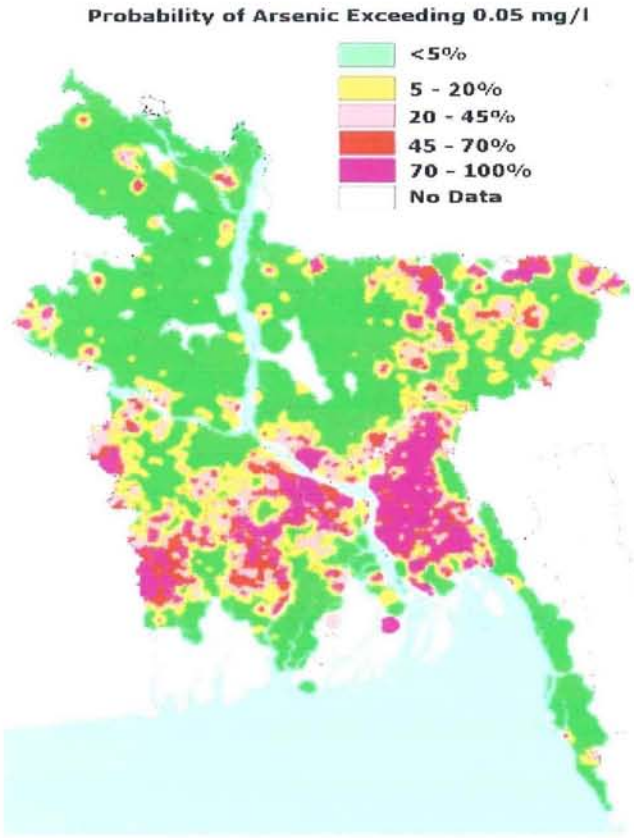


Plate 1.2 Map of Bangladesh showing the As contaminated areas

1.9 Uses of Arsenic

Arsenic compounds are mainly used in agriculture, forestry and industrial processes. Arsenic tri-oxide is used in manufacturing of agricultural chemicals (pesticides), glass and glassware, industrial chemicals, copper and lead alloys and pharmaceuticals. In agriculture, As compounds such as lead arsenate, copper aceto arsenite, sodium arsenate, calcium arsenate, and organic As compounds are used as pesticides. Substantial amount of monomethylarsonic acid (MMAA) and dimethylarsinic acid (DMAA) are used as selective herbicides. Chromated copper arsenate, sodium arsenate and zinc arsenate are used as wood preservatives. Some phenyl As compounds such as arsenal acid are used as feed additives for poultry and swine.

Small amount of As compounds continue to be used as drugs in some countries. As a medicine As is used since the fifth century BC when Hypocrites recommended the use of As sulfide for the treatment of abscess. Arsenic preparation was used for the treatment of skin disorder, tuberculosis, leukemia, asthma, leprosy, syphilis, amoebic dysentery etc. Homeopaths are also using As as drug. Besides these, As is used for preparation of dyes, poisonous gas, transistor, as a component of semiconductor, as a preservative in tanning and in the industry of textile and paper etc..

1.10 Plant Responses to Arsenic Toxicity

Since 1884, As has been known to us as a phytotoxic agent (Stiles 1958; Steevens et al. 1972). Arsenic toxicity in plants was described by Machlis (1941) as consisting of root plasmolysis and leaf wilting followed by root discoloration and necrosis of leaf tips and margins. Plants can develop toxicity symptoms while they are exposed to excess As either in soil or in solution culture such as: inhibition of seed germination (Liebig 1966); decrease in plant height (Carbonell-Barrachina et al. 1995; Marin et al. 1992; Tsutsumi 1980), reduction in root growth (Tang and Miller 1991), wilting and necrosis of leaf blades (Odanaka et al. 1987), reduction in leaf area and photosynthesis (Knauer et al. 1999; Marin et al. 1993); decreased in shoot growth (Tang and Millar 1991); and lower fruit and grain yield (Carbonell-Barrachina et al. 1995; Tsutsumi 1980).

Significant reduction of root length with increasing As concentration is due to the fact that plant roots are the first point of contact for As in the nutrient solution. It was found that corn dry matter production decreased significantly in sand and silt loam soil at 80 ppm (parts

per million) As level where sandy soil showed the severe toxicity (Jacobs and Keeney 1970). Arsenite has a high toxicity for radicular membranes (Sachs and Michael 1971), because As^{3+} reacts with sulfhydryl groups of proteins (Speer 1973), causing disruption of root functions (Orwick et al. 1976) and cellular death. Arsenate is a well-known decoupler of phosphorylation in mitochondria (Ter-Welle and Slater 1967) and it can inhibit leaf uptake of other chemicals (Sargent and Blackman 1969) and seed germination (Wauchope 1983).

Low concentrations of As have been reported to increase growth of maize (Woolson et al. 1971a) and potatoes (Jacobs et al. 1970). Arsenic form is more important than the As level in solution in determining the phytotoxic effect to rice. When plants were grown in nutrient solution containing As^{5+} at levels ranging from 0.05 to 0.8 mg As L^{-1}, the total dry matter production was not affected (Marine et al. 1992). Both As^{3+} and MMAA were phytotoxic to rice. Arsenic (As^{3+}) caused a significant reduction of growth when applied at the maximum rate (0.8 mg L^{-1}). The MMAA was the most toxic form of As with respect to total dry matter production. Total dry matter production was significantly reduced at any of the applied MMAA rates (Marine et al. 1992).

In a hydroponic experiment where DMAA (a sodium salt) was used in rice (*Oryza sativa* L. cv. Mercury), photosynthesis, photosynthesis activity, leaf area and dry matter production were significantly reduced at 0.8 and 1.6 mg As L^{-1}. But at the lower rate (0.2 mg As L^{-1}) of DMAA application, there was no significant reduction of photosynthesis, photosynthesis activity and growth (Marin et al. 1993). Marin et al. (1992) hypothesized that DMAA may influence the allocation of carbohydrates and decreased the growth. It was also reported that organic arsenical metabolite may block protein synthesis or some other biosynthetic pathway (Sckerl and Frans 1969) which may decrease the growth. Arsenic is not phytotoxic for all plants, rather the growth of some plants increased in presence of As. Growth of Chinese brake fern (*Pteris vittata* L.) increased in presence of As and could accumulate huge amounts of As in the fronds (Ma et al. 2001).

1.11 Relationship between Arsenic and Phytosiderophores

Reports describing the relationship between As and PS are not available. It is well established that PS production is related to Fe-deficiency symptom. We found that As-induced whitish chlorosis in rice (Shaibur et al. 2006) and barley (Shaibur et al. 2008b). Therefore, we

tried to set up a relationship between As-induced chlorosis and PS production and release. Release of PS is inhibited by the presence of KCN and DCCD, suggesting that the release is highly dependent on metabolic energy (Takagi 1990). The PS-mediated Fe uptake is also inhibited strongly by metabolic inhibitors (Takagi et al. 1984). Arsenic is a phytotoxic agent, therefore PS formation and release could be affected in presence of As.

1.12 Rationale of the Study

Arsenic is known to have severe phytotoxic effect on plant growth. Plants response to As toxicity in different ways. Physiological and mineralogical properties of plants are changed by As toxicity. Physiology can be seen with eyes; however, quantitative analysis is required for the determination of mineralogical properties. Among the physiology, visible symptom is the important characteristic. In this report, I tried to describe the physiological and mineralogical properties of rice and barley as affected by As, in step wise.

This PhD dissertation was started with introduction chapter **(CHAPTER 1)**, where the background of the research and problem of As have been discussed. General methodology of the research has been described in **CHAPTER 2**. Subsequently I described the research in the following ways.

Physiological and mineralogical response of rice seedlings at elevated concentration of As was described in **CHAPTER 3**, where I suggested that As may induced Fe-chlorosis. In the second experiment **(CHAPTER 4)**, I tried to prove that As-induced chlorosis in rice was due to Fe-translocation problem in presence of citrate-Fe^{3+}. Second experiment **(CHAPTER 4)** showed that additional citrate-Fe^{3+} could not ameliorate As-induced chlorosis. Third experiment was conducted to prove that As-induced chlorosis was Fe-chlorosis in presence of sufficient EDTA-Fe^{3+} **(CHAPTER 5)**. In this experiment **(CHAPTER 5)**, **I** found that additional EDTA-Fe^{3+} could effective ameliorate As-induced chlorosis. After that the 4^{th} experiment was conducted to determine the efficiency of EDTA-Fe^{3+} and citrate-Fe^{3+} to ameliorate As-induced chlorosis **(CHAPTER 6)**. I found that EDTA-Fe^{3+} effectively ameliorated As-induced chlorosis but citrate-Fe^{3+} could not. Fifth experiment was conducted to observe the effect of As on PS production and release **(CHAPTER 7)**. A feeding experiment with ^{59}Fe was conducted for measuring absorption and translocation activity of ^{59}Fe in related with As toxicity and PS **(CHAPTER 8)**. Seventh experiment was conducted to observe the

effect of P on the formation of reddish color Fe-plaque **(CHAPTER 9)**. Subsequently, the apoplastic-Fe was removed (Bienfait et al. 1985) to observe the concentration of P, Fe and As in the Fe-plaque and in root cells **(CHAPTER 10)**. In the last experiment, I also tried to measure the content of P, Fe and As in rice roots **(CHAPTER 11)**. Finally, I summarized the results of my experiments in **CHAPTER 12**.

CHAPTER 2

MATERIALS AND METHODS

2.1 Experimental Plants

Rice (*Oryza sativa* L. cv. Akitakomachi) and barley (*Hordeum vulgare* L. cv. Minorimugi) were used as the test plants in this research work. Two types of nutrient solutions were used for rice and barley individually.

2.2 Composition of Nutrient Solution

For rice, ½-strength nutrient solution was used to prepare the seedling. At the time of treatment application, full-strength solution was used in every experiment of rice. However, barley seedlings were introduced with 1/5-strength nutrient solution to prepare the seedlings and latter on ½-strength solution was used at the treatment.

Table 2.1 Composition of full strength modified Hoagland-Arnon solution (Takagi 1993).

----------Solution for Rice ----------		------Solution for Barley (+Fe medium)------	
Salt	Strength	Salt	Strength
NH_4NO_3	1 mM	KNO_3	6.0 mM
K_2SO_4	1 mM	$Ca(NO_3)_2$	4.0 mM
$MgSO_4$	0.8 mM	$NH_4H_2PO_4$	1.0 mM
NaH_2PO_4	0.5 mM	*NaH_2PO_4	----------
$CaCl_2$	0.5 mM	$MgSO_4$	2.0 mM
$MnSO_4$	10 µM	H_3BO_3	3.0 µM
$CuSO_4$	1 µM	$MnSO_4$	0.5 µM
$ZnSO_4$	1 µM	$CuSO_4$	0.2 µM
H_3BO_3	3 µM	$ZnSO_4$	0.4 µM
H_2MoO_4	0.05 µM	H_2MoO_4	0.05 µM
Citrate-Fe^{3+}	10 µM	EDTA-Fe^{3+}	20 µM

The pH of the nutrient solution was adjusted to 5.5 for rice and +Fe barley, however, for –Fe barley the pH was 6.5. *In –Fe barley, similar concentration of NaH_2PO_4 was used instead of $NH_4H_2PO_4$. The pH was adjusted by the addition of 1 M HCl and or 1 M NaOH.

2.3 Seed Germination and Plant Culture

2.3.1 Rice Seed Germination and Plant Culture

Rice (*Oryza sativa* L. cv. Akitakomachi) seeds were surface sterilized with 2% chlorinated lime [$Ca(OCl)_2$] for 45 min and washed with tap water for 1 h. After being washed, seeds were wrapped between moistened towels and were kept in an incubator at 25 ± 2°C for 72 h. Germinated seeds were transferred on a net in a box containing 2% $CaCl_2$ for 7 days. After that, seedlings were transferred in ½-strength nutrient solution for another 7 days (2 times) in the greenhouse. Twenty one days after germination (at 4-5th leaf stage of the seedlings), seedlings were transplanted in groups of five (referred to as a hill) in one bunch and each bucket (10 L) containing 16 bunches. Treatments were started with full-strength nutrient solution. Arsenic was added as sodium meta-arsenite ($NaAsO_2$). The pH (pH 5.5) was adjusted daily with a digital pH meter (Horiba Korea, Seoul, Korea) and with 1 M HCl and/or 1 M NaOH at around 4 p.m. during the experiment. Solution was renewed every week and was not aerated.

2.3.2 Barley Seed Germination and Plant Culture

Barley seeds (*Hordeum vulgare* L. cv. Minorimugi) were surface-sterilized with 2% (w/v) chlorinated lime [$Ca(OCl)_2$] for 45 min, rinsed with tap water continuously for 1 h and soaked between moistened towels covered with wrapping paper at 25 ± 2°C for 24 h. Germinated seeds were placed on a plastic net of the seed box containing 2 mM $CaCl_2$ solution in the phytotron (14 h photoperiod, 280 μmol m^{-2} s^{-1} light density; and 17/10°C day/night temperatures) or in the greenhouse at the Faculty of Agriculture, Iwate University, Japan. After 7 days, the solution in the seed box was replaced with 1/5-strength modified Hoagland-Arnon solution (Hoagland and Arnon 1938) containing 4.0 μM EDTA-Fe^{3+}. Seedlings were allowed to grow until the length of the second leaf was about 20% of that of the first leaf. Seedlings were then transplanted in bunches (3 plants were wrapped with sponge rubber) and 16 bunches were placed in one pot (10 L/liters) filled with ½-strength modified Hoagland-Arnon solution containing 10 μM EDTA-Fe^{3+}. Plants were grown up to 14 or 21 DAT (days after treatments) and harvested when nutrient deficiencies symptoms or inhibitory effects were most apparent. Arsenic was used as sodium meta-arsenite ($NaAsO_2$). The pH (5.5 or 6.5) of the solutions was

monitored daily with a digital pH meter and adjusted with 1 M HCl and or 1 M NaOH at around 4 p.m. during the experiment. Solutions were renewed every week, aerated throughout the experiment and the solution level was maintained by adding deionized water.

2.4 Measurement of PS Accumulated in Barley Roots

Three bunches seedlings from each treatment were collected in the morning (8.30 a.m.), 30 min later after the onset of day time of the growth chamber, to assay for PS accumulation in roots. Roots were carefully washed with deionized water as early as possible and kept in the refrigerator (-20°C). Plants were lyophilized, separated into shoots and roots and the weight was measured. Lyophilized roots were homogenized with a mortar and pestle in 10 mL ethanol (80%, v/v) making volume 50 mL. Root extract was filtered and concentrated by evaporation at 55°C under vacuum. Compounds extracted from roots were dissolved in MQ water (18.2 MΩ cm^{-1}), purified by Milli-RO 60 (Millipore corporation, USA), introduced to an Amberlite IR-120B cation exchange resin column and the resin was washed with deionized water. Cationic fraction absorbed to the resin was eluted with 100 mL of 1 M NH_4OH, concentrated under vacuum and assayed for PS (Kawai et al. 1993). The PS content in roots was measured by the Fe solubilizing assay of Takagi (1976).

2.5 Collection and Measurement of PS Released by the Barley Roots

Roots of a bunch of seedlings were soaked in beakers containing 500 mL deionized water for 3 h starting from 8 a.m. on 14 or 21 DAT. Seedlings were transferred to the respective pots after collection of root washings. Approximately 10-15 mg thymol (Kanto Chemical Company, Tokyo, Japan) was added to each beaker for preventing microbial degradation of PS. Root washings were introduced to an Amberlite IR-120B cation exchange resin similarly to the procedure for the determination of PS accumulation and the amount of released PS was measured.

2.6 Chlorophyll Index (SPAD value)

Chlorophyll index of fully developed (3^{rd} leaves of barley or 5^{th} leaves of rice) new leaves on harvest was measured using a SPAD-502 chlorophyll meter (Minolta Camera Company, Tokyo, Japan).

2.7 Analysis of Plant Samples

Rice and barley seedlings were collected and washed with deionized water. Shoots and roots were separated and dried at 60 ± 5°C for 48 h. For mineral nutrition, oven dried samples were digested with a nitric acid-perchloric acid mixture (Piper, 1942) and analyzed. Amount of K, Ca, Mg, Fe, Mn, Zn and Cu were determined with atomic absorption spectroscopy (AAS) and As was measured by Hydride Generation Technique (Hitachi HFS-3). Phosphorus was determined colorimetrically using a UV-visible Spectrophotometer (model UV mini 1240, Shimadzu Corporation, Kyoto, Japan) at 420 nm wavelengths after developing the yellow color with vanadomolybdate as described by Barton (1948) and Jackson (1958).

2.8 Experimental Design

The experiment was a completely randomized block design with 3 replications. Data were analyzed by analysis of variance (SAS 1988).

2.9 Statistical Analysis

All the data were subjected to an analysis of variance (SAS 1988) by the computer "sas" at Iwate University. Differences between means were evaluated by using the Ryan-Einot-Gabriel-Welsch multiple range test ($p < 0.05$).

2.10 Reagents

All chemicals used were of analytical reagent grade. All solutions were prepared previously with MQ water. Stock solution of As was prepared by dissolving $NaAsO_2$ (Kanto Chemical Company, Tokyo, Japan) in MQ water and was kept at room temperature 25 ± 2°C in acid washed reagent bottle.

CHAPTER 3

CRITICAL TOXICITY LEVEL OF ARSENIC AND ELEMENTAL COMPOSITION OF ARSENIC-INDUCED CHLOROSIS IN HYDROPONIC RICE

ABSTRACT

A hydroponic experiment with rice (*Oryza sativa* L. cv. Akitakomachi) was conducted to observe the response of rice at elevated concentration of arsenic (As) in presence of citrate-Fe^{3+}. The treatments were 0, 6.7, 13.4 and 26.8 μM As (equal to 0, 0.5, 1.0 and 2.0 mg As L^{-1}) from sodium meta-arsenite (Na_2AsO_2) for 14 days in the greenhouse. Shoot dry weight (DW) decreased by 1.75, 16.2 and 40.1% for 6.7, 13.4 and 26.8 μM As treatments, respectively. The calculated critical toxicity levels (CTL) of As in shoot was 40.2 μg g^{-1} DW and in root the value was 577 μg g^{-1} DW, indicating that the shoot was more sensitive to As than the roots in rice. Arsenic toxicity induced chlorosis symptom in the fully developed young leaves at 13.4 and 26.8 μM As treatments by decreasing chlorophyll index. Leaf number decreased at 13.4 and 26.8 μM As treatments but leaf blade decreased at 26.8 μM As treatment. Among the elements, iron (Fe) concentration, accumulation and translocation were the most decreased at 6.7, 13.4 and 26.8 μM As treatments. It was suggested that the chlorosis was due to Fe-deficiency induced by As and was not due to heavy metal induced Fe-deficiency. Roots contained almost 6-14 times higher As concentration than the shoots in the As treated plants. Arsenic translocation (%) decreased at 13.4 and 26.8 μM As treatments as compared to 6.7 μM As treatment. Arsenic and Fe were mostly concentrated in the roots of rice seedlings suggesting co-existence of these two elements.

Abbreviations: CDL (critical deficient level); CTL (critical toxic level); DAT (days after treatments); DW (dry weight)

3.1 INTRODUCTION

Arsenic contaminated water is being used for agricultural purpose which could be one of the major expose routes for As-toxicity to human and animals. Around the time of 1978, rice growing during the dry season was started in Bangladesh under the "green revolution" to increase food production. Bangladesh is one of the major rice growing countries and rice is the staple food crop (Rahman et al. 2007). The average background concentration of As in Bangladesh is much below 10 mg kg^{-1} dry soil. However, in some areas where soils receive As-contaminated groundwater irrigation, the concentration has been found to be as much as 80 mg kg^{-1} dry soil (Huq et al. 2003). Many crops receiving As contaminated water for irrigation have

been found to accumulate As at levels that exceed the minimum allowable daily limit (MADL) of 0.2 mg kg^{-1} DW. Rice and wheat (*Triticum aestivum* L.) receiving As-contaminated irrigation water have been found to absorb the toxic metalloid into roots and stems (Huq et al. 2003). However, the amount of As obtained through rice grain per person per day may, in many instants, surpass the MADL in Bangladesh.

Most groundwater used for irrigation in Bangladesh is contaminated with As (Khan et al. 1998). If the groundwater contaminated with As is applied as irrigation water, it may reduce the growth as well as production of the crops. Deep tubewells were dug and a large volume of groundwater has been withdrawn for irrigation especially in dry season. It is considered that the withdrawal of groundwater may change the geo-chemical and physical changes of the underground, causing As contamination in turn. Generally, groundwater contains 50% arsenate and 50% arsenite (Samanta et al. 1999) which may convert from one form to another. Redox potential is mainly governing this transformation (Masscheleyn et al. 1991; Onken and Hossner 1995). Arsenite is the dominating form of As in flooded paddy soil (Takamatsu et al. 1982), which is considered as the most toxic form. Arsenic toxicity is responsible for shorter plant height, thinner leaf-coloring, earlier root-coloring to yellowish brown or brown and curled leaves under sunlight in rice plant (Shaibur et al. 2006). Some data have already been published regarding plant response at high As level in, for example, rice (Abedin et al. 2002a), bush bean (*Phaseolus vulgaris* L.; Wallace et al. 1980) and tomato (*Lycopersicum esculentum* L.; Xu et al. 2007). However, there is little data on critical toxicity level of As and elemental composition of As-induced chlorosis in hydroponic rice under As-toxicity. It is, therefore, necessary to observe the effect of As on the response of rice and As concentration in plant tissues.

3.2 MATERIALS AND METHODS

3.2.1 Seed Germination and Plant Culture

Seed germination and plant culture has been described in **CHAPTER 2**. Twenty one days after germination at 4-5th leaf stage treatments were started. Arsenic treatments were 0, 6.7, 13.4 and 26.8 µM (0, 0.5, 1.0 and 2.0 mg As L^{-1}) for 14 days. Arsenic was added as sodium meta-arsenite ($NaAsO_2$). The pH (pH 5.5) was adjusted daily with a digital pH meter at around 4 p.m. during the experiment (June-July, 2005).

3.2.2 Chlorophyll Index (SPAD value)

Chlorophyll index (SPAD value) of fully developed (fifth leaf) new leaves was measured on 14 DAT as described in section 2.6 of **CHAPTER 2**. In each leaf, SPAD values of 3 points were measured and the average was calculated. Means of each bunch were obtained. Average of the data of 3 bunches was calculated.

3.2.3 Analysis of Plant Samples

Seedlings were collected on 14 DAT and washed with deionized properly. Shoots and roots were analyzed as described in section 2.7 of **CHAPTER 2**.

3.2.4 Determination of Arsenic

Arsenic was measured by Hydride Generation Atomic Absorption Spectrophotometric (HGAAS) technique by using the instrument (Hitachi HFS-3). We digested the samples with nitric-perchloric acids mixture and the volume of the solution was around 5 mL. After that, the volume of the digested solution was made 50 mL with MQ water (18.2 $M\Omega\ cm^{-1}$), purified by Milli-RO 60 (Millipore Corporation, USA). For As determination, the samples were further diluted up to 100-2000 times. As a result, the interference of nitrate on As determination might be minimized. It was reported that reduced nitrogen oxides (resulting from HNO_3 digestion) and nitrite could suppress instrumental response for As (Huang and Fujii 2001).

3.2.5 Calculation for the Parameters

Concentration in mg or μg of element g^{-1} DW; accumulation in mg or μg of element $plant^{-1}$ shoot or root; and translocation (%) in nutrient accumulation in shoot/ total accumulation (shoot + root) × 100. Tu et al. (2004) defined translocation factor (TF) as the ratio of As concentration in fronds of Chinese Brake fern to that in the roots of the plant.

3.2.6 Statistical Analysis

The experiment was arranged in randomized blocks with 3 replications. Data were subjected to analysis of variance as described in section 2.9 of **CHAPTER 2**.

3.3 RESULTS

3.3.1 Visible Symptoms

Arsenic induced little interveinal chlorosis in the old leaves and whitish chlorosis in the fully developed young leaves of rice seedlings on 14 DAT at 13.4 and 26.8 μM As levels in which the chlorosis was more pronounced at 26.8 μM As treatment **(Fig. 3.1)**. Not only chlorosis but also necrosis (burning of leaf tip) was also observed in the old leaves. At the early stage of As treatments, the leaves showed curling symptom at the day time and the youngest leaves failed to unfold. However, this symptom was not found in control plants. These symptoms may indicate water deficit on rice seedlings under As-toxicity (Yamane 1989; Shaibur et al. 2006). The most common visible symptom was growth reduction both in shoots and roots. Reduction of shoot height and root length due to As-toxicity could be termed as "little shoots or roots". The reddish color along the root length was found and felt slippery to the touch due to As-toxicity where the toxicity was higher at 26.8 μM As treatment.

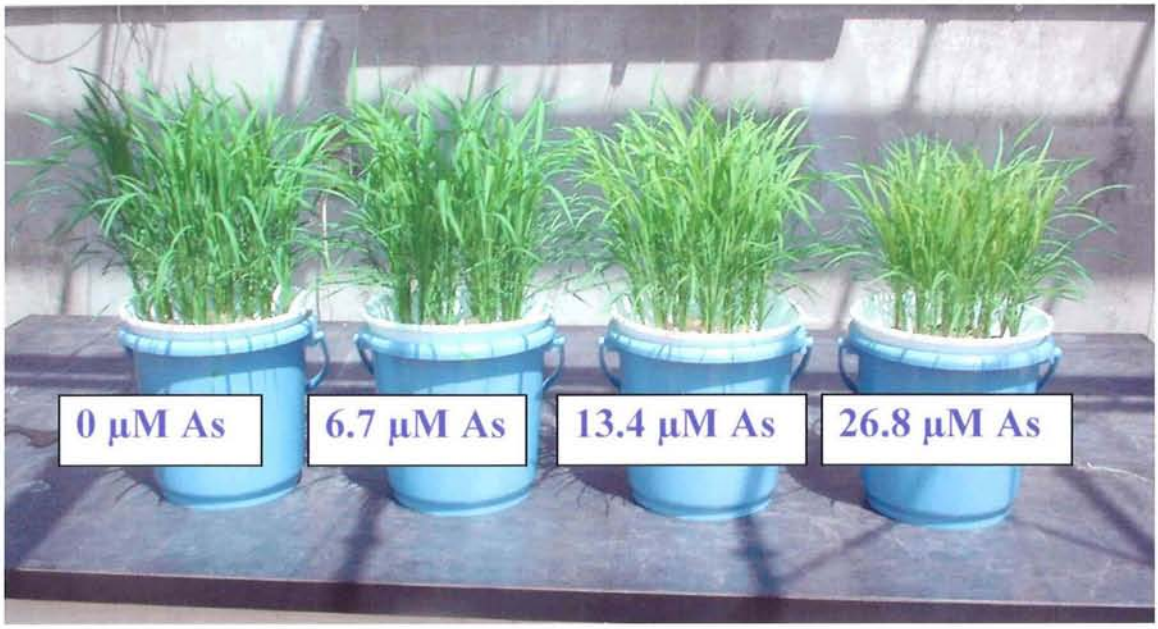


Figure 3.1 Photograph of rice seedlings at elevated concentrations of As. Seedlings produced whitish chlorosis in the fully developed young leaves at 13.4 and 26.8 μM As treatments. This picture was taken after 14 days of As treatments

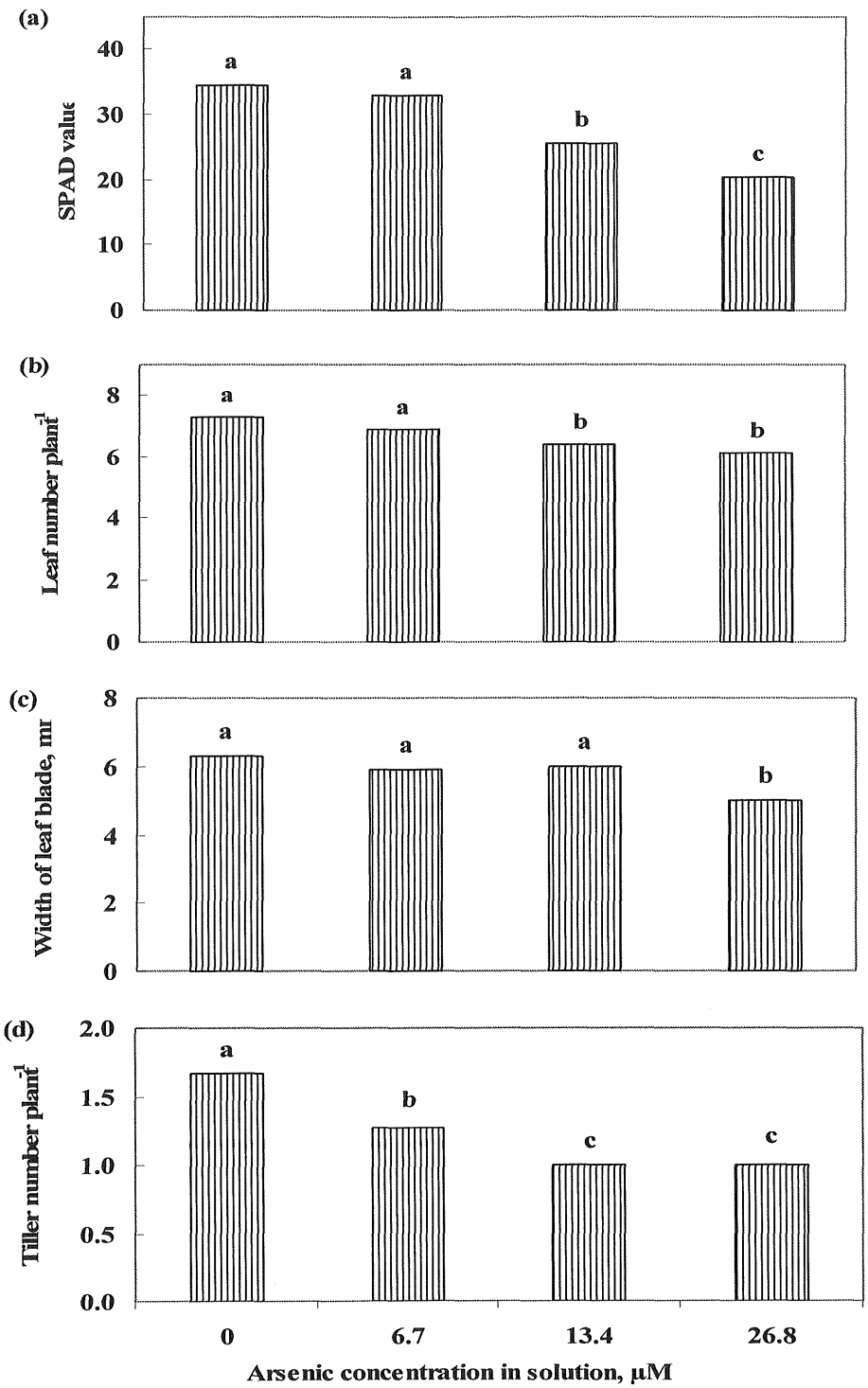


Figure 3.2 (a) SPAD value, (b) leaf number, (c) width of leaf blade and (d) tiller number of rice seedlings with different levels of As. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

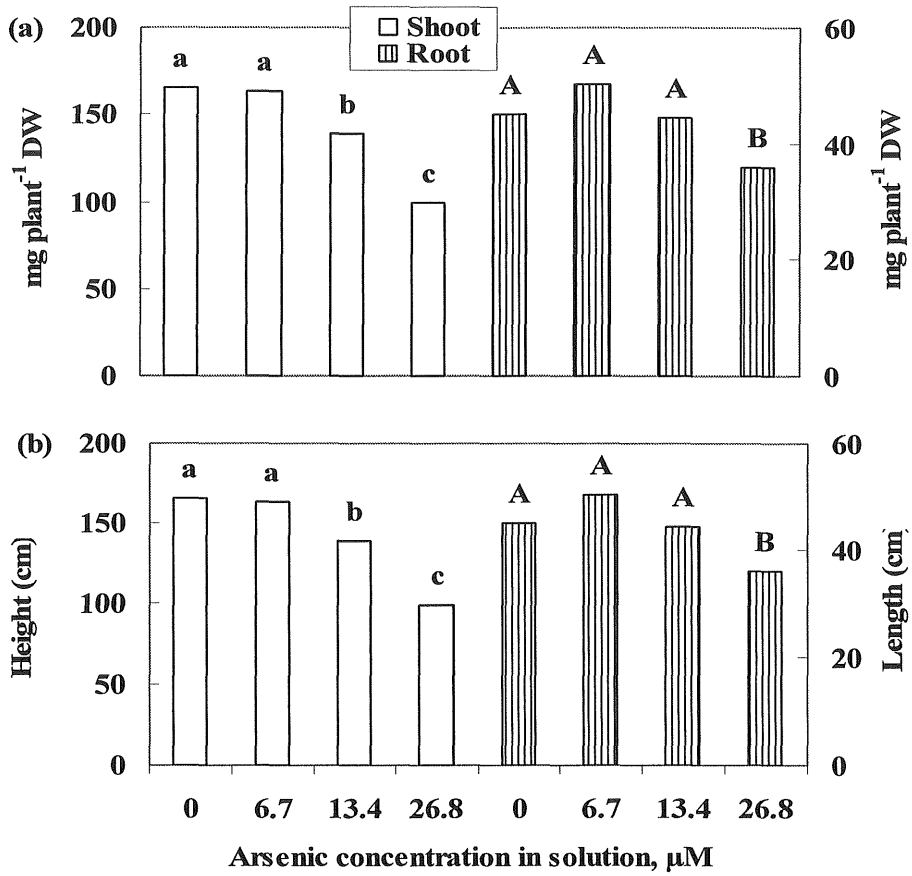


Figure 3.3 (a) Dry weight (DW) and (b) shoot height and root length of rice seedlings with different levels of As. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

3.3.2 Chlorophyll Index (SPAD value)

Chlorophyll index in the fully developed fifth young leaves decreased at 13.4 and 26.8 µM As treatments as compared to control **(Fig. 3.2a)**. The lowest value was recorded at 26.8 µM As treatment. We found that the concentration of Fe in shoot decreased in the As treatments **(Table 3.1)**. Although Fe is not a component of the chlorophyll molecule, it is essential for the synthesis of chlorophyll (Weier et al. 1982). Chlorosis may be caused by Fe-deficiency as well as by Mg-deficiency (Weier et al. 1982). In this experiment, Fe concentration decreased, therefore, the reduction of Fe concentration could be highly responsible for the reduction of chlorophyll index. It was considered that As-induced chlorosis

was due to Fe-deficiency because the chlorosis was found in the fully developed young leaves (Mengel and Kirkby 2001).

3.3.3 Leaf Number, Width of Leaf Blade and Tiller Number

Leaf number decreased at 13.4 and 26.8 μM As treatments and width of leaf blade decreased at 26.8 μM As treatment **(Figs. 3.2bc)**. Leaf blade was measured as the width of the middle position of the fifth leaves of the seedlings. Tiller number decreased significantly with increasing As in nutrient solution **(Fig. 3.2d)**. Recently, it was reported that during the duration of 35 days Akihikari rice variety did not produce any new tiller even in control plants (Shaibur et al. 2006). Reduction of leaf number, width of leaf blade and tiller number were most probably responsible for the reduction of DW **(Fig. 3.3a)**. Abedin et al. (2002a) found lower number of tiller of rice at 8 mg As L^{-1} treatment in their greenhouse pot experiment. Marin et al. (1993) reported that rice leaf area decreased at 0.8 and 1.6 mg As (dimethylarsinic acid, DMAA) L^{-1} treatments.

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

Shoot DW was not affected at all by 6.7 μM As treatment but decreased at 13.4 and 26.8 μM As treatments as compared to control **(Fig. 3.3a)**. Root DW decreased at 26.8 μM As treatment only, indicating that shoots were more sensitive to As than the roots in rice. Similar results were also found in the case of shoot height and root length **(Fig. 3.3b)**. Shoot height decreased by 6.56, 16.8 and 34.4%, while the values for root length were 1.42, 7.51 and 42.3% for 6.7, 13.4 and 26.8 μM As treatments, respectively. Arsenite and arsenate decreased shoot height and root length of rice seedlings in a greenhouse pot experiment (Abedin and Meharg 2002; Abedin et al. 2002a). Tsutsumi (1980) observed, no reduction of plant height up to 125 mg As kg^{-1} in rice but observed 63% reduction at 312.5 mg As kg^{-1} dry soil.

3.3.5 Critical Toxicity Level (CTL) of Arsenic

Typical polynomial two order growth curves of rice to As-toxicity were presented in Figs. 4ab. The CTL of As in shoots and in roots of hydroponic rice was calculated from the growth curves considering 10% DW reduction (Ohki 1984). The calculated CTL of As in shoots was 40.2 μg g^{-1} DW and the value in roots was 577 μg g^{-1} DW. These calculated values

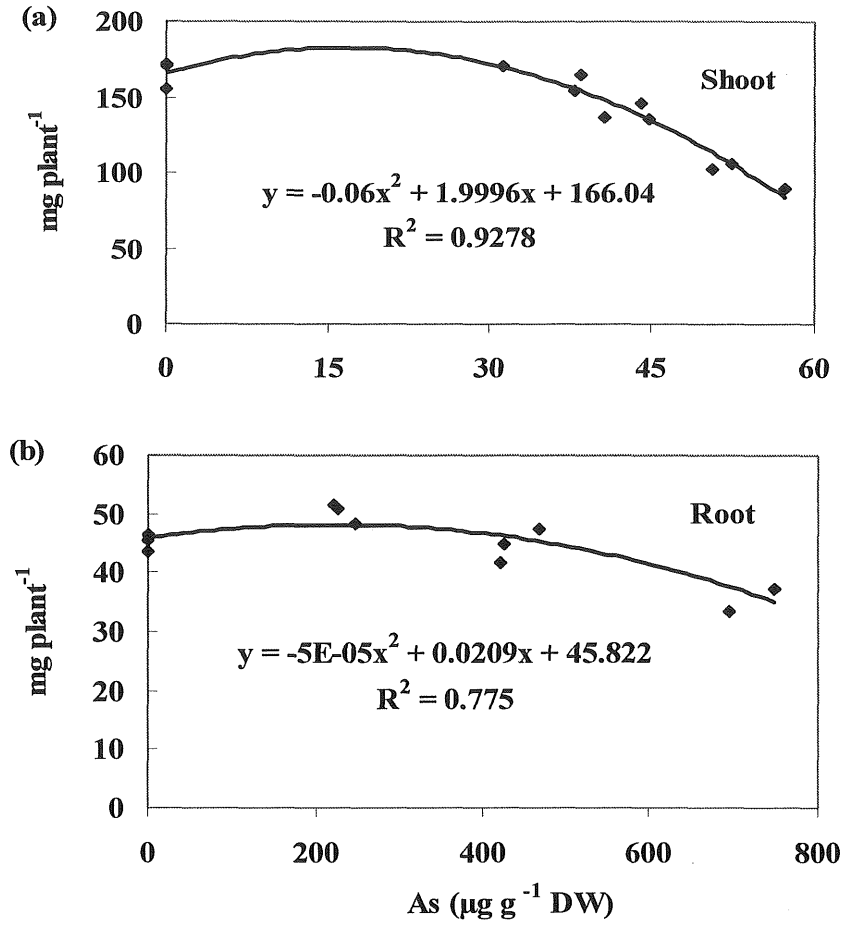


Figure 3.4 Two order polynomial growth curve (a) shoots and (b) roots of rice seedlings containing different concentration of As in plant tissues.

may be used to suggest the CTL of other elements in other plants. It was reported that in Akihikari rice variety the CTL of As was 21.0 µg g^{-1} DW in shoots and 325 µg As g^{-1} DW in roots (Shaibur et al. 2008a). It seemed that Akitakomachi rice variety was more resistant to As-toxicity than Akihikari rice variety, though this needed to be verified with experiments. These differences were most probably due to the differences of variety, environment and experimental methods. The CTL may be dependent on the source of Fe, content of mineral nutrients in growth medium, species of the plants and also the species of As used. Recently, it was reported that the CTL of As in sorghum as 11.7 µg g^{-1} DW in shoots and 367 µg As g^{-1} DW in roots. In barley, the CTL of As was 1.20 µg g^{-1} DW in shoots and 75.3 µg As g^{-1} DW in roots that could reduce 10% DW (Shaibur et al. 2008a).

3.3.6 Macro and Micronutrients

We observed that the concentration of P increased in shoots at 6.7, 13.4 and 26.8 μM As treatments as compared to control **(Table 3.1)**, which might be the concentration effect because the DW decreased **(Fig. 3.3a)**. Accumulation in shoots and translocation of P from roots to shoots were not much affected by the As-toxicity though it decreased at the higher As treatments **(Tables 3.2 & 3.3)**. The polynomial two order relationship between P and As concentration in plant tissues was significantly related with $R^2 = 0.9814$ for shoots and $R^2 = 0.5088$ for roots **(Figs. 3.5a,b)**, although the effect of As on P concentration in roots did not appear to be much affected.

Table 3.1 Concentrations of elements in shoots and roots of rice seedlings grown in nutrient solution 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	5.90c	47.9a	2.64a	3.93a	101.1a	671b	101a	26.8b	nd
6.7	7.62b	50.8a	2.45a	3.96a	81.2b	692b	87a	26.9b	36.0c
13.4	8.27b	50.0a	2.38ab	3.48a	72.3b	812a	111a	30.1a	43.2b
26.8	9.07a	46.4a	2.17b	2.90b	83.4b	855a	109a	32.9a	53.4a
	Concentrations in roots								
0	6.19a	51.4a	0.71a	1.80a	2088b	74.9b	56.8b	241b	nd
6.7	6.34a	41.4b	0.73a	2.19a	2156b	120.5a	120a	240b	232c
13.4	6.60a	40.4b	0.75a	1.73a	2378a	114.1a	113a	260ab	439b
26.8	6.72a	32.4c	0.62b	1.26b	2506a	109.0a	102a	278a	731a

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

Concentration of K was not much affected in shoots by the As treatments; but K concentration in roots decreased with increasing As **(Table 3.1)**. Accumulation of K decreased in shoots at 13.4 and 26.8 μM As treatments **(Table 3.2)**, but translocation (%) of K was not markedly affected **(Table 3.3)**. Yamane (1989) reported that the K concentration decreased in

rice plants due to As-toxicity. It was also reported that K concentration decreased significantly in shoots but marginally in roots of rice seedlings at 0.8 and 1.6 mg As (DMAA) L^{-1} treatments (Marin et al. 1993).

Table 3.2 Accumulation of elements in shoots and roots of rice seedlings grown in nutrient solution with different levels of As.

	----------mg plant^{-1} ----------				----------µg plant^{-1} ----------				
Treatment	P	K	Ca	Mg	Fe	Mn	Zn	Cu	As
(µM As)	Accumulation in shoot								
0	0.98ab	7.94a	0.44a	0.65a	16.8a	111.3a	16.7a	4.46a	nd
6.7	1.24a	8.28a	0.40a	0.64a	13.2b	112.7a	14.2a	4.39a	5.85a
13.4	1.15a	6.95b	0.33b	0.48b	10.1c	113.0a	15.4a	4.19a	6.01a
26.8	0.90b	4.60c	0.22c	0.29c	8.25d	84.7b	10.8b	3.27b	5.29a
	Accumulation in root								
0	0.28b	2.32a	0.032a	0.081b	94.1b	3.37b	2.56c	10.9a	nd
6.7	0.32a	2.10ab	0.037a	0.110a	108.3a	6.05a	5.98a	12.0a	11.7c
13.4	0.29b	1.81b	0.033a	0.077b	105.7a	5.07a	5.06a	11.6a	19.6b
26.8	0.24c	1.16c	0.022b	0.045c	90.2b	3.91b	3.65b	10.0a	26.4a

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

In shoots, Ca concentration was almost constant in the range between 0 and 13.4 µM As, but decreased significantly at 26.8 µM As treatment as compared to control. Similar results were also found in roots at 26.8 µM As treatment **(Table 3.1)**. Calcium accumulation in shoots decreased with increasing As **(Table 3.2)** but translocation (%) of Ca from roots to shoots was not much affected **(Table 3.3)**.

It was clearly observed that Mg concentration in shoots was similar in the range between 0 and 13.4 µM As treatments, however, decreased significantly at 26.8 µM As treatment as compared to other treatments **(Table 3.1)**. Similar result was also obtained in roots at 26.8 µM As treatment. Accumulation of Mg in shoots was also negatively influenced with increasing As **(Table 3.2)**. We observed that the translocation (%) of Mg was not influenced by As-toxicity **(Table 3.3)**.

Table 3.3 Translocation (%) of elements from roots to shoots in rice seedlings grown in nutrient solution with different levels of As.

Treatment (μM As)	P	K	Ca	Mg	Fe	Mn	Zn	Cu	As
0	78a	77a	93a	89a	15a	97a	87a	29a	nd
6.7	80a	80a	92a	85a	11b	95a	70b	27ab	33a
13.4	80a	79a	91a	86a	9c	96a	75b	27ab	24b
26.8	79a	80a	91a	86a	8c	96a	75b	25b	17c

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

In shoots, Fe concentration decreased in As-treated plants as compared to control **(Table 3.1)**. However, Fe concentration increased in roots with increasing As and the highest value was that for 26.8 μM As treatment **(Table 3.1)**. Our result showed that Fe was mostly concentrated in roots of As treated seedling. The two order polynomial relationship between Fe and As concentration was correlated with $R^2 = 0.8656$ for shoots and $R^2 = 0.8244$ for roots, respectively **(Figs. 3.6a,b)**. The relationships were exactly opposite for the two organs. The relationships suggested that As might play a role in the uptake and translocation of Fe in rice at elevated As concentration. Similar relationships have also been reported by Porter & Peterson (1977), De Koe et al. (1988) and Shaibur et al. (2008a).

Manganese concentration increased in shoots with increasing As and the highest value was at 26.8 μM As treatment **(Table 3.1)**. Similar to shoots, Mn concentration was also increased in roots of As treated plants as compared to control **(Table 3.1)**. Yamane (1989) reported that Mn concentration increased both in shoots and roots of rice due to the applied As^{3+} or As^{5+} at the rate of 33.5, 67 and 134 mg kg^{-1} dry soil. Recently, we reported that As decreased Mn concentration in shoots of As-induced whitish chlorosis in Akihikari rice variety (Shaibur et al. 2006). We found that Mn accumulation decreased in shoots at 26.8 μM As treatment **(Table 3.2)** but translocation (%) of Mn was not much affected by the applied treatments **(Table 3.3)**.

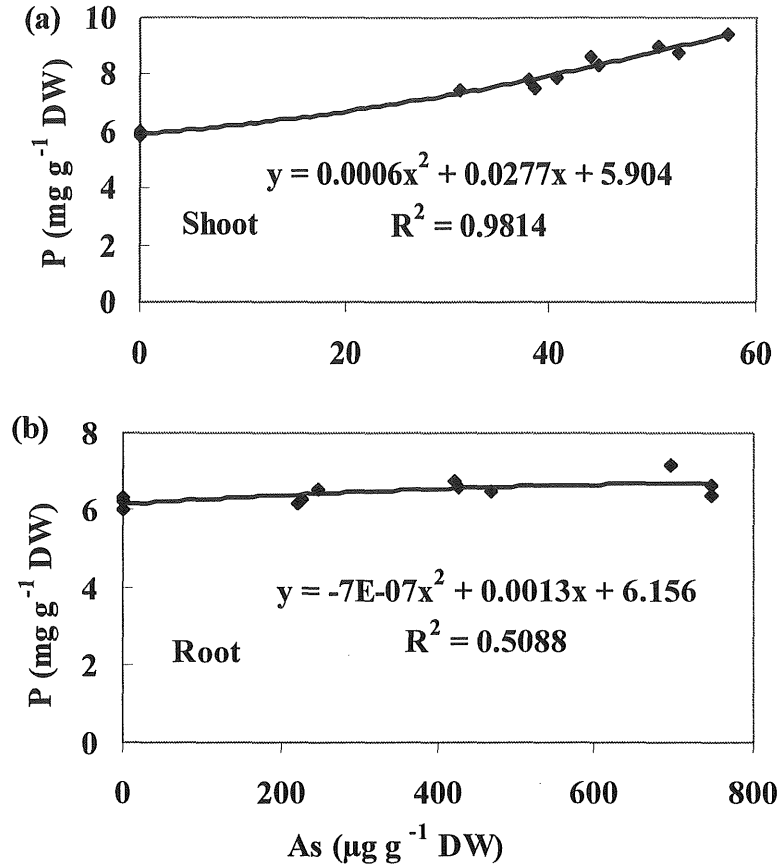


Figure 3.5 Polynomial two order correlation between As and P content (a) in shoots and (b) in roots varies with the As treatments in rice seedlings.

Zinc concentration was not much affected in shoots by the As treatments, but it increased in roots of all the As treated plants as compared to control **(Table 3.1)**. Zinc accumulation decreased in shoots at 26.8 µM As treatment as compared to control but increased in roots resulting in lower translocation **(Tables 3.2 & 3.3)**.

Copper concentration increased both in shoots and in roots at 26.8 µM As treatment as compared to control **(Table 3.1)**. However, accumulation and translocation (%) of Cu was not much affected though it showed decreasing tendency in shoots at 26.8 µM As treatment **(Tables 3.2 & 3.3)**.

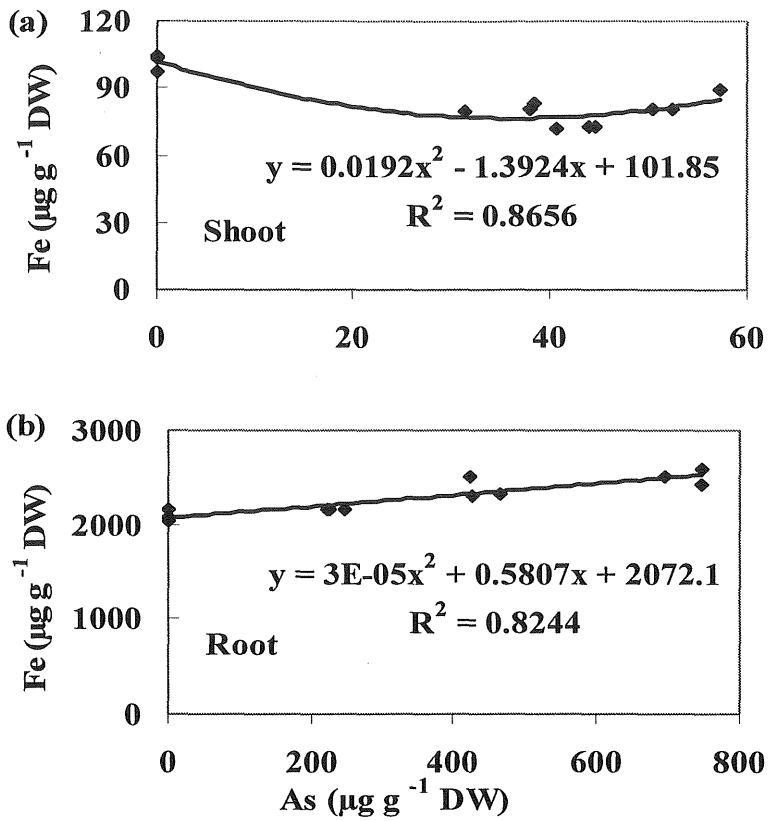


Figure 3.6 Polynomial two order correlation between As and Fe content (a) in shoots and (b) in roots varies with As treatments in rice seedlings.

3.3.7 Arsenic

Arsenic concentration increased both in shoots and in roots with increasing As **(Table 3.1)**. Arsenic concentration in roots were 6.44, 10.2 and 13.7 times higher than that of shoots at 6.7, 13.4 and 26.8 µM As treatments, respectively, indicating that As was mostly concentrated in roots. Yamane (1989) also reported a similar result that the roots of rice (*Oryza sativa* L. cv. Nihonbare) accumulated almost 90% of As. Arsenic accumulation was not much affected in shoots though it increased in roots with increasing As in the medium **(Table 3.2)**. We found that As translocation decreased at 13.4 and 26.8 µM As treatments as compared to 6.7 µM As treatment **(Table 3.3)**, suggesting that the toxicity was very severe and reduced the translocation (%) of As. It has already been reported that arsenite translocation from roots to shoots is limited by its high toxicity to root membranes (Sachs and Michael 1971). Liu et al.

(2005) concluded that the main barrier to uptake and translocation of As, fed in the form of arsenite, might be the root tissue rather than Fe-plaque.

3.4 DISCUSSION

We assumed that the chlorotic symptom induced by As in rice was most probably Fe-chlorosis because the symptom was observed in the young leaves (Mengel and Kirkby 2001). If the chlorotic symptom is found in the old leaves, it is Mg-chlorosis (Maynard 1979). We found that the chlorophyll index was also the lowest in the chlorotic leaves **(Fig. 3.2a)**. Visible symptom in roots was also prominent. Reddish color was observed in the As-treated plants. The depth of reddish color increased with increasing As in the medium. The reddish color of the roots may be due to the formation of Fe precipitates or Fe-plaque on the root surface of rice (Batty and Younger 2003).

Shoot DW decreased by 1.75, 16.2 and 40.1% at 6.7, 13.4 and 26.8 μM As treatments, respectively, while 11.3% increased and 1.33 and 20.2% decreased for the same treatments, respectively. This result indicated that As caused greater reduction of shoot DW than that of the root DW. Our result suggested that the threshold value of As sensitivity in hydroponic rice was between 0 and 13.4 μM As (1 mg As L^{-1}) in shoots, considering that elemental concentration inducing >10% reduction of DW, is CTL (Ohki 1984). Abedin et al. (2002a) found considerable reduction in straw and root biomass of rice due to 8 mg As L^{-1} concentration in the greenhouse pot experiment. In the presence of As, the activity of enzyme or protein or plant growth regulators may be decreased and decreasing the plant growth. This speculation needs verification. Reduction of DW may also be associated with the reduction of leaf number, leaf blade, tiller number, shoot height and root length **(Figs. 3.2bcd & 3.3b)**. Arsenic might destroy the root structure, resulting in decreased root length. It is known that arsenite reacts with sulfhydryl groups of proteins of roots (Speer 1973) causing disruption of root function (Isensee et al. 1971; Orwick et al. 1976) and even cellular death.

Our results indicated that As concentrated Fe in roots **(Table 3.1)** and blocked Fe translocation from roots to shoots **(Table 3.3)**. Iron translocation (%) was the most reduced by As among the metal micronutrients **(Table 3.3)**. This result suggested that Fe may be inactivated by As in or at the root surface. By using X-ray micro analyzer, Yamane (1989) suggested that Fe and As accumulated on the root surface of rice. It is well established that Mg-

chlorosis is found first in old leaf (Maynard 1979) but Fe-chlorosis is found in young leaves (Mengel and Kirkby 2001). In our experiment, the chlorotic symptom was observed in the fully developed young leaves, indicating that As-induced chlorosis was Fe-chlorosis. The CDL (critical deficient levels) of Fe is almost similar for C_3 and C_4 plants and ranging from 66 to 72 μg g^{-1} DW (Marschner 1998). We found that plants treated with As contained Fe concentration slightly higher than the deficient level, this may be because we digested the green old leaves together with chlorotic new leaves and determined the Fe concentration.

The ratios (%) of Fe:P concentrations in shoots were 1.71, 1.07, 0.87 and 0.92 at 6.7, 13.4 and 26.8 μM As treatments, respectively **(from Table 3.1)**. It meant that Fe concentrations decreased much more than the P concentrations in shoot tissues. The higher reduction of Fe concentrations was mostly responsible for the lower chlorophyll index **(Fig. 3.2a)**, resulting in whitish chlorosis symptom in the young leaves. It was reported that the lower the Fe:P values were, the lower the chlorophyll indices were (Ladouceur et al. 2006).

The CTL of Mn in plants is between 200-5300 μg g^{-1} DW in fully expanded leaves (Mingle and Kirkby 2001). Our experimental plants contained 671 to 855 μg Mn g^{-1} DW in shoots, indicating that our control plants together with other plants were Mn-toxic. Reports have shown that Mn-toxicity induced Fe-deficiency in hydroponic rice at the concentration of 9181 μg Mn g^{-1} DW (Alam et al. 2003). Therefore, our experimental plants were not Mn-toxic that induced Fe-deficiency. It is therefore suggested that Mn was not related for the induction of whitish chlorosis in this experiment.

In leaves, the CDL of Zn is below 15-20 μg g^{-1} DW (Marschner 1998). It has been shown that Zn-toxicity leads to chlorosis in young leaves (Marschner 1998). The CTL level of Zn of crop plants are between 100 and more than 300 μg Zn g^{-1} DW (Ruano et al. 1988), where the latter values seem to be more typical (Marschner 1998). Our data was within the normal level ranging from 87 to 111 μg Zn g^{-1} DW **(Table 3.1)**. Therefore, it could be considered that Zn-toxicity was not involved in the induction of chlorosis symptom in this experiment.

Our experimental plants contained Cu from 26.8 to 32.9 μg g^{-1} DW **(Table 3.1)** which is within the CTL. For most crop species, the CTL of Cu in the leaves is above 20-30 μg g^{-1} DW (Robson and Reuter 1981). It meant that the control plants itself contained higher content of Cu in shoot tissues. Based on the fact, it was considered that Cu concentration in shoots might not be involved in the production of chlorosis in leaves.

Some anions are strongly adsorbed to the membrane surface of roots. Arsenic anions (arsenite and arsenate) may rapidly adsorb to the root surface, leading to the intense high As concentration, especially in hydroponic culture (Wauchope 1983). That may be the reason why the highest levels of As are found in roots. Formation of Fe-plaque in roots also might be involved. Iron-plaque, coating of Fe hydroxides/oxides is commonly formed on the roots of rice. It is the consequence of oxidation of roots by release of oxygen and oxidants into the rhizosphere (Armstrong 1967; Chen et al. 1980). There may be two pathways by which arsenite enter into rice roots. In the primary way, part of arsenite may be oxidized to arsenate in the root rhizosphere, which has high affinity for Fe-plaque, co-precipitate with Fe^{3+} and adsorb on the plaque (Otte et al. 1991). At the root-plaque interface, siderophores by microbes or phytosiderophores exuded by rice roots, may form complex with Fe^{3+} and mobilize Fe-bound arsenate, take up through phosphate co-transporters (Liu et al. 2005). This may stimulates uptake of Fe and arsenate in/on the root surface and may increase As and Fe concentration in arsenite treated plants in the present experiment. In the second possible pathway, arsenite may be accumulated on the Fe-plaque in the form of $H_3AsO_3^0$ and then transported into rice roots via aquaporins (Meharg and Jardine 2003). In our experiment, we found that in presence of higher As, As accumulation was higher in roots but lower in shoots **(Table 3.2)** as compared to the lower concentration, this may be due to the fact that Fe-plaque can act as a barrier to the uptake of toxic metals (Batty et al. 2000; Chen et al. 2005) on the roots.

3.5 CONCLUSIONS

Arsenic toxicity decreased dry weight, shoot height, root length, leaf number, width of leaf blade and tiller number. Shoots were more sensitive to As than the roots. The CTL for As of this rice cultivar in hydroponic culture may be between 0 to 6.7 μM As (0 to 0.5 mg L^{-1}). The CTL of As in this rice variety was 40.2 μg g^{-1} DW in shoots and value was 577 μg g^{-1} DW in roots that could reduce 10% DW. Arsenic induced whitish chlorotic symptom in the fully developed young leaves. Concentration and accumulation in shoots; and translocation of Fe among the metal micronutrients were the most affected. Therefore, it was suggested that the chlorosis was most probably Fe-chlorosis, caused by Fe deficiency induced by As, and was not heavy metal induced Fe-deficiency. This chlorosis symptom may be due to Fe-translocation

problem. Arsenic and Fe were mostly concentrated in or on the roots of rice. Our experimental results suggested that Fe might be inactivated by As in or on roots.

CHAPTER 4

ARSENITE AND CITRATE-Fe^{3+} INTERACTION: EFFECT OF PHYTOSIDEROPHORES ON ^{59}Fe ABSORPTION AND TRANSLOCATION IN ARSENIC TOXIC RICE

ABSTRACT

Two experiments were conducted by taking rice (*Oryza sativa* L. cv. Akitakomaci) as the test plant. In both experiments, arsenic (As) was used as sodium meta-arsenite ($NaAsO_2$) and iron (Fe) was used as citrate-Fe^{3+} for 14 days. Seedlings were grown in the greenhouse at pH 5.5. First experiment was conducted to observe the effect of additional citrate-Fe^{3+} on physiological and mineralogical properties 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 citrate-Fe^{3+}). It was found that As-induced conspicuous whitish chlorosis in the fully developed young leaves. Chlorophyll index was the lowest in As-treated seedlings. Iron concentration in shoots and translocation from roots to shoots were the most affected among the nutrient elements in As-treated seedlings. Additional citrate-Fe^{3+} could not recover As-induced chlorosis in rice. Second experiment was conducted to confirm the result of the first experiment. The treatments were 0 μM As + 10 μM citrate-Fe^{3+} (control) and 26.8 μM As + 10 μM citrate-Fe^{3+} (As-treated). It was also found that among the nutrient elements Fe concentration in shoots and translocation from roots to shoots were the most decreased, confirming the result of the first experiment. Furthermore, in the second experiment, control and As-treated seedlings were fed with 10 μM labeled ^{59}Fe in absence or presence of 10 μM PS (phytosiderophores) for 4 h starting from 10.30 am to 2.30 p.m. to observe the efficiency of PS on ^{59}Fe absorption and translocation in As-treated seedlings. Absorption and translocation of ^{59}Fe increased in PS treated control plants 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 increased the absorption of ^{59}Fe in roots as compared to that without PS treated plants but could not increase ^{59}Fe translocation to shoots, indicating that ^{59}Fe can not be easily translocated to shoots by PS in 26.8 μM As-treated seedlings. Our result confirmed that As-induced chlorosis in young leaves were due to Fe-translocation problem from roots to shoots.

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

4.I INTRODUCTION

Arsenic (As) could decrease rice growth even at 6.7 μM level in the growth medium (Shaibur et al. 2006). Elevated concentrations of As in rooting medium have been shown to be associated with necrosis in the leaf tip and whitish chlorosis in the fully developed young leaves of rice (Shaibur et al. 2006). Rice shoots containing around 100 μg As g^{-1} DW shows toxicity symptom (Shaibur et al. 2006), but some ferns are able to grow in high As contaminated conditions and could accumulate extremely high content of As in the aerial parts without showing visible toxicity symptoms (Ma et al. 2001; Zhao et al. 2002).

Underground water of Bangladesh contains high concentrations of As and mostly exceeded WHO's guidelines, 0.01 mg As l^{-1} (Tanabe et al. 2001). Long term use of As contaminated water for irrigation has resulted in elevated As levels in agricultural soils (Alam and Sattar 2000). Plants grown on As contaminated condition may contain higher As concentrations in the rice grain (Abedin et al. 2002a). The mean As level of Bangladeshi rice is 0.13 mg kg^{-1} DW (range 0.03-0.30) and the predominant form of As in Bangladesh rice is inorganic (Williams et al. 2005).

Arsenite and arsenate are the predominant form of As in anaerobic and aerobic soil, respectively. In anaerobic condition, arsenite is the predominant form of As, but arsenate As and organic As are also prevailed (Abedin et al. 2002). Arsenite and arsenate are inter converted each other where chemical kinetics play an important role (Carbonell-Barrachina et al. 1998; Meharg 2004). Tomato and rice roots have a high capacity to reduce arsenate to arsenite (Xu et al. 2007). Arsenate could be reduced to arsenite within 24 h if tomato is grown in nutrient solution. However, arsenite remained stable in the solution for up to 3 days. Root exudates and microbes have little contribution to arsenate reduction (Xu et al. 2007). Arsenite and arsenate are transported by different As transporters. Arsenate is transported via phosphate transporter in higher plants (Asher and Reay 1979; Meharg and Hartley-Whitaker 2002) but arsenite is transported across the plasma membrane via MIPs (major intrinsic protein)/aquaporins (*see* Meharg and Jardine 2003). In microbes, arsenate is also taken up into the cells by phosphate transporter (Rosen 2002; Silver and Phung 2005) and arsenite is transported by aquaglycerolporin channels (Rosen 2002; Silver and Phung 2005).

Numerous inorganic amendments e.g. Fe oxides and hydroxides are used to reduce As availability (Hartley et al. 2004). Iron oxides are well known for As absorption from soils

(Lumsdon et al. 1984; Waychunas et al. 1993). A report shows that Fe oxides could decrease almost 50% water soluble As in a garden soil (Mench et al. 1998). Amorphous Fe hydroxide (am-Fe(OH_3) could effectively adsorb As. Sometimes Fe grit is used for As immobilization in garden soil (Vangronsveld et al. 1994). Carbonell-Barrachina et al. (1999) reported that Fe hydrous oxides played an important role to control As adsorption–desorption reaction in sludge. It means Fe effectively controls As solubility in soils.

Mechanisms of Fe absorption and translocation in plants have received much attention, because they are the key process in the supply of Fe to plants (Alam et al. 2005). Iron is abundant in soils, but its deficiency is a common phenomenon in plants growing in neutral to alkaline soils. The reason is that the availability of Fe^{2+} and Fe^{3+} is low in neutral soils. Iron deficiency in plants occur if some factors inhibits Fe absorption and translocation or factors impairing its utilization in metabolic processes in plant tissues, rather than scarcity of Fe in the root rhizosphere. If the Fe nutrition hampers plants response, the following steps should be considered (1) availability of indigenous Fe should be increased, (2) external Fe should be increased and (3) efficiency of plants like absorption and translocation should be increased (Shenker and Chen 2005). In our study, we used additional Fe as citrate-Fe^{3+} to increase external Fe and used phytosiderophores (PS) to increase the efficiency of rice roots of the plants showing As-induced whitish chlorosis.

Phytosiderophores secreted by grasses (Strategy II plants) are well known for their Fe acquisition capacity in Fe-deficient condition (Takagi et al. 1984; Römheld and Marschner 1986). Iron acquisition mechanisms in grasses is characterized by two process (1) grasses release low molecular weight substances called PS, have high affinity for Fe^{3+} chelation, solubilize sparingly soluble Fe^{3+} in the root rhizosphere and (2) then the Fe^{3+}-PS is transported by an unknown transport system (Takagi et al. 1984; Römheld and Marschner 1986). Evidence showed that Fe uptake enhanced in rice by the addition of PS to the medium, resulting in greening of the chlorotic leaves of rice (Takagi et al. 19984). Recently, Alam et al. (2005) found that ^{59}Fe absorption and translocation were enhanced in barley by the addition of PS in the medium.

Arsenate is the dominant form of plant available As in upland soils (Meharg and Hartley-Whitaker 2002) and it is readily reduced to arsenite in anaerobic condition as undissociated arsenious acid, H_3AsO_3 (Abedin et al. 2002b). Considering the fact that the

arsenate and arsenite are present concomitantly convertible and therefore we used arsenite in our study. Recently, we reported that arsenite As showed its toxicity in rice at 13.4 and 26.8 μM levels (Shaibur et al. 2006). We tried to observe if As-induced chlorosis in rice could be ameliorated with additional citrate-Fe^{3+} or not, because As induced chlorosis at lower concentration of citrate-Fe^{3+}. To the best of our knowledge, there is no information regarding $NaAsO_2$ and citrate-Fe^{3+} interaction in rice grown hydroponically. The objectives were (1) to evaluate the efficiency of citrate-Fe^{3+} on rice treated with higher As, (2) to observe the physiological and mineralogical properties of As-induced chlorotic rice and (3) to observe the effectiveness of PS in the absorption and translocation of ^{59}Fe in rice grown under higher As concentration in Fe-sufficient condition.

4.2 MATERIALS AND METHODS

4.2.1 Seed Germination and Plant Culture

Seedlings of rice were grown as described in section 2.3.1 of **CHAPTER 2.**

4.2.2 Applied Treatments

Treatments of the first experiment (10 July to 16 August, 2005) 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+}). In the second experiment (16 July to 22 August, 2005), the treatments were 0 μM As + 10 μM citrate-Fe^{3+} (control) and 26.8 μM As + 10 μM citrate-Fe^{3+} (As-treated). In addition, the plants of the first experiment were fed with 10 μM $FeCl_3$ leveled with ^{59}Fe in presence or absence of 10 μM PS.

4.2.3 Chlorophyll Index (SPAD value)

Chlorophyll index was measured as described in section 2.6 of **CHAPTER 2.**

4.2.4 Analysis of Plant Samples

Plants were harvested on 14 DAT. After harvesting, seedlings were analyzed as described in section 2.7 of **CHAPTER 2.**

4.2.5 Radio Isotope (RI) Experiment with PS

Seedlings of the second experiment were transferred to RI laboratory from greenhouse on 11 DAT 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 seedlings were washed carefully with deionized water, then transferred to freshly prepared 100 mL full-strength nutrient solution containing 10 μM ^{59}Fe as $^{59}FeCl_3$, adjusted to pH 5.5 with HCl, was added 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 seedlings (5 plants). This was done with 3 replications. 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). Seedlings were the washed with tap water. Shoots were separated from the roots, air dried for 24 h, then oven dried at 70 ± 2°C for 24 h and weighed.

Twenty mL of concentrated nitric acid (HNO_3) was used to digest the plant samples (Zarcinas et al. 1987). Radioactivity of ^{59}Fe in the root apoplast or digested plant tissues was determined using a gamma scintillation counter (Auto Well Gamma System, AccuFLEX ARC-7000, Aloka, Tokyo, Japan). Adsorbed ^{59}Fe in the root apoplast (apoplastic ^{59}Fe) was not included to calculate root ^{59}Fe. Adsorbed and absorbed ^{59}Fe was calculated based on the molar radioactivity of the elements supplied after correction for their half-life decay.

4.2.6 Source of PS and Radioactive ^{59}Fe

Phytosiderophores known as mugineic acid was collected from the root washing of barley seedlings grown in Fe-depleted medium as described previously (Takagi 1993). Radioactive ^{59}Fe was purchased from Perkin Elmer Life and Analytical Science (Boston, MA, USA).

4.2.7 Terminologies Used

Concentration of an element is defined as the amount of element g^{-1} DW (mg or μg element g^{-1} DW), while accumulation refers to the total amount of element $plant^{-1}$ shoots or

roots (mg or µg of element plant^{-1}). Total absorption of ^{59}Fe refers to the sum of the total amount of ^{59}Fe (shoots plus roots accumulation). ^{59}Fe translocation refers to the total amount of ^{59}Fe in shoots and translocation activity of shoots refers to the content of ^{59}Fe in shoot per gram shoot DW. Absorption activity of roots was calculated by dividing the total amount of ^{59}Fe (shoots plus roots) with the root DW.

4.2.8 Environmental Condition

The temperature of the greenhouse was relatively high ranging from 23 to 37ºC in night and day, respectively.

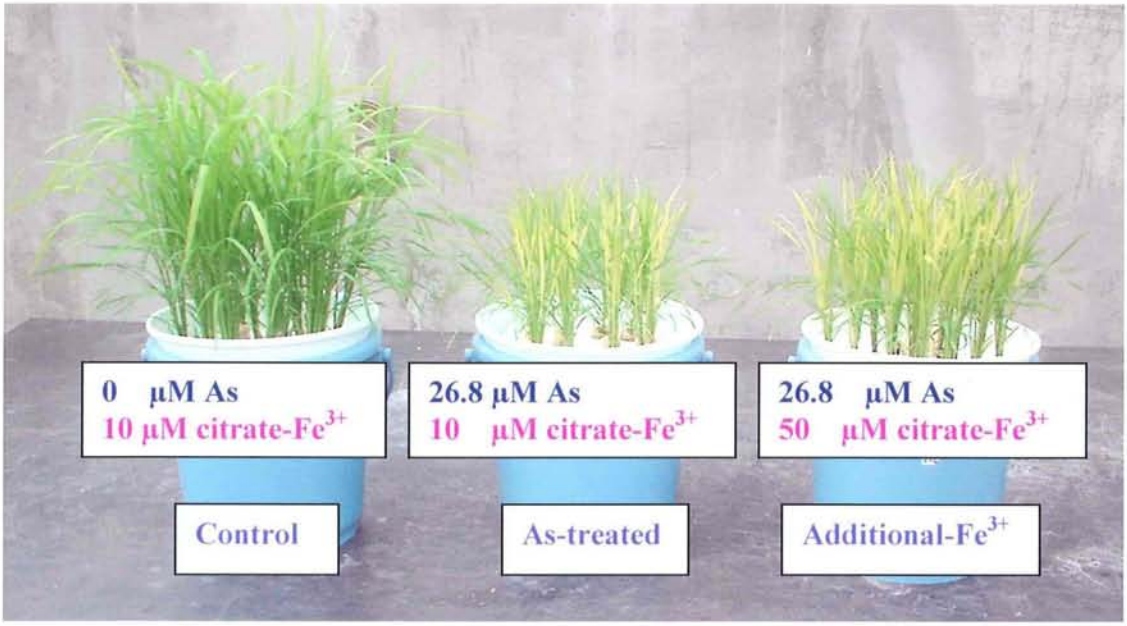


Figure 4.1 Photograph of rice seedlings at elevated concentration of As and or in presence of additional citrate-Fe^{3+} **(first experiment)**. This picture was taken after 14 days of As exposure.

4.3 RESULTS

4.3.1 Visible Symptoms

In control plants, dew like water drops appeared in the leaf tip of young and old leaves in the evening to early morning; however, it was absent in As and additional-Fe^{3+} treated plants. Plants showed whitish chlorotic symptom in the fully developed young leaves of As and

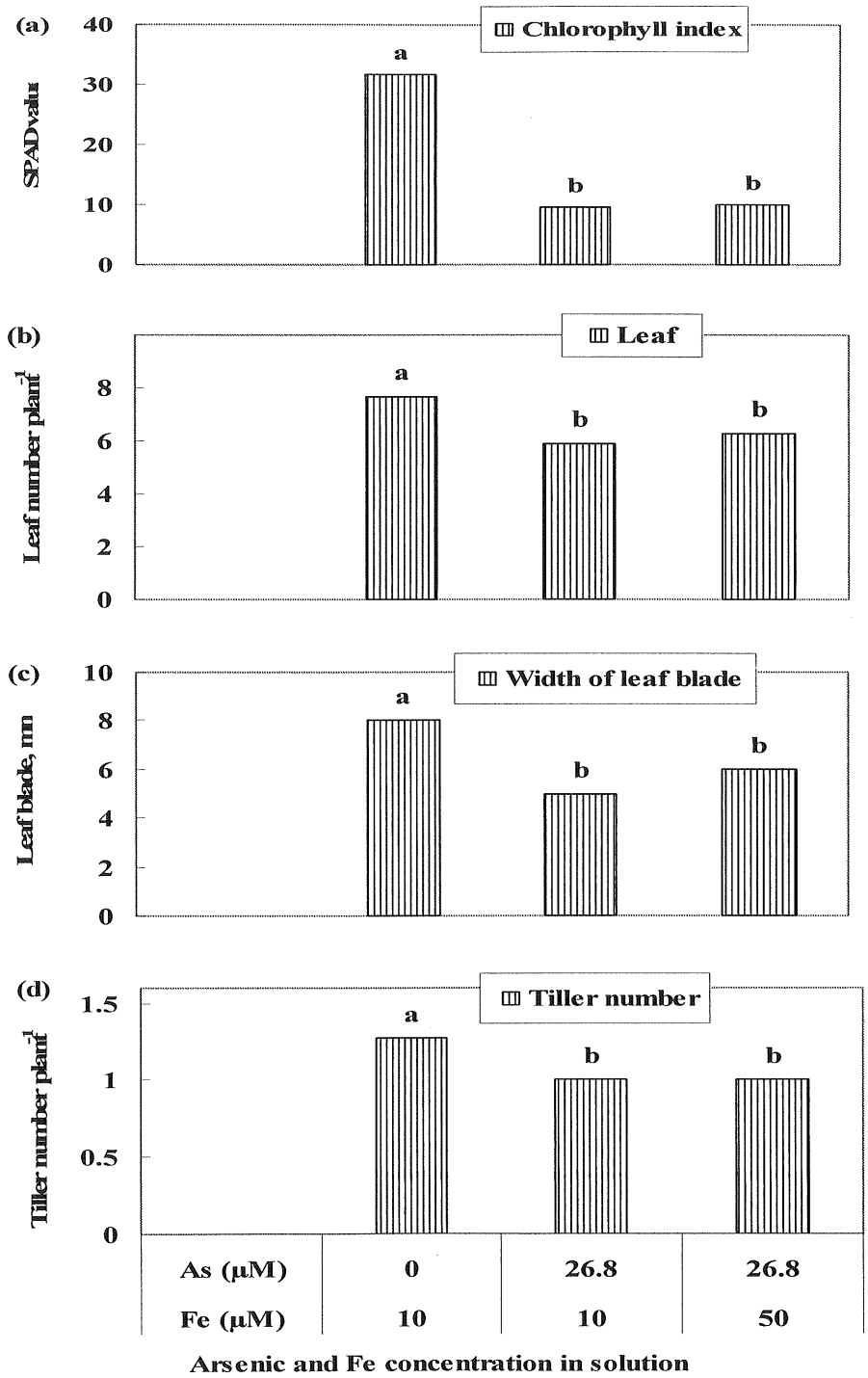


Figure 4.2 (a) SPAD value, (b) leaf number, (c) width of leaf blade and (d) tiller number of rice seedlings in two levels of citrate-Fe^{3+} and 26.8 μM As **(first experiment)**. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

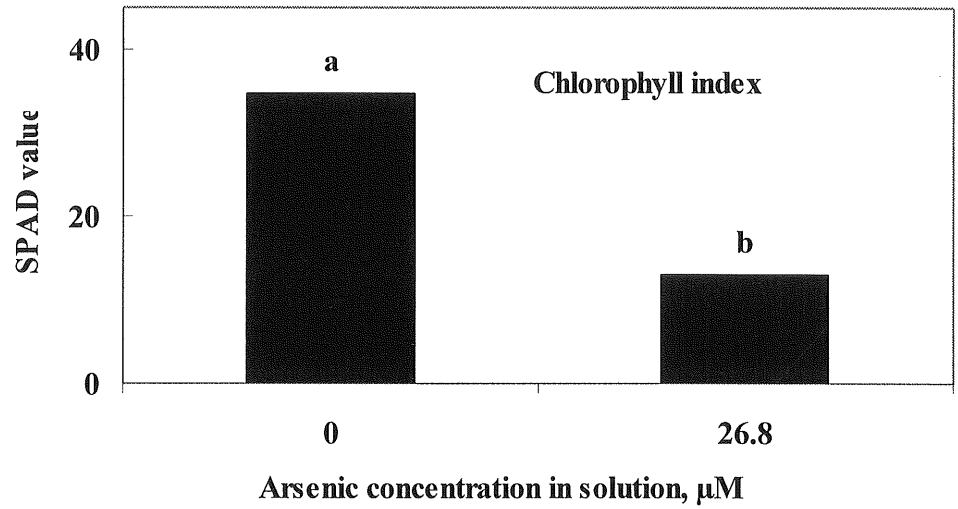


Figure 4.3 Chlorophyll index (SPAD value) of rice seedlings in two levels of As in solution **(second experiment)**. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

additional citrate-Fe^{3+} treated plants, in which the symptom seemed to be more pronounced in As-treated plants compared to additional-Fe^{3+} plants **(Fig. 4.1)**. Chlorotic symptom induced by 26.8 µM As in rice grown in citrate-Fe^{3+} condition could not be recovered by additional citrate-Fe^{3+}. Our result showed that rice responded well in warmer season (second half of July and August) in Morioka, Iwate, Japan. Mechanism of chlorophyll index reduction in the grasses needs to be investigated. Interveinal chlorosis appeared in the old leaves of As-treated plants. Green color in the old leaves of As-treated plants was not as pronounced as it was in control plants. Sometimes, necrotic symptom was found in the old leaves of As-treated plants. At sunlight, seedlings were curled in As and additional citrate-Fe^{3+} treated plants. Turgidity also decreased in As and additional citrate-Fe^{3+} treated plants as compared to control with increasing sunlight intensity. These results suggested that As might depress water movement from roots to shoots.

Arsenic changed the color of roots from white to reddish brown. Arsenic-treated roots were white up to 72 h and after that the reddish color appeared. Iron plaques were visible clearly as the reddish coating on the root surface of rice grown hydroponically (Armstrong 1967; Chen et al. 1980). Similar, visible symptoms were observed in the second experiment.

4.3.2 Chlorophyll Index (SPAD value)

In the first experiment, chlorophyll index decreased in As-treated plants as compared to control plants **(Fig. 4.2a)**. Additional citrate-Fe^{3+} could not increase the chlorophyll index as compared to As-treated plants **(Fig. 4.2a)**. Similar to the first experiment, chlorophyll index was also decreased by the 26.8 μM As treatment in the second experiment **(Fig. 4.3)**.

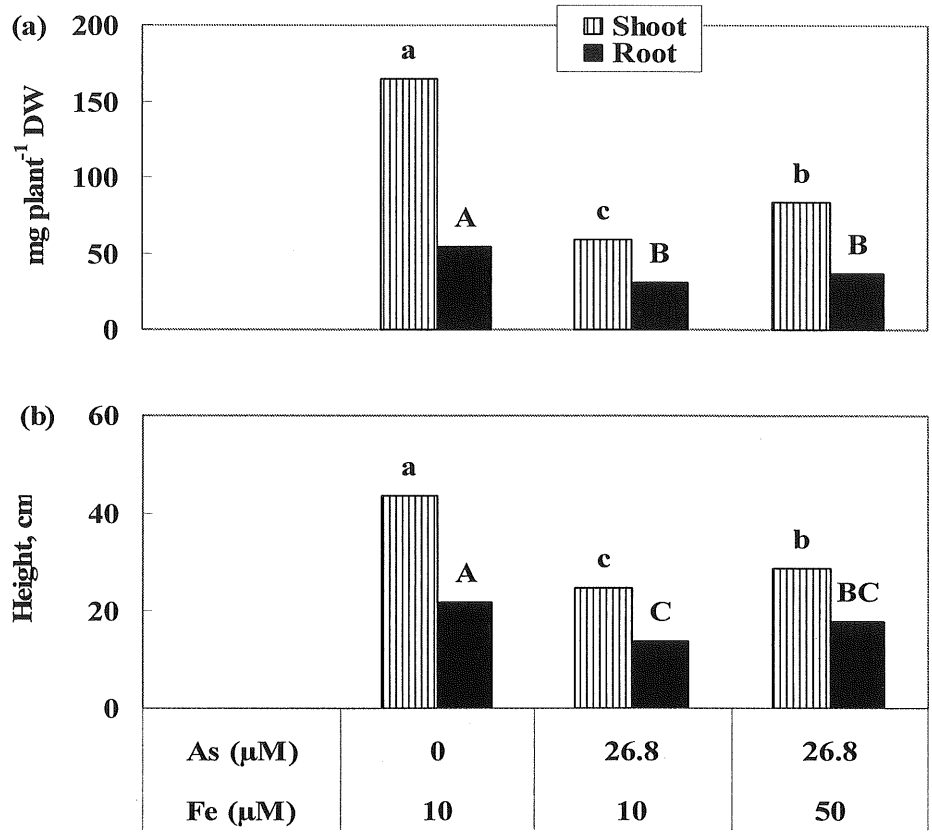


Figure 4.4 (a) Dry weight, DW (b) shoot height and root length of rice seedlings in two levels of citrate-Fe^{3+} and 26.8 μM As (first experiment). Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

4.3.3 Leaf Number, Width of Leaf Blade and Tiller Number

In the first experiment, leaf number, width of leaf blade and tiller number decreased in As-treated plants as compared to control plants **(Figs. 4.2bcd)**. Report showed that tillering was severely depressed by As as in the case of P deficiency (Kitagishi and Yamane 1981; Shaibur et al. 2009b). Additional citrate-Fe^{3+} could not recover all these physiological parameters as

compared to As-treated plants. Similar to the first experiment, all those physiological parameters were decreased in As-treated plants in second experiment (data were not shown).

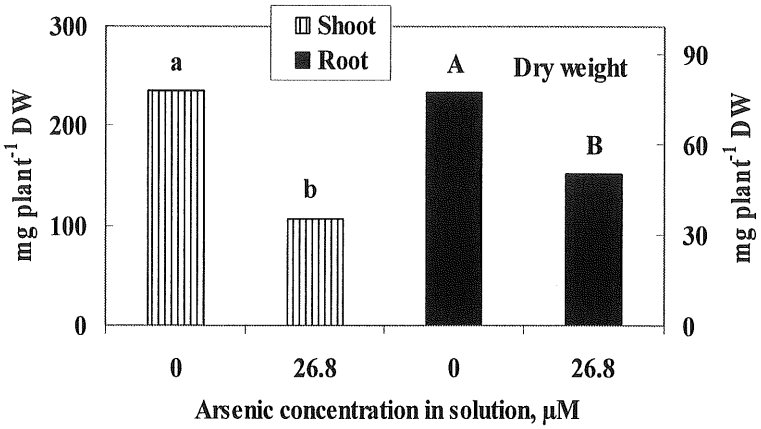


Figure 4.5 Dry weight (DW) of rice seedlings in two levels of As in solution (second experiment). Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

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

In the first experiment, DW was the highest in control but decreased in As-treated plants **(Fig. 4.4a)**. Arsenic at 26.8 µM level decreased the DW by 65% in shoots and 44% in roots, respectively. However, in additional citrate-Fe^{3+} plants, the DW reduction was 50% in shoots and 34% in roots as compared to control. In additional citrate-Fe^{3+} plants, the DW increment was 41% in shoots and 17% in roots as compared to As-treated plants. Our result indicated that additional citrate-Fe^{3+} could decrease As-toxicity little, resulting in enhancement of DW both in shoots and roots. Additional citrate-Fe^{3+} in the rooting medium did not show any toxic effect on rice growth. In the second experiment, it was also found that shoots and roots DW decreased in As-treated plants as compared to control plants **(Fig. 4.5)**.

In the first experiment, shoot height and root length decreased in As-treated plants as compared to control plants **(Fig. 4.4b)**. Arsenic-toxicity decreased shoot height by 43% as compared to control; however, the decrement in additional citrate-Fe^{3+} plants was 34%. Additional citrate-Fe^{3+} increased the height by 16% as compared to As-treated plants. Similarly, As-toxicity decreased root length by 35% as compared to control; however, the decrease in

Table 4.1 Concentration and accumulation of nutrients in shoots and roots of rice seedlings grown in nutrient solution with As and or As + citrate-Fe^{3+} **(first experiment)**.

Treatments (μM)		P	K	Ca	Mg	Fe	Mn	Zn	Cu
As	citrate-Fe^{3+}	----------mg g^{-1} DW ----------				----------μg g^{-1} DW ----------			
		Concentrations in shoots							
0	10	9.43b	48.1a	2.98a	4.07a	67.4a	616b	102b	36.7a
26.8	10	18.7a	40.5b	2.22b	3.45b	54.4b	853a	155a	31.8b
26.8	50	16.0a	40.5b	2.18b	3.21b	66.3a	800a	144a	32.6b
		Concentrations in roots							
0	10	7.41a	25.1a	0.80b	1.86a	1034c	115b	77.7b	219a
26.8	10	7.67a	23.6a	1.26a	1.40b	1729b	573a	185a	269a
26.8	50	8.57a	24.4a	1.40a	1.36b	3007a	589a	160a	232a
		Concentrations in young leaves							
0	10	8.39b	26.2a	3.03a	1.50a	44.6a	172b	60.7c	24.5b
26.8	10	16.6a	27.5a	2.88a	1.12b	8.83c	584a	192a	28.7a
26.8	50	15.2a	26.3a	2.57b	1.13b	13.4b	505a	130b	25.3b
		----------mg plant $^{-1}$ ----------				----------μg plant $^{-1}$----------			
		Accumulations in shoots							
0	10	1.55a	7.95a	0.46a	0.67a	11.1a	101a	16.8a	6.04a
26.8	10	1.10b	2.38c	0.11b	0.20b	3.20c	50.1c	9.13c	1.87c
26.8	50	1.33a	3.37b	0.12b	0.27b	5.52b	66.6b	12.0b	2.72b
		Accumulations in roots							
0	10	0.40a	1.37a	0.03a	0.10a	56.5b	6.23c	4.22b	11.8a
26.8	10	0.23c	0.72b	0.02b	0.04b	53.0b	17.5b	5.66a	8.18b
26.8	50	0.31b	0.87b	0.03a	0.05b	108.0a	21.1a	5.75a	8.32b
		Translocation (%)							
0	10	79a	85a	94a	87a	16a	94a	80a	34a
26.8	10	82a	77a	85ab	83a	6b	74b	62b	19c
26.8	50	81a	79a	81b	85a	5b	76b	68b	25b

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.

Table 4.2 Concentration and accumulation of nutrients in shoots and roots of rice seedlings grown in nutrient solution with As and or As + citrate-Fe^{3+} **(second experiment).**

Treatments (μM)		P	K	Ca	Mg	Fe	Mn	Zn	Cu
As	citrate-Fe^{3+}	----------mg g^{-1} DW ----------				----------μg g^{-1} DW ----------			
		Concentrations in shoots							
0	10	7.20b	47.8a	4.63a	4.01a	93.7a	458.6a	54.8a	18.8a
26.8	10	11.2a	33.3b	3.36b	2.97b	77.3b	184.5b	34.5b	12.0b
		Concentrations in roots							
0	10	4.39a	22.8a	1.05a	1.91a	698.6b	66.7a	61.1a	137.8a
26.8	10	2.69b	9.91b	1.19a	0.83b	947.0a	37.6b	62.0a	140.9a
		Concentrations in young leaves							
0	10	4.87a	20.1a	1.51a	1.99a	56.8a	76.5a	56.7a	7.56a
26.8	10	5.53a	19.0a	1.32b	1.59b	14.6b	67.0a	38.7b	6.54a
		----------mg plant $^{-1}$ ----------				----------μg plant $^{-1}$----------			
		Accumulations in shoots							
0	10	1.69a	11.3a	1.09a	0.94a	22.1a	108.1a	13.0a	4.44a
26.8	10	1.19b	3.52b	0.36b	0.30b	8.22b	19.5b	3.66b	1.27b
		Accumulations in roots							
0	10	0.34a	1.76a	0.08a	0.15a	54.0a	5.10a	4.78a	10.6a
26.8	10	0.13b	0.49b	0.06b	0.04b	47.6b	1.88b	3.11b	7.08b
		Translocation (%)							
0	10	83a	86a	93a	86a	29a	95a	73a	30a
26.8	10	90a	88a	86a	88a	15b	91a	54b	15b

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.

additional citrate-Fe^{3+} plants was 18% as compared to control. Additional citrate-Fe^{3+} increased the root length by 26% as compared to As-treated plants. Similar to the first experiment, shoot height and root length decreased in As-treated plants (data were not shown).

4.3.5 Macro and Micronutrients

In the first experiment, in most of the cases, K, Ca and Mg concentrations decreased in shoots of As-treated plants as compared to control plants, but P concentration increased **(Table 4.1)**. Phosphorus concentration was also increased in the young leaves of As-treated plants but Mg concentrations decreased for the same **(Table 4.1)**. Translocations of macronutrients were not much affected by the applied treatments **(Table 4.1)**. Additional citrate-Fe^{3+} could not increase the concentration but in some cases accumulation of some elements increased as compared to As-treated plants **(Table 4.1)**. The second experiment showed a similar result to the first experiment in As-treated plants **(Table 4.2)**.

Table 4.3 Concentrations, accumulations and translocations of arsenic in hydroponic rice seedlings as affected by the different treatments **(first experiment)**.

Treatments (μM)		Concentrations			Accumulations		Translocations
		-----μg As g^{-1} DW -----			---μg As plant $^{-1}$ ---		(%)
As	citrate-Fe^{3+}	shoot	root	YL	shoot	root	
0	10	nd	nd	nd	nd	nd	nd
26.8	10	75.1a	909a	9.30b	22.2b	140a	13.8b
26.8	50	69.7b	851a	12.3a	29.0a	152a	16.0a

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, YL = young leaves. nd = not detected.

In the first experiment, Fe concentrations decreased in shoots of As-treated plants as compared to control, but increased in roots, resulting in lower translocations **(Table 4.1)**. Iron concentrations increased both in shoots and roots of additional citrate-Fe^{3+} plants as compared to As-treated plants. Similar to the first experiment, Fe concentration was also decreased in shoots but increased in roots of As-treated plants as compared to control **(Table 4.2)**. Concentrations of Mn and Zn increased both in shoots and in roots of As-treated and additional-Fe^{3+} plants as compared to control; however, copper concentration decreased in shoots for the same **(Table 4.1)**. In the second experiment, Mn and Zn concentration decreased

in shoots **(Table 4.2)**. Manganese and Zn concentration were not affected similarly in shoots by the As treatments.

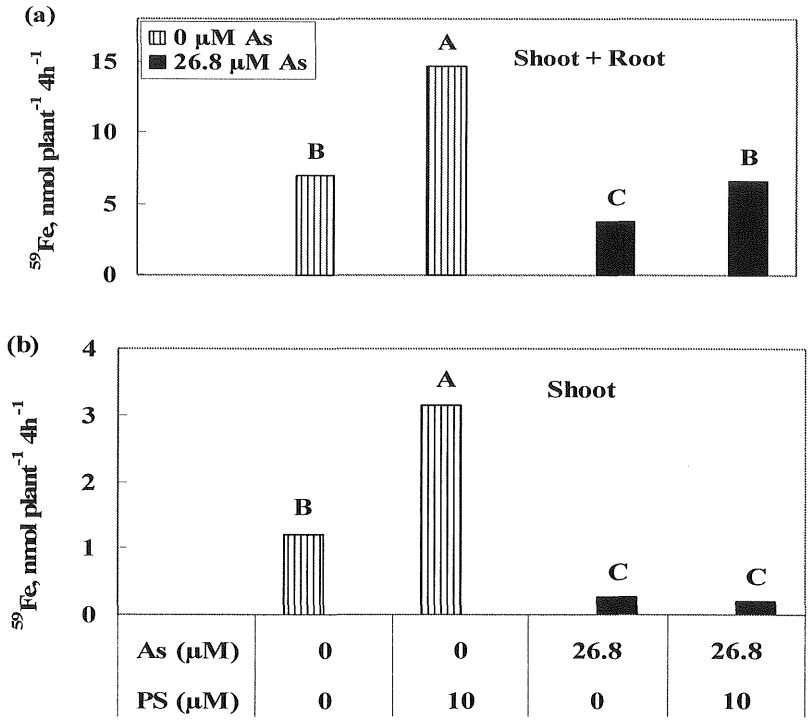


Figure 4.6 (a) Total absorption (shoot + root) and (b) translocation of ^{59}Fe to shoots of rice seedlings with two levels of As. Seedlings were fed with 10 µM $FeCl_3$ labeled with ^{59}Fe in presence or absence of phytosiderophores (PS).

4.3.6 Arsenic

Arsenic concentration was 75.1 µg g $^{-1}$ DW in shoots of As-treated plants; however, the value was 69.7 µg As g $^{-1}$ DW in additional-Fe^{3+} plants **(Table 4.3)**. Arsenic concentration in roots was not much affected with the treatments **(Table 4.3)**. Roots concentrated almost 12.1 and 12.2 times higher As as compared to shoots in As-treated and additional-Fe^{3+} plants, respectively **(Table 4.3)**.

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

In the RI experiment, total absorption of ^{59}Fe was found to be the highest in PS treated control plants as compared to without PS treated plants **(Fig. 4.6a)**. Similarly, PS also enhanced

total ^{59}Fe absorption in As-treated plants **(Fig. 4.6a)**. Translocation data showed that in presence of PS, ^{59}Fe translocation was enhanced by 240% in control plants; however, the translocation was not enhanced at all in As-treated plants **(Fig. 4.6b)**.

4.3.8 Translocation Activity of Shoots

Phytosiderophores enhanced translocation activity almost 3 times in control plants; however, translocation activity was not enhanced at all in As-treated seedlings **(Fig. 4.7)**.

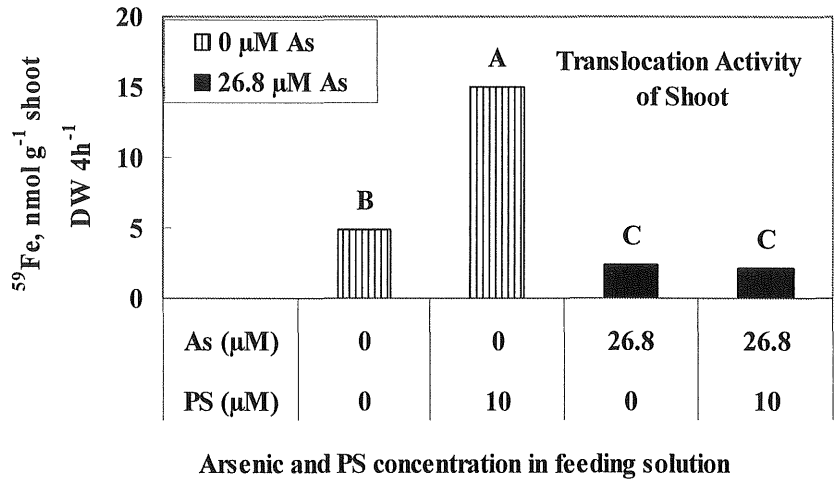


Figure 4.7 Translocation activity of shoots (nmol g $^{-1}$ shoot DW) to ^{59}Fe in rice seedlings as affected by two levels of As with or without PS. Seedlings were fed with 10 µM $FeCl_3$ labelled with ^{59}Fe in presence or absence of PS.

4.3.9 Absorption Activity of Roots

Absorption activity of roots was the highest in presence of PS in control plants **(Fig. 4.8a)**. It was clear that As-treated plants showed a lower absorption activity as compared to plants grown in absence of As. This was most probably due to the fact that in As-treated plants, root DW decreased.

Absorption activity in PS treated control plants was 2.55 times higher as compared to without PS treated plants; however, it was 1.96 times in As-treated plants. It meant that the magnitude of absorption activity of roots was lower in As-treated plants as compared to without As-treated plants.

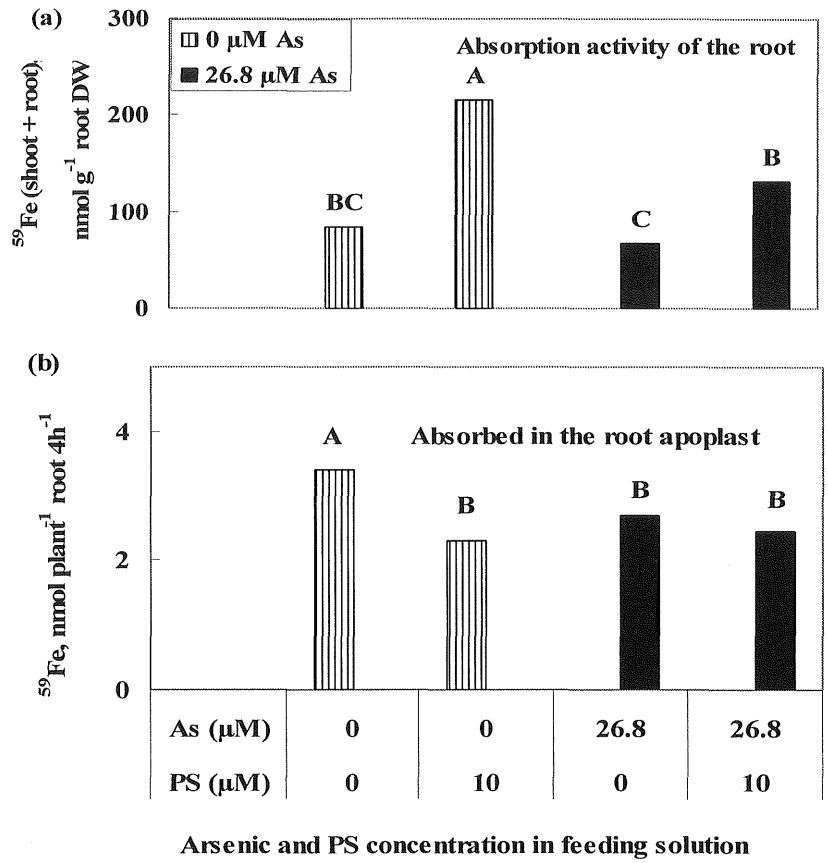


Figure 4.8 (a) Absorption activity of roots; total absorption (shoots + roots) per gram root dry weight (DW); and (b) apoplastic root ^{59}Fe (adsorbed in root apoplast) in rice seedlings as affected by two levels of As. Seedlings were fed with 10 µM $FeCl_3$ labeled with ^{59}Fe in presence or absence of phytosiderophores (PS).

4.3.10 Apoplastic ^{59}Fe in Roots

The highest content of ^{59}Fe in the root apoplast was observed in control plants that were not treated with PS **(Fig. 4.8b)**. There was a decrease in the concentration of ^{59}Fe in the root apoplast of plants treated with PS as compared to without PS treated plants. In As-treated plants, the content was similar in presence or absence of PS.

4.4 DISCUSSION

In the first experiment, Fe concentration was 54.4 µg g^{-1} DW in the shoots of As-treated plants **(Table 4.1)** and this lower Fe concentration was mostly responsible for the induction of

whitish chlorosis in the fully developed young leaves **(Fig. 4.1)**. Iron concentration in shoots was measured after digesting the whole shoots (stems, leaves and young leaves together). We separated the young leaves from the old leaves in all treatments to measure Fe concentration in young leaves. In young leaves, the Fe concentrations were 44.6, 8.83 and 13.4 μg g^{-1} DW in control, As-treated and additional-Fe^{3+} plants, respectively **(Table 4.1)**. Lower Fe concentrations in the chlorotic young leaves were mostly responsible for the induction of whitish chlorosis. Concentrations ratios (%) of Fe:P were 0.71, 0.29 and 0.41 in shoots of control, As-treated and additional citrate-Fe^{3+} plants, respectively **(from Table 4.1)**. Similarly, ratios (%) of Fe:P were 0.53, 0.05 and 0.09 in the young leaves, respectively **(from Table 4.1)**. It meant that Fe concentrations decreased much more than the P concentrations. The higher reduction of Fe concentrations was mostly responsible for the lower chlorophyll indices in As-treated plants **(Fig. 4.2a)**. The ratio of Fe to P in plant tissues has been reported as one of the regulators of Fe chlorosis in shoot tissues. It was reported that the lower the Fe:P values were, the lower the chlorophyll index were (DeKock and Alexander 1955; Pushnik et al. 1984; Ladouceur et al. 2006).

Additional citrate-Fe^{3+} in the medium increased Fe concentration in shoots **(Table 4.1)**, but this value was still within the CDL, resulting in no increased chlorophyll index **(Fig. 4.2a)**. The CDL of Fe was almost similar for C_3 and C_4 species and ranging from 66-72 μg g^{-1} DW (Marschner 1998). Present experiment did not confirm that As-induced chlorosis was Fe-chlorosis caused by Fe-deficiency. In presence of citrate-Fe^{3+}, As might break Fe transporter severely and therefore Fe can not be easily translocated to shoots. If the chlorosis symptom in the young leaves is disappeared by the additional-Fe, then the chlorosis is considered Fe-chlorosis. In this current experiment, additional citrate-Fe^{3+} in the medium did not show any toxic effect on rice growth as the concentration of Fe in the shoots was almost similar to the normal concentration. The normal concentrations of Fe in the leaf tissues are 50-100 μg g^{-1} DW (Mengel and Kirkby 2001) and the concentration >500 μg Fe g^{-1} DW in leaf tissues is considered to be the toxic for plant growth (Marschner 1998).

In the second experiment, Fe concentrations in shoots were within the CDL (65 μg Fe g^{-1} DW), resulting in the whitish chlorosis in the fully developed young leaves. Similar to the first experiment, it was also found that the concentration ratios (%) of Fe:P were 1.17 and 0.26

in the second experiment **(from Table 4.2)**. The lowest chlorophyll index **(Fig. 4.3)** was obtained for the lowest ratios of Fe:P (0.26 ; from **Table 4.2)**.

Arsenic treated roots contained higher content of Fe compared to control roots. Reddish color Fe-plaque was visible in As-treated plants. In anaerobic condition, rice releases oxygen to the root rhizosphere, resulting in the formation of Fe-oxyhydroxide plaque (Armstrong 1964). Arsenate has high affinity to form complex with Fe^{3+}-plaque to produce high insoluble Fe-arsenate in the root rhizosphere. Rice exhibit Fe-deficiency as a result of Fe-plaque formation (Meharg 2004).

In the first experiment, enhancement of Mn and Zn concentration in shoots is unknown. In most of the cases, it was found that Mn and Zn concentrations decreased in the higher As-treated hydroponic rice (Shaibur et al. 2006; Shaibur et al. 2008c), that we found in our second experiment. However, enhancement of Mn concentration was found in hydroponic sorghum (Shaibur et al. 2008a) and in some hydroponic rice experiment (unpublished). Additional citrate-Fe^{3+} had hardly positive effect on the enhancement of the concentration of those elements in shoot tissues. Additional citrate-Fe^{3+} in the medium increased Fe, Mn, Zn and Cu accumulations in shoots as compared to As-treated plants (Table 4.1), indicating that Fe had some positive effect over As-toxicity.

Our result indicated that additional-Fe^{3+} reduced As concentration in shoots **(Table 4.3)**. Arsenic concentration in young leaves increased in additional-Fe^{3+} plants as compared to As-treated plants **(Table 4.3)**. It is possible because the translocation of As increased in additional-Fe^{3+} plants. Arsenic has high affinity to react with sulfhydryl group of protein in root (Speer 1973) and Fe^{3+} has high affinity to As absorption (Hartley et al. 2004). We found that higher As was translocated in additional citrate-Fe^{3+} plants **(Table 4.3)**. Accumulations of As in roots were not much affected with the treatments **(Table 4.3)**.

Total absorption data indicated tat PS effectively played a role in the absorption of ^{59}Fe in both control and As-treated plants **(Fig. 4.6a)**. However, the magnitude of total ^{59}Fe absorption in As-treated plants was not as high as it was in control plants. In control plants, PS enhanced 109% higher total absorption, but the absorption increased 77% in As-treated plants. The reduction of ^{59}Fe absorption (%) might be due to the reduction of PS activity by the As. Arsenic effectively reduced PS production and synthesis in barley grown in Fe-depleted medium (Shaibur et al. 2009a). Phytosiderophores effectively enhanced ^{59}Fe translocation in

control plants but failed it in As-treated seedlings **(Fig. 4.6b)**, indicating that ^{59}Fe can not be easily translocated with the chelators PS in the As-treated plants. Our result suggested that As-induced chlorosis might be due to the problem of Fe translocation mediated with PS.

It is reported that absorption and translocation of ^{59}Fe in plants fed with ^{59}Fe and PS were increased relatively to plants fed solely with Fe^{3+} in control plants (Alam et al. 2005). Increase in ^{59}Fe absorption and translocation in PS treated plants compared to plants treated without PS, indicated that the PS-^{59}Fe complex was absorbed at the root surface via an unknown transport system and then translocated to the aerial parts (Takagi et al., 1984; Römheld and Marschner, 1986). *Yellow stripe* 1 (ys1) is membrane protein in maize root (*Zea mays* L.), mediates the absorption of PS-Fe^{3+} complex (Curie et al. 2001) and the levels of *ys1* mRNA levels increase in both shoots and roots plants grown under Fe-deficient condition. Translocation of Fe in rice plants in the form of PS-Fe^{3+}-complex was 12%; however, the other Fe might be translocated as citrate-Fe^{3+}-complex or something else (Mori et al. 1991). Lindsay and Scwab (1982) suggested that Fe chelators do not increase the solubility of Fe^{3+} or Fe^{2+} but only serve to hold Fe in a soluble form at a sufficient concentrations resulting in increasing diffusion of Fe to the root surface.

Phytosiderophores effectively enhanced the translocation activity of shoot in control plants **(Fig. 4.7)**. However, PS failed to enhance the translocation activity in As-treated shoots, suggesting that As at 26.8 μM level may inactivate the Fe transporter in rice roots.

Absorption activity was almost 3 times higher in control plants fed with PS but it was almost 2 times in As-treated plants **(Fig. 4.8a)**, indicating that As-toxicity decreased the absorption activity of PS in roots. The reduction of apoplastic ^{59}Fe in PS treated control plants was most probably due to the fact that ^{59}Fe was solubilized by PS and not precipitated on the surface of roots **(Fig. 4.8b)**. It was reported that the reduction of root apoplastic ^{59}Fe was more pronounced in the roots of PS treated plants (Alam et al. 2005).

The results of our study are generally quite favorable in supporting an important role of PS in Fe absorption and translocation in As-treated rice. It has been reported that As reduced Fe absorption and translocation in rice (Shaibur et al. 2006). However, the efficiency of PS in the absorption and translocation of Fe in rice has not been established.

4.5 CONCLUSIONS

The first experiment implied that As might induce Fe-chlorosis in the fully developed young leaves of rice grown in citrate-Fe^{3+} containing medium. Reduced Fe concentration in the young leaves and reduction of Fe translocation from roots to shoots might be the most vital factors for the induction of whitish chlorosis. The second experiment confirmed the result of the first experiment. The experiment with radioactive ^{59}Fe confirmed that even Fe chelator PS failed to carry ^{59}Fe from roots to shoots of rice in higher As treated condition. Our result demonstrated that As-induced chlorosis in rice was due to Fe translocation and absorption problem, but translocation of Fe was more damaged by As.

CHAPTER 5

ALLEVIATION OF ARSENIC-INDUCED CHLOROSIS WITH ADDITIONAL EDTA-Fe^{3+} IN HYDROPONIC RICE

ABSTRACT

A hydroponic experiment was carried out in the greenhouse to investigate the responses of rice (*Oryza sativa* L. cv. Akitakomachi) seedlings at elevated concentrations of arsenic (As; sodium meta-arsenite, $NaAsO_2$) in presence or absence of additional EDTA-Fe^{3+} (ethylene diamine tetraacetic acid-Fe^{3+}). The main objectives of this experiment were to prove that As-induced chlorosis was Fe-chlorosis and to observe physiological and mineralogical properties of As-induced Fe-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+}) for 14 days. Arsenic-induced Fe-chlorosis was more pronounced in the fully developed young leaves of As-treated plants. Chlorophyll index and iron (Fe) concentrations decreased in the shoots of As-treated plants as compared to control plants. However, chlorosis disappeared and Fe concentration was elevated in high-Fe^{3+} plants as compared to As-treated and medium-Fe^{3+} plants, confirming that As-induced chlorosis was Fe-chlorosis caused by Fe-deficiency. Dry weight (DW) decreased in As-treated plants as compared to control plants, but increased in high-Fe^{3+} plants, indicating that As-toxicity at 13.4 μM level largely depended on the concentration of EDTA-Fe^{3+} in the medium.

Abbreviations: CTL (critical toxicity level); CDL (critical deficient level); DAT (days after treatments); DW (dry weight); EDTA-Fe^{3+} (ethylene diamine tetraacetic acid-Fe^{3+}).

5.1 INTRODUCTION

Iron compounds are used to reduce the availability of arsenic (As) in As contaminated soil (Hartley et al. 2004). Iron oxide surfaces are known to be involved in As adsorption from soils (Elbassam et al. 1975; Jacobs et al. 1970). Arsenic adsorption effectivity is not same for all Fe materials e.g. the efficiency of As adsorption was as Fe^{3+}>Fe^{2+}>iron grit>goethite>lime (Hartley et al. 2004). This is because As has high affinity for oxidic surfaces, preferentially attaching itself from the soil solution to Fe oxides (Akins and Lewis 1976; Wauchope 1975). Arsenic adsorption is also brought about due to charges that exist on the Fe-oxide surfaces (Parfait 1980). Ferrous sulphate (Artiola et al. 1990) has an extremely high adsorptive capacity for As (Vangronsveld et al. 1994).

Experimental data of As-toxic plants are available e.g.- rice (Marin et al. 1993; Abedin et al. 2002; Imamul-Huq et al. 2007; Rahman et al. 2007), sorghum and barley (Shaibur et al. 2008ab), ornamental arum (Imamul-Huq et al. 2005), fern (Ma et al. 2001; Zhao et al. 2002; Meharg 2003) and mung bean (Singh et al. 2007). In our primary experiment, we found that sodium meta-arsenite ($NaAsO_2$) at 13.4 and 26.8 μM level induced whitish chlorosis in the fully developed young leaves of Akitakomachi rice variety when the nutrient solution contained EDTA-Fe^{3+}. So far we know, there is no information about the alleviation of As-toxicity with additional EDTA-Fe^{3+} in hydroponic rice. The main objectives of this experiment were to prove that As-induced chlorosis was Fe-chlorosis and to observe physiological and mineralogical properties of As-induced Fe-chlorosis in rice.

5.2 MATERIALS AND METHODS

5.2.1 Seed Germination and Plant Culture

Seed germination and plant culture has been described in section 2.3.1 of **CHAPTER 2**. Duration of the experiment was from 24 June to 3 August 2008. 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 greenhouse.

5.2.2 Environmental Condition

The experiment was 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.

5.2.3 Sample Preparation and Analysis

Sample preparation and analysis has been described in section 2.7 of **CHAPTER 2**. Additionally, the young leaves were separated from the old leaves to analyze the concentration of macro and micronutrients. This was done to find out which element in the young leaves was the most affected by the treatments, because the chlorosis was found in the young leaves.

5.2.4 Determination of Arsenic

Arsenic was measured by using Hydride Generation Atomic Absorption Flame Emission Spectrophotometer (HGAAFES, AA-6200; Shimadzu Corporation, Kyoto, Japan). The volume of the primary digested solution was around 5 mL which was made at 50 mL with MQ water (18.2 MΩ cm^{-1}, and purified by Milli-RO 60, Millipore Corporation, USA). Solutions were further diluted up to 100-2000 times to determine As.

5.2.5 Other Parameters

Determination of chlorophyll indices, calculation for the parameters and statistical analysis were described elsewhere (Shaibur et al. 2006).

5.3 RESULTS

5.3.1Visible Symptoms

Dew like water drops appeared in the leaf tip of control and high EDTA-Fe^{3+} treated plants in the evening and in the early morning. However, it was absent in As-treated and medium-Fe^{3+} plants. Control plants were the greenest **(Fig. 5.1)**. Plants showed whitish chlorosis in the fully developed young leaves of As-treated and medium-Fe^{3+} seedlings at 10 days of As treatment, in which the chlorosis was more pronounced in As-treated plants **(Fig. 5.1)**. Chlorosis disappeared almost completely at high-Fe^{3+} treatment and the plants were almost similar to the control in color **(Fig. 5.1).** Chlorosis was clearer in the warmer season (June-August, 2008) in Morioka, Iwate, Japan. At sunlight, seedlings were curled and showed water deficiency symptom in As-treated plants. Turgidity decreased in As-treated plants as compared to control plants with increasing intensity of sunlight.

Roots of As treated plants were white up to 72 h and after that the reddish color appeared. Iron-plaques were visible clearly as the reddish coating on the root surface of rice seedlings in As-treated, medium-Fe^{3+} and high-Fe^{3+} treatments. Roots also contained algae like green plaque on its surface.

5.3.2 Chlorophyll Index (SPAD value)

After exposure to As-toxicity, chlorophyll index decreased in As-treated seedlings as compared to control **(Fig. 5.2a)**. Additional EDTA-Fe^{3+} (medium-Fe^{3+} or high-Fe^{3+}) seemed to

increase the chlorophyll index as compared to As-treated plants but was still lower than control plants **(Fig. 5.2a)**.

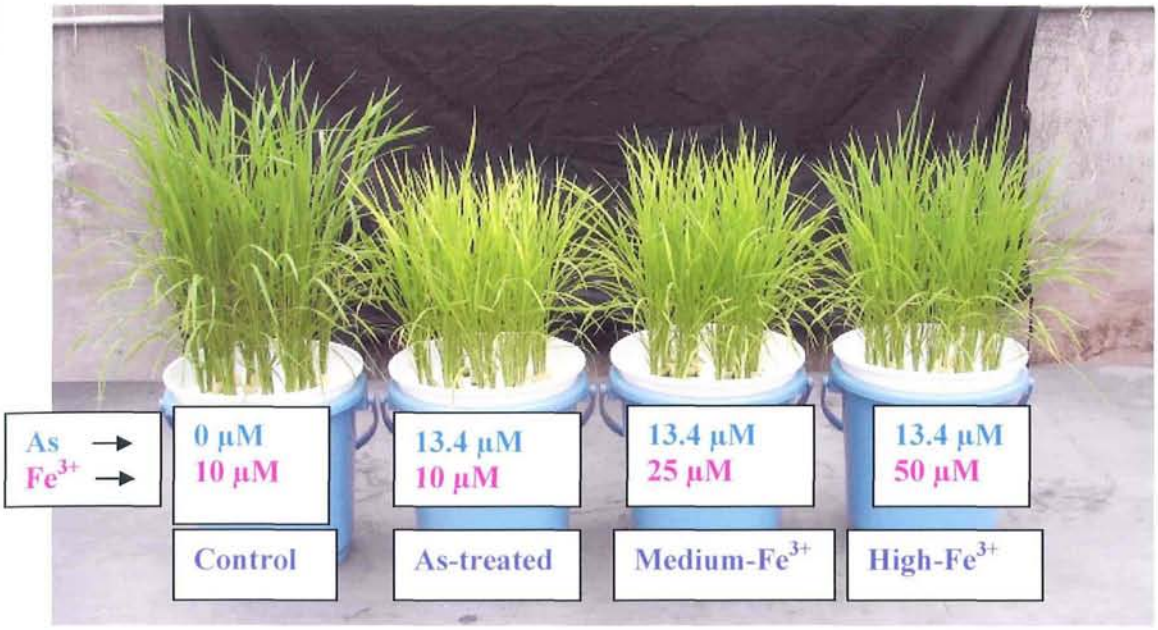


Figure 5.1 Photograph of rice seedlings at elevated concentration of As and in presence of additional EDTA-Fe^{3+}. Arsenic induced conspicuous whitish chlorosis in the young leaves at 13.4 µM level. Whitish chlorosis disappeared partially at medium-Fe^{3+} and almost completely at high-Fe^{3+} treatment. This picture was taken after 14 days of As exposure.

5.3.3 Leaf Number, Width of Leaf Blade and Tiller Number

After exposure to As-toxicity at 13.4 µM level, leaf number and width of leaf blade decreased as compared to control **(Figs. 5.2bc)**. Leaf number increased in high-Fe^{3+} plants as compared to As-treated and medium-Fe^{3+} plants, however, the width of leaf blade was not much affected by high-Fe^{3+} treatment **(Figs. 5.2bc)**. In this experiment any new tiller was not observed even in control plants.

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

Dry weight was lower in shoots of As-treated and medium-Fe^{3+} plants as compared to control. However, DW increased in high-Fe^{3+} plants as compared to As-treated and medium-Fe^{3+} plants **(Fig. 5.3a)**. Similar trends were also found in root DW and shoot height **(Figs. 5.3ab)**. Root length was not much affected by the treatments **(Fig. 5.3b)**.

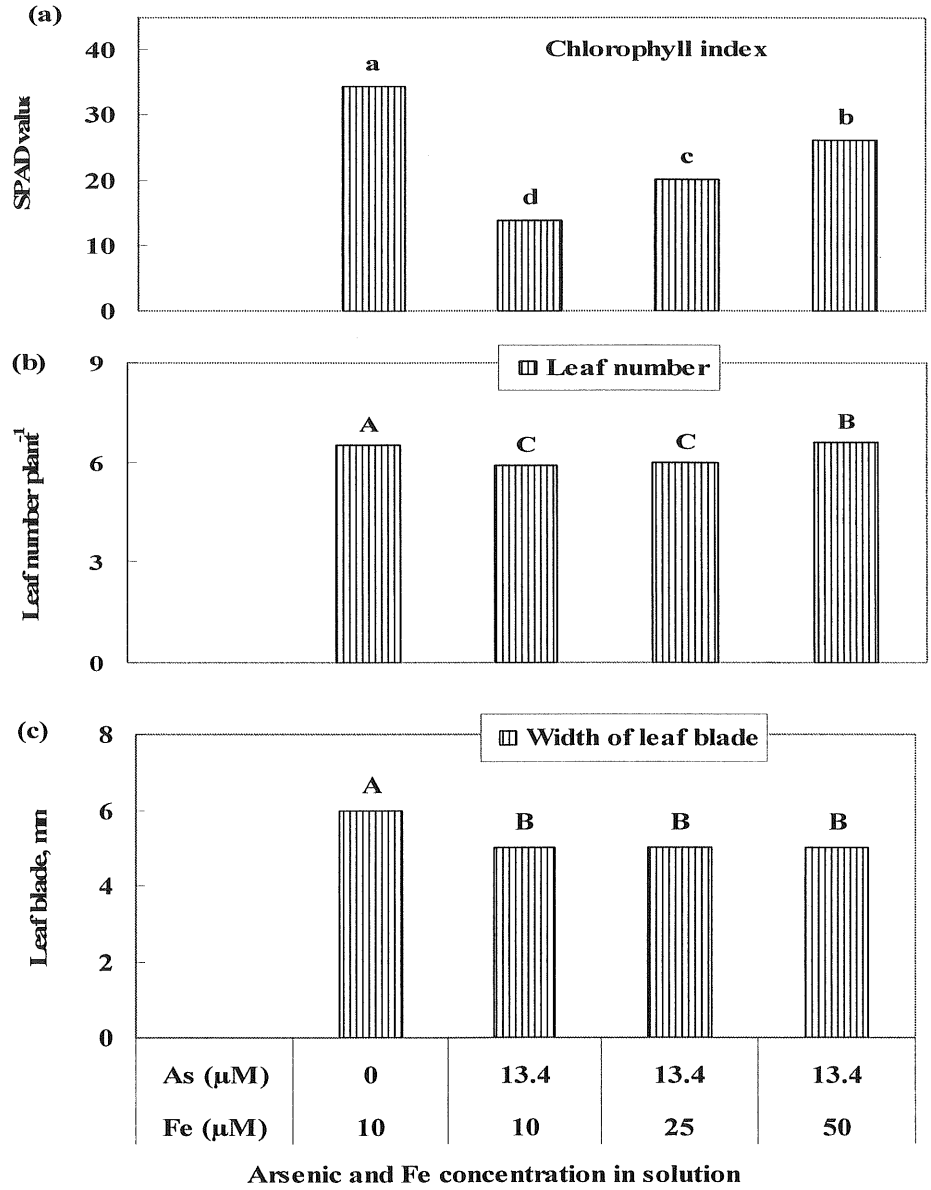


Figure 5.2 (a) SPAD value, (b) leaf number and (c) width of leaf blade of rice seedlings at elevated concentration of As and or in presence of additional EDTA-Fe^{3+}. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

5.3.5 Macro and Micronutrients

Arsenic increased P concentration but decreased Mg concentration in shoots as compared to control **(Table 5.1)**. Phosphorus concentration was similar in the shoots of

As-treated, medium-Fe^{3+} and high-Fe^{3+} plants. Potassium concentration was not much affected in shoots with the treatments. Phosphorus concentration decreased in roots of high-Fe^{3+} plants as compared to the others **(Table 5.1)**. Phosphorus accumulation increased in shoots but Ca and Mg accumulation decreased in shoots of high-Fe^{3+} plants as compared to the others **(Table 5.1)**.

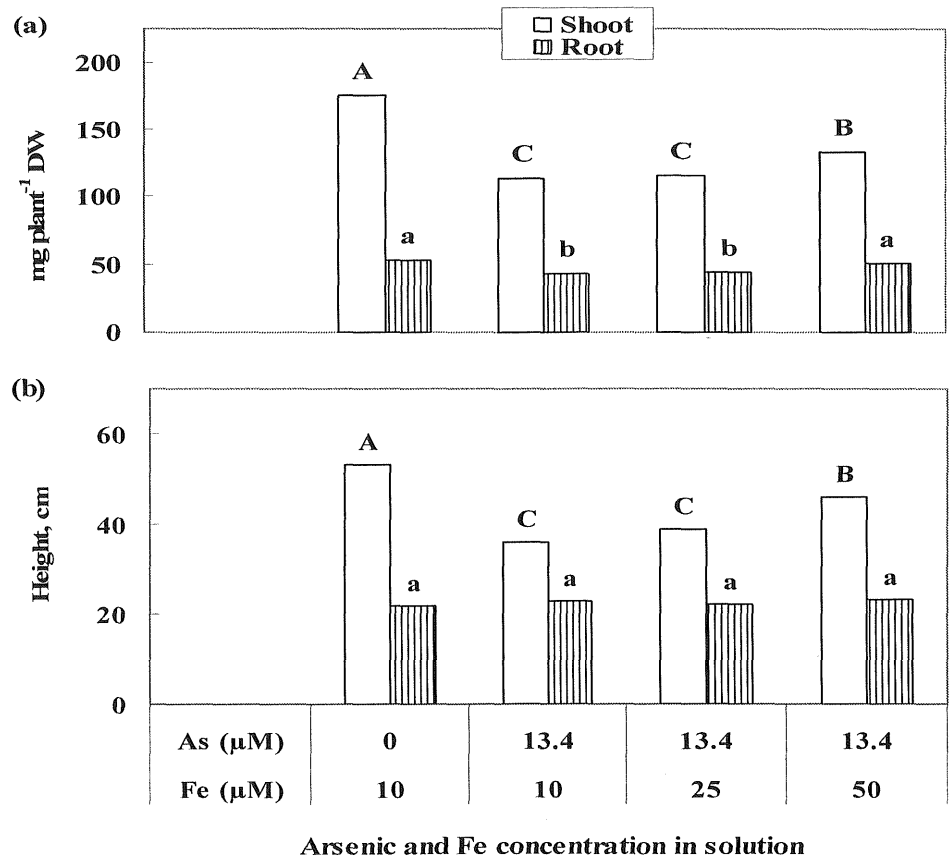


Figure 5.3 (a) Dry weight, DW and (b) shoot height and root length of rice seedlings at elevated concentration of As and or in presence of additional EDTA-Fe^{3+}. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test.

Translocations of macro elements were not much affected with the treatments **(Table 5.1)**. Arsenic increased P concentrations in the young leaves, though K concentrations were not much affected in the young leaves **(Table 5.2)**. In general, P concentration was the most affected among the macro elements with the treatments **(Tables 5.1 & 5.2)**.

Table 5.1 Concentrations, accumulations and translocations of elements in shoots and roots of rice seedlings grown in different treatments of As and EDTA-Fe^{3+}.

Treatments (μM)		P	K	Ca	Mg	Fe	Mn	Zn	Cu
		----------mg g^{-1} DW ----------				----------μg g^{-1} DW ----------			
As	EDTA- Fe^{3+}	Concentrations in shoots							
0	10	8.15b	43.4a	2.06a	3.35a	71.6a	735.7a	54.7a	22.0a
13.4	10	14.3a	46.3a	1.96ab	2.90b	40.6c	651.3b	55.5a	25.0a
13.4	25	13.6a	44.9a	1.81b	2.76b	50.4b	592.7b	48.1a	24.6a
13.4	50	13.4a	46.4a	1.80b	2.78b	66.9a	627.4b	47.4a	23.7a
		Concentrations in roots							
0	10	4.70a	33.0a	0.47b	1.23a	282.4c	80.4b	43.9c	241.5a
13.4	10	4.60a	23.4c	0.53a	1.08b	422.8b	136.0a	62.4a	128.7b
13.4	25	5.20a	24.4c	0.50ab	0.95b	455.4b	99.2ab	50.3bc	105.9bc
13.4	50	3.75b	27.5b	0.53a	1.04b	771.6a	105.5a	46.5c	88.4c
		----------mg $plant^{-1}$ ----------				----------μg $plant^{-1}$----------			
		Accumulation in shoots							
0	10	1.42b	7.59a	0.36a	0.59a	12.5a	129.1a	9.63a	3.86a
13.4	10	1.62b	5.26c	0.22b	0.33b	4.60d	74.1c	6.30b	2.84c
13.4	25	1.58b	5.22c	0.21b	0.32b	5.86c	68.9c	5.59b	2.86c
13.4	50	1.79a	6.19b	0.24b	0.37b	8.91b	83.5b	6.30b	3.16b
		Accumulation in roots							
0	10	0.25a	1.74a	0.025b	0.06a	15.0c	4.27b	2.35ab	11.3a
13.4	10	0.20b	0.99c	0.022c	0.05ab	17.9bc	5.75a	2.65a	5.45b
13.4	25	0.22b	1.07c	0.022c	0.04b	20.1b	4.40b	2.21b	4.69b
13.4	50	0.19b	1.39b	0.027a	0.05ab	39.0a	5.33a	2.35ab	4.47b
		Translocation (%)							
0	10	85.1a	81.3a	93.6a	90.0a	45.7a	96.8a	80.5a	25.4c
13.4	10	89.2a	84.2a	90.9a	87.8a	20.5b	92.8a	70.5b	34.2b
13.4	25	87.8a	82.9a	90.4a	88.5a	22.6b	94.0a	71.6b	38.0ab
13.4	50	90.4a	81.6a	89.9a	87.5a	18.6b	94.0a	72.9b	41.4a

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. 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.

Table 5.2 Concentrations of elements in young and old leaves of rice seedlings grown in different treatments of As and EDTA-Fe^{3+}.

Treatments (μM)		----------mg g^{-1} DW ----------				----------μg g^{-1} DW ----------			
		P	K	Ca	Mg	Fe	Mn	Zn	Cu
As	EDTA-Fe^{3+}	Concentrations in young leaves							
0	10	6.56b	38.7a	1.72a	2.60a	87.2a	358.5b	29.6a	24.8a
13.4	10	15.6a	43.5a	1.23b	2.29b	37.7d	453.6a	23.3b	28.6a
13.4	25	15.0a	39.1a	1.16bc	2.26b	47.6c	466.9a	20.3b	29.4a
13.4	50	12.7a	41.8a	0.96c	2.01b	69.5b	385.4b	20.8b	25.9a
		Concentrations in old leaves							
0	10	10.4b	25.9b	5.29a	5.63a	106.3a	1796.3a	51.6a	26.4b
13.4	10	17.2a	34.4a	3.27c	4.34b	60.1bc	853.0b	46.3b	31.1a
13.4	25	18.5a	34.5a	4.01b	5.50a	58.2c	987.7b	43.4b	35.3a
13.4	50	16.7a	33.8a	3.42c	4.17b	68.3b	831.9b	33.9c	30.1ab

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.

Arsenic decreased Fe concentrations in shoots but increased in roots, resulting in lower translocations **(Table 5.1)**. Iron concentration increased in shoots of medium-Fe^{3+} and high-Fe^{3+} plants as compared to As-treated plants. Iron concentrations were higher in roots of As-treated plants as compared to control **(Table 5.1)**, which increased further in roots of medium-Fe^{3+} and high-Fe^{3+} treated plants as compared to control and As-treated plants **(Table 5.1)**. Manganese concentration was higher in shoots of control plants as compared to the others and were similar in shoots of As-treated, medium-Fe^{3+} and high-Fe^{3+} treated plants **(Table 5.1)**. Zinc and Cu concentrations were not much affected in the shoots with treatments. Most of the cases, high-Fe^{3+} failed to accumulate higher nutrient contents in shoots and roots.

5.3.6 Arsenic

Arsenic concentration decreased in shoots of medium-Fe^{3+} and high-Fe^{3+} plants as compared to As-treated plants but the As concentration in roots was not much affected **(Table 5.3)**. Arsenic concentrations in the old leaves were higher as compared to the young leaves in all the treatments **(Table 5.3)**. Accumulations and translocations were also not much affected with the treatments **(Table 5.3)**.

5.4 DISCUSSION

Arsenic-treated plants showed chlorosis in the fully developed young leaves **(Fig. 5.1)**. Green color in old leaves of As-treated plants was not as pronounced as in control plants, suggesting that the deep green of the old leaves in As-treated plants was turned into mild green with As. It is well known that As first attacks in the old leaves compared to the young leaves. Chlorosis induced in As-treated (13.4 μM) plants was slightly disappeared in medium-Fe^{3+} plants but was disappeared almost completely in the high EDTA-Fe^{3+}, indicating that As-induced chlorosis was Fe-chlorosis. Shenker and Chen (2005) noted that no ultimate practice was available for completely overcoming Fe-deficiency. If the chlorosis of the young leaves is partially disappeared by the additional Fe, then the chlorosis is considered as Fe-chlorosis (Shenker and Chen 2005). Enhancement of chlorophyll index was taken place in additional-Fe^{3+} plants as compared to As-treated plants, confirming that As-induced chlorosis was Fe-chlorosis. Arsenic is known to have several phytotoxic effects and one of them is the reduction in the chlorophyll index (Shaibur et al. 2006; Shaibur et al. 2008a; Shaibur et al. 2009b). In spite of slight decreasing chlorophyll indices the Kalmi does not show whitish chlorosis in the leaf tissues (Shaibur et al. 2009b). Determination of chlorophyll content was often accomplished to assess the impact of most environmental stresses, as pigment content was linked to the visual symptoms and photosynthetic plant productivity (Jain and Gadre 1997).

Decrease of turgidity was most probably due to the fact that As might depress water movement from roots to shoots (Yamane 1989; Shaibur et al. 2006). The explanation of reddish color in roots has been given in previous chapters. Greening of the roots was most probably due to the fact that the pots were a little bit light transparent, resulting in the formation of algae plaque.

Chlorophyll index, leaf number, shoot and root DW and shoot height increased in high-Fe^{3+} plants as compared to As-treated plants, indicating that additional-Fe^{3+} might reduce As-toxicity at high concentration. Enhancement of chlorophyll index, leaf number and shoot height were most probably responsible for the enhancement of shoot DW in high-Fe^{3+} plants. On the contrary, reduction of these physiological parameters was mostly responsible for the reduction of DW in the As-treated plants. Our physiological data showed that As-toxicity at 13.4 μM level was very much dependent on the EDTA-Fe^{3+} concentration in the nutrient

solution. The higher was the EDTA-Fe^{3+} concentration in the solution, the lower was the As-toxicity.

Table 5.3 Concentrations, accumulations and translocation of As in plant parts of rice seedlings grown in different treatments of As and EDTA-Fe^{3+}.

Treatments (μM)		----------As (μg g^{-1} DW)----------				----As (μg $plant^{-1}$)---		Translocation
As	EDTA- Fe^{3+}	Shoot	Root	Young Leaves	Old Leaves	Shoot	Root	(%)
0	10	nd	nd	nd	nd	nd	nd	nd
13.4	10	39.9a	470.9a	21.1b	41.5a	4.53a	20.2a	19.1a
13.4	25	31.5b	358.1b	26.4a	43.9a	3.66a	15.9a	18.9a
13.4	50	32.5b	444.5a	15.7c	43.4a	4.33a	22.6a	16.8a

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 As in shoot to the total accumulation (shoot + root). The translocation was expressed in %. DW = dry weight. nd = not detected.

Shoot DW decreased by 35.1, 34.1 and 23.8% and root DW by 19.7, 15.9 and 4.34% in As-treated, medium-Fe^{3+} and high- Fe^{3+} treatments as compared to control. The DW increased by 1.58 and 17.4% in shoots; and 4.71 and 19.1% in roots in medium-Fe^{3+} and high-Fe^{3+} treatments, respectively as compared to As-treated plants, indicating that As-toxicity decreased with increasing Fe concentration in the medium. Similar to the DW, shoot height increased by 8.33 and 27.8% in medium-Fe^{3+} and high-Fe^{3+} treatments, respectively as compared to As-treated plants.

In shoots, Fe concentration was 40.6 μg g^{-1} DW in As-treated plants and this lower Fe concentration was mostly responsible for the induction of whitish chlorosis in the fully developed young leaves. In this case, the whole shoots (stems, old leaves and young leaves) were digested together and the Fe concentration was measured. Among the macro and micronutrients, Fe concentration in the shoots and in the young leaves was the most decreased **(Tables 5.1 & 5.2)**. Iron concentrations were 87.2, 37.7, 47.6 and 69.5 μg g^{-1} DW in young leaves of control, As-treated, medium-Fe^{3+} and high-Fe^{3+} plants, respectively **(Table 5.2)**. The lowest concentration of Fe (37.7 μg g^{-1} DW) in the chlorotic young leaves was the most

responsible factor for the induction of whitish chlorosis. Our data showed that the old leaves contained comparatively high concentration of Fe **(Table 5.2)** as compared to young leaves and kept the old leaves green **(Fig. 5.1)**. Concentrations ratios (%) of Fe:P in shoots were 0.88, 0.28, 0.37 and 0.50 **(from Table 5.1)**. Similarly, the concentrations ratios (%) Fe:P were 1.33, 0.24, 0.31 and 0.54 in the young leaves **(from Table 5.2)**. It meant that Fe concentrations decreased much more than the P concentrations both in shoots and in young leaves. The higher reduction of Fe concentrations was mostly responsible for the induction of whitish chlorosis in the young leaves **(Fig. 5.1)** and lower chlorophyll index **(Fig. 5.2a)**. It was reported that the lower was the Fe:P values in the shoot tissues, the lower was the chlorophyll index (Dekock and Alexander 1955; Pushnik et al. 1984; Ladouceur et al. 2006; Shaibur et al. 2008c; Shaibur et al. 2009a).

Arsenic decreased Fe concentrations both in shoots and in young leaves which was within the CDL (30-50 μg Fe g^{-1} DW; Bergmann 1988), resulting in whitish chlorosis in the fully developed young leaves. Additional EDTA-Fe^{3+} increased Fe concentration in the shoots as well as in the young leaves as compared to As-treated plants **(Tables 5.1 & 5.2)**, resulting in increased chlorophyll index **(Fig. 5.2a)** and disappearing the chlorosis of the young leaves **(Fig. 5.1)**. Our result confirmed that As-induced chlorosis was Fe-chlorosis caused by Fe-deficiency. These findings have not been reported yet. In this current experiment, additional EDTA-Fe^{3+} did not show any toxic effect on rice growth as the concentration of Fe in the shoots was almost similar to the normal concentration. The normal concentrations of Fe in the leaf tissues are 50-100 μg g^{-1} DW (Mengel and Kirkby 2001) and concentration >500 μg Fe g^{-1} DW in leaf tissues is considered to be the toxic for plant growth (Marschner 1998). Previous chapter showed that additional citrate-Fe^{3+} can not overcome chlorosis induced by As. Further research needs to be done to find out the reason why additional EDTA-Fe^{3+} could recover chlorosis induced by As but citrate-Fe^{3+} could not.

Manganese concentration was lower in the shoots of As-treated, medium-Fe^{3+} and high-Fe^{3+} plants as compared to control **(Table 5.1)**. However, the Mn concentration was higher in the chlorotic young leaves of As-treated and medium-Fe^{3+} plants as compared to the green young leaves of control and high-Fe^{3+} plants **(Table 5.3)**. The higher Mn concentration in the young leaves might also be involved for the induction of chlorosis. Our result showed that the concentration of P, Fe and Mn were affected much compared to other elements, in which Fe concentration, accumulation and translocation was the most affected **(Tables 5.1 & 5.2)**.

Arsenic concentration decreased in the shoots significantly in medium-Fe^{3+} and high-Fe^{3+} plants as compared to As-treated plants, though this decrease was not much affected **(Table 5.3)**. Arsenic concentrations in the young leaves were 21.1, 26.4 and 15.7 μg g^{-1} DW in As-treated, medium-Fe^{3+} and high-Fe^{3+} plants, respectively **(Table 5.3)**. However, the values were 41.5, 43.9 and 43.4 μg g^{-1} DW in the old leaves, respectively, indicating that old leaves contained high concentration of As as compared to young leaves. Arsenic concentration was not affected in roots also by the additional-Fe^{3+}, suggesting that additional EDTA-Fe^{3+} may not be effective to reduce much amount of As in root tissues. Accumulation and translocations were not much affected with the treatments, therefore, could be said that additional EDTA-Fe^{3+} might not be very effective to reduce As concentration and accumulation in hydroponic rice.

Root concentrated almost 11.8, 11.4 and 13.7 times higher As concentrations as compared to shoots. This was because As has high affinity to react with sulfhydryl group of protein in root (Speer 1973) and Fe^{3+} has high affinity to As absorption (Hartley et al. 2004). It is considered that, at high EDTA-Fe^{3+} concentration, root absorbs higher As together with high EDTA-Fe^{3+}, resulting in higher Fe and As in roots. Probably, in this way As blocks Fe translocation from roots to shoots and mostly concentrated in roots. The current data showed that Fe and As had a positive relationships in roots but the negative relationship in shoots.

5.5 CONCLUSIONS

It could be concluded that As might induce Fe-chlorosis in the fully expanded young leaves of rice grown in EDTA-Fe^{3+} containing medium. We found that the chlorosis in the young leaves was disappeared partially by the additional-Fe^{3+}, confirming that As-induced chlorosis was Fe-chlorosis caused by Fe-deficiency. This result also indicated that As-toxicity largely depended on the EDTA-Fe^{3+} concentration in the hydroponic medium regarding growth enhancement.

CHAPTER 6

EFFECTIVITY OF CITRATE-Fe^{3+} AND EDTA-Fe^{3+} TO AMELIORATE ARSENIC-INDUCED IRON-CHLOROSIS IN HYDROPONIC RICE

ABSTRACT

We showed that arsenic (As) could induce whitish chlorosis in the fully developed young leaves of rice (*Oryza sativa* L. cv. Akitakomachi) seedlings at 13.4 and 26.8 μM level, containing 10 μM citrate-Fe^{3+} or EDTA-Fe^{3+} in the medium. We also showed that the As-induced chlorosis could not be ameliorated with additional citrate-Fe^{3+}, but it could be ameliorated with additional EDTA-Fe^{3+}. In order to clarify the effectivity of citrate-Fe^{3+} and EDTA-Fe^{3+} to ameliorate As-induced chlorosis, we did this succeeding experiment. 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+}). Whitish chlorosis was found in the fully developed young leaves of As-treated plants. Additional citrate-Fe^{3+} could not recover the whitish chlorosis. However, additional EDTA-Fe^{3+} recovered the chlorosis almost completely, indicating that the effectivity of EDTA-Fe^{3+} was much more pronounced compared to citrate-Fe^{3+} to ameliorate As-induced chlorosis. The EDTA-Fe^{3+} treated plants were greener as compared to additional citrate-Fe^{3+} plants. Iron concentration in the shoots of additional EDTA-Fe^{3+} plants was much higher as compared to additional citrate-Fe^{3+} plants, suggesting that EDTA-Fe^{3+} might be translocated easily to the shoots as compared to citrate-Fe^{3+}. Leaf number, width of leaf blade, tiller number, dry weight (DW) and shoot height were decreased in As-treated plants as compared to control. Additional citrate-Fe^{3+} and additional EDTA-Fe^{3+} plants partially recovered leaf number, DW and shoot height. Arsenic concentration seemed to be decreased in additional citrate-Fe^{3+} and EDTA-Fe^{3+} plants where EDTA-Fe^{3+} was more effective than the citrate-Fe^{3+}. Our result showed that As-toxicity was largely dependent on the concentration of Fe in the medium.

Abbreviations: CTL (critical toxicity level); CDL (critical deficient level); DAT (days after treatments); DW (dry weight); EDTA-Fe^{3+} (ethylene diamine tetraacetic acid-Fe^{3+}).

6.1 INTRODUCTION

In the area where As contaminated groundwater was applied for rice cultivation in Bangladesh, As concentration was found to be from 3.2 to 27.5 mg kg^{-1} dry soil in the top 75-150 mm. However, the values were from 0.10 to 2.75 mg kg^{-1} dry soil if receiving irrigation

water without As contamination (Ali et al. 2003). Total concentrations of determined trace elements were much lower in calcareous soils of Bangladesh except As (Jahiruddin et al. 2000). Arsenic concentration was higher than the maximum acceptable limit for agricultural soils (Jahiruddin et al. 2000). The allowable limit of As in soil is proposed as 20 mg kg^{-1} dry soil for crop production (Kabata-Pendias and Pendias 1992). Generally, surface soils (0-15 cm) contained higher concentration of As than the subsurface soils (15-30 cm; Huq et al. 2003). Vegetables grown on As contaminated soils contained higher content of As as compared to plants grown on uncontaminated soils (Farid et al. 2003). Plants show As-toxicity symptom when it is grown in As contaminated condition.

Owing to the known visible toxicity symptoms of As in hydroponic rice and the discovery of the reason of whitish chlorosis in the young leaves, As-Fe interactions has had highly impressive interest to the academic community. Therefore, it is now necessary to find out if different Fe sources have different capacity to ameliorate As-induced chlorosis in hydroponic rice. It is why three succeeding experiments (**CHAPTER 3**; **CHAPTER 4** and **CHAPTER 5**) were conducted. Firstly, we found that As-induced whitish chlorosis in the young leaves of rice in presence of citrate-Fe^{3+} and EDTA-Fe^{3+}. Arsenic-induced chlorosis could not be ameliorated with additional citrate-Fe^{3+}. However, additional EDTA-Fe^{3+} could recover As-induced chlorosis. The present experiment was conducted to verify the effectivity of additional citrate-Fe^{3+} and EDTA-Fe^{3+} to ameliorate As-induced chlorosis.

6.2 MATERIALS AND METHODS

6.2.1 Seed Germination and Plant Culture

Seedlings of rice (*Oryza sativa* cv. Akitakomachi) were grown as described in section 2.3.1 of **CHAPTER 2**. Before transplantation, the seedlings were washed with RO water. 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+}) for 14 days.

6.2.2 Environmental Condition

Temperature fluctuation was around 22 to 32°C. The duration of the experiment was 2005.08.25 to 2005.09.29.

6.2.3 Chlorophyll Index (SPAD value)

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

6.2.4 Sample Preparation and Analysis

Plants were harvested on 14 DAT. Sample preparations and analysis has been described in section 2.7 of **CHAPTER 2**.

Figure 6.1 Photograph of rice seedlings at elevated concentration of As in presence of additional citrate-Fe^{3+} or EDTA-Fe^{3+}. Arsenic induced chlorosis was effectively be alleviated with additional EDTA-Fe^{3+} but additional citrate-Fe^{3+} failed to alleviate As-induced chlorosis. This photograph was taken after 14 days of As exposure.

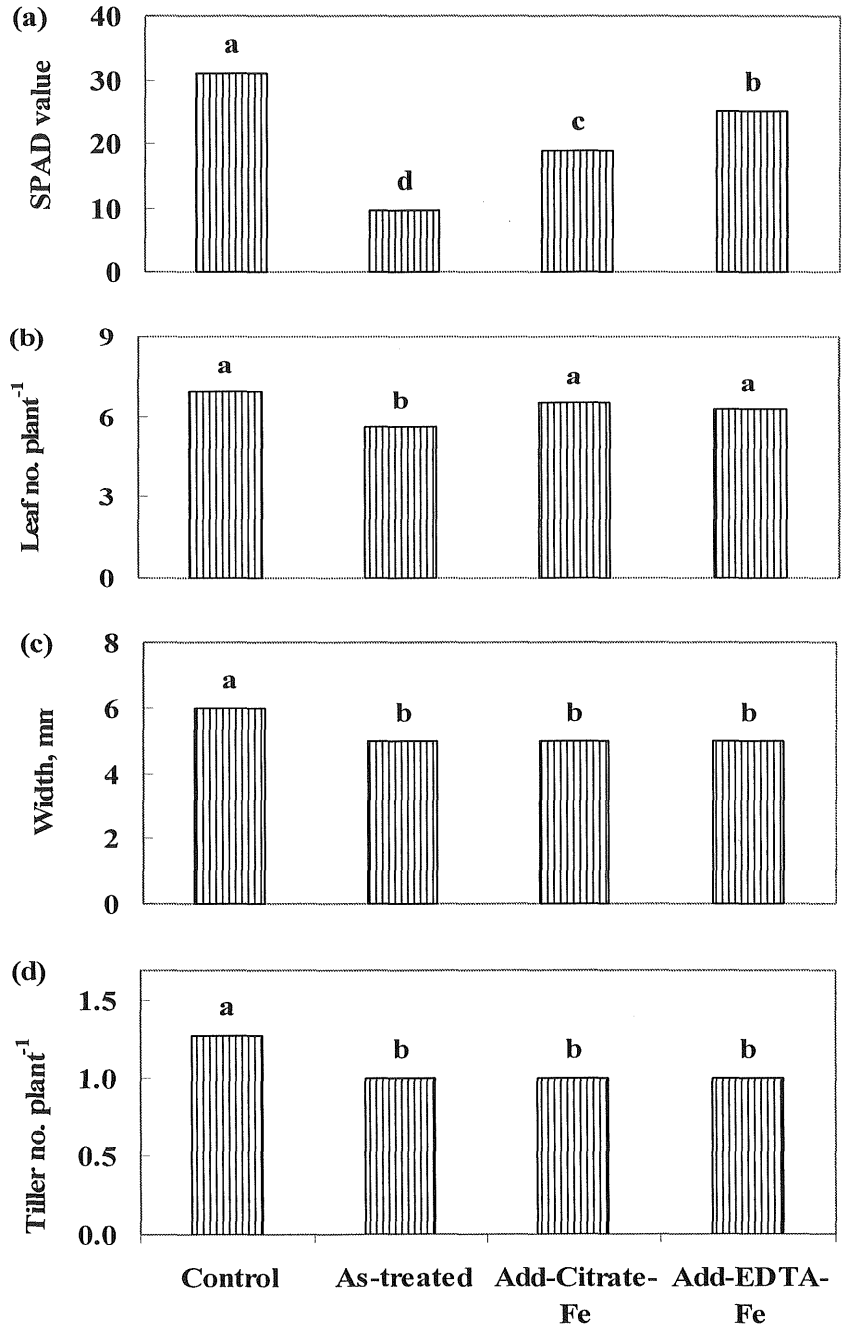


Figure 6.2 (a) Chlorophyll index (SPAD value), (b) leaf number, (c) width of leaf blade and (d) tiller number of rice seedlings at elevated concentration of As in presence of additional citrate-Fe^{3+} or EDTA-Fe^{3+}. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test. Add-Citrate-Fe = additional citrate-Fe^{3+}, Add-EDTA-Fe = additional EDTA-Fe^{3+}.

6.3 RESULTS

6.3.1 Visible Symptoms

Interveinal and whitish chlorosis was observed in the fully developed youngest leaves in As-treated seedlings. Leaves of As-treated plants showed lack of turgidity within 12 h of As application. Fully developed youngest leaves showed whitish chlorosis in As-treated plants.

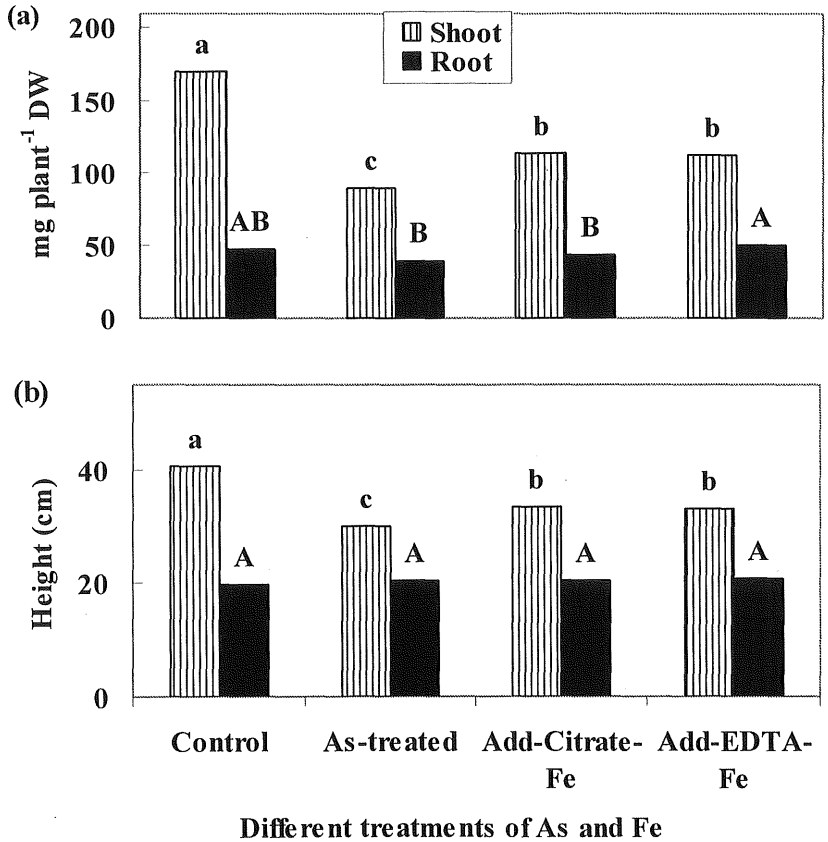


Figure 6.3 (a) Dry weight, DW and (b) shoot height and root length of rice seedlings at elevated concentration of As in presence of additional citrate-Fe^{3+} or EDTA-Fe^{3+}. Bars with different letters are significantly different ($p < 0.05$) according to a Ryan-Einot-Gabriel-Welsch multiple range test. Add-Citrate-Fe = additional citrate-Fe^{3+}, Add-EDTA-Fe = additional EDTA-Fe^{3+}.

Additional citrate-Fe^{3+} did not have conspicuous effect on the alleviation of chlorosis, however, additional EDTA-Fe^{3+} alleviated chlorosis almost completely **(Fig. 6.1)**. Visible growth seemed to be the higher in additional citrate-Fe^{3+} and additional EDTA-Fe^{3+} as

compared to the As-treated plants **(Fig. 6.1)**. Reddish brown color was observed in the roots of As-treated plants as compared to control plants.

Table 6.1 Concentrations, accumulations and translocations of elements in shoots and roots of rice seedlings grown in different treatments of As, citrate or EDTA-Fe^{3+}.

Treatments	P	K	Ca	Mg	Fe	Mn	Zn	Cu
	----------mg g^{-1} DW ----------				----------µg g^{-1} DW ----------			
				Concentrations in shoots				
Control	8.66b	49.9a	2.30a	2.70a	69.0b	822a	120.1a	29.4a
As-treated	16.2a	48.3a	1.93b	2.25b	45.7d	440c	71.8b	23.8b
Add-Citrate-Fe	6.90c	48.3a	1.99b	2.44ab	58.5c	662b	75.2b	26.4ab
Add-EDTA-Fe	3.25d	43.2a	1.79b	2.27b	87.1a	594b	66.9b	21.8b
				Concentrations in roots				
Control	4.71b	46.5a	0.36b	1.65a	1152c	128c	89.0b	240a
As-treated	3.39c	33.0c	0.41a	1.07b	1401b	254b	129.9a	204a
Add-Citrate-Fe	6.18a	38.4b	0.47a	1.11b	3470a	335a	130.7a	233a
Add-EDTA-Fe	5.38b	39.9b	0.46a	1.26b	3004a	367a	110.2a	83.0b
	----------mg $plant^{-1}$ ----------				----------µg $plant^{-1}$----------			
				Accumulation in shoots				
Control	1.41a	8.49a	0.39a	0.46a	11.7a	139.7a	20.4a	4.99a
As-treated	1.44a	4.28b	0.17c	0.20c	4.06c	39.0c	6.37b	2.11b
Add-Citrate-Fe	0.78b	5.43b	0.22b	0.27b	6.58b	74.5b	8.48b	2.97b
Add-EDTA-Fe	0.36c	4.83b	0.20bc	0.25b	9.77a	66.6b	7.48b	2.44b
				Accumulation in roots				
Control	0.22b	2.17a	0.017b	0.078a	53.6b	5.98c	4.17b	11.2a
As-treated	0.13c	1.27d	0.016b	0.041b	54.0b	9.81b	5.02a	7.83b
Add-Citrate-Fe	0.27a	1.68c	0.020ab	0.048b	151.4a	14.6a	5.71a	10.1a
Add-EDTA-Fe	0.27a	1.97b	0.023a	0.062a	148.2a	18.1a	5.44a	4.07c
				Translocation (%)				
Control	87.0a	79.7a	95.9a	85.6a	18.0a	95.8a	83.0a	30.9b
As-treated	91.6a	77.1a	91.5a	82.8a	7.05b	79.9b	56.0b	21.3c
Add-Citrate-Fe	74.2b	76.4a	91.6a	84.9a	4.16c	83.6b	59.7b	22.6c
Add-EDTA-Fe	57.9c	71.1a	89.8a	79.9a	6.20b	78.6b	57.9b	37.5a

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. Add-Citrate-Fe = additional citrate-Fe^{3+}, Add-EDTA-Fe = additional EDTA-Fe^{3+}.

6.3.2 Chlorophyll Index (SPAD value)

Chlorophyll index decreased in As-treated plants as compared to control **(Fig. 6.2a)**. Chlorophyll index increased slightly in additional citrate-Fe^{3+} plants as compared to As-treated plants, though this increase was not much. However, chlorophyll index increased much in additional EDTA-Fe^{3+} plants as compared to As-treated plants and was almost near to control plants **(Fig. 6.2a)**.

6.3.3 Leaf Number, Width of Leaf Blade and Tiller Number

Leaf number decreased significantly in As-treated plants as compared to the control **(Fig. 6.2b)**. Additional citrate-Fe^{3+} or EDTA-Fe^{3+} enhanced leaf number as compared to As-treated plants. Arsenic decreased width of leaf blade and tiller number. Additional citrate-Fe^{3+} or EDTA-Fe^{3+} failed to enhance leaf number and tiller number as compared As-treated plants **(Figs. 6.2cd)**.

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

Dry weight decreased in shoots of As-treated plants as compared to control, however, DW increased in additional citrate-Fe^{3+} and additional EDTA-Fe^{3+} plants as compared to As-treated plants **(Fig. 6.3a)**. Similar tendency was observed in root DW and shoot height, in which the response was clearer in case of shoot height **(Fig. 6.3b)**.

6.3.5 Macro and Micronutrients

Arsenic increased P concentration in shoots but decreased in roots as compared to control **(Table 6.1)**. Phosphorus concentration decreased in the shoots of additional citrate-Fe^{3+} or additional EDTA-Fe^{3+} treated plants as compared to the others **(Table 6.1)**. Phosphorus accumulation in shoots of additional citrate-Fe^{3+} and additional EDTA-Fe^{3+} treated plants decreased as compared to the others **(Table 6.1)**. Phosphorus translocation decreased in additional citrate- Fe^{3+} and additional EDTA-Fe^{3+} plants as compared to control and As-treated plants. Potassium and Mg concentration were not much affected in shoots with the treatments though Ca concentration decreased in As-treated and additional citrate-Fe^{3+} or additional EDTA-Fe^{3+} as compared to control.

Potassium concentration in shoots did not seem to be affected much in As-treated plants. However, K concentration decreased in roots of As-treated, additional citrate-Fe^{3+} or additional EDTA-Fe^{3+} treated plants **(Table 6.1)**. Potassium concentration increased in the roots of additional citrate-Fe^{3+} or EDTA-Fe^{3+} as compared to As-treated plants. In this experiment, the effect of citrate-Fe^{3+} and EDTA-Fe^{3+} were more or less similar regarding K concentration in roots. Potassium accumulations decreased both in shoots and roots of As-treated and citrate-Fe^{3+} or EDTA-Fe^{3+} plants as compared to control **(Table 6.1)**. Potassium translocation was not affected much by the applied treatments.

Table 6.2 Concentrations, accumulations and translocation of As in plant parts of rice seedlings grown in different treatments of As, citrate or EDTA-Fe^{3+}.

Treatments	-----As (μg g^{-1} DW)----		-----As (μg $plant^{-1}$)-----		Translocation
	Shoot	Root	Shoot	Root	(%)
Control	nd	nd	nd	nd	nd
As-treated	59.6a	1126a	5.29a	43.3a	10.9a
Add-Citrate-Fe	52.3b	930b	5.89a	40.6a	12.6a
Add-EDTA-Fe	42.8b	859b	4.83a	42.3a	10.2a

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 As in shoot to the total accumulation (shoot + root). The translocation was expressed in %. DW = dry weight. nd = not detected. Add-Citrate-Fe = additional citrate-Fe^{3+}, Add-EDTA-Fe = additional EDTA-Fe^{3+}.

Calcium concentration decreased in shoots of As-treated plants as compared to control. Calcium concentration was similar in shoots of As-treated and additional citrate-Fe^{3+} or additional EDTA-Fe^{3+} plants **(Table 6.1)**. In roots, Ca concentrations were higher in As-treated and additional citrate-Fe^{3+} or additional EDTA-Fe^{3+} plants as compared to control **(Table 6.1)**. Additional citrate-Fe^{3+} or additional EDTA-Fe^{3+} seemed to have positive effect over As-treated plants on Ca accumulation both in shoots and roots **(Table 6.1)**. Calcium translocation was not much affected in the applied treatments **(Table 6.1)**.

Magnesium concentration decreased in shoots and roots of As-treated plants as compared to control **(Table 6.1)**. Accumulation of Mg was also decreased in As-treated shoots and roots as compared to control. Additional citrate-Fe^{3+} or additional EDTA-Fe^{3+} seemed to has positive effect on Mg accumulation both in shoots and roots as compare to As-treated plants. Magnesium translocation was not much affected by the used treatments **(Table 6.1)**.

Iron concentration decreased in shoots of As-treated plants as compared to control plants. However, Fe concentration increased in shoots of additional citrate-Fe^{3+} or additional EDTA-Fe^{3+} plants as compared to As-treated plants **(Table 6.1)**. Iron concentration was much enhanced in shoots of EDTA-Fe^{3+} treated plants and the enhancement was almost 2 fold as compared to As-treated plants **(Table 6.1)**. In roots, As-treated seedlings had higher content of Fe as compared to control plants. Iron concentrations further increased in roots of additional

citrate-Fe^{3+} or additional EDTA-Fe^{3+} plants as compared to As-treated plants, in which Fe concentration was higher in additional citrate-Fe^{3+} plants **(Table 6.1)**.

Iron accumulation was the highest in the shoots of control plants. Accumulation was almost similar in control and EDTA-Fe^{3+} treated shoots **(Table 6.1)**. In roots, the highest accumulation was in the additional citrate-Fe^{3+} treated plants. Iron translocation was the most decreased in As-treated plants as compared to the other elements **(Table 6.1)**.

Manganese concentration decreased in shoots of As-treated plants as compared to control plants. However, Mn concentration increased in shoots of additional citrate-Fe^{3+} and EDTA-Fe^{3+} plants as compared to As-treated plants **(Table 6.1)**. Similar trends were found in the Mn accumulation in shoots. In roots, Mn concentration was higher in As-treated and additional citrate-Fe^{3+} or EDTA-Fe^{3+} plants as compared to control **(Table 6.1).** Similar results were found in case of Mn accumulation **(Table 6.1)**. Manganese translocation was lower in As-treated and additional citrate-Fe^{3+} or EDTA-Fe^{3+} plants as compared to control **(Table 6.1)**. Similar to the Mn concentration, Zn and Cu concentrations were affected in As-treated and additional citrate-Fe^{3+} or EDTA-Fe^{3+} plants **(Table 6.1)**.

6.3.6 Arsenic

Arsenic concentration **(Table 6.2)** seemed to be decreased both in shoots and roots of additional citrate-Fe^{3+} and EDTA-Fe^{3+} treated plants as compared to As-treated plants, in which the effect seemed to be higher in additional EDTA-Fe^{3+} plants. However, the accumulation and translocation of As were not much affected with the treatments **(Table 6.2)**.

6.4 DISCUSION

Reduction of turgidity under sunlight implied that As was an indicative of a sudden decrease in water mobility. Reduction of chlorophyll index was the sign of As-toxicity. It was reported that determination of chlorophyll content was often accomplished to assess the impact of most environmental stress, as the pigment content was linked to the visual symptoms and photosynthetic plant productivity (Jain and Gadre 1997).

Additional citrate-Fe^{3+} seemed to decrease As-toxicity as compared to As-treated plants but the reduction was not very pronounced. However, additional EDTA-Fe^{3+} effectively enhanced the chlorophyll concentration as compared to As-treated and additional citrate-Fe^{3+}

plants, indicating that EDTA-Fe^{3+} may be absorbed more easily by the plants as compared to citrate-Fe^{3+} **(Table 6.1)**.

Arsenic-toxicity decreased chlorophyll index as compared to control plants **(Fig. 6.2a)** and produced whitish chlorosis in the fully developed young leaves, however, in spite of decreasing chlorophyll indices by higher As concentration Kalmi did not show any chlorosis (Shaibur et al. 2009b). Enhancement of chlorophyll index in the EDTA-Fe^{3+} plants as compared to As-treated and additional-Fe^{3+} plants indicated that the effectivity of EDTA-Fe^{3+} was higher than that of citrate-Fe^{3+}. Chlorosis could be different types e.g.- Mg-chlorosis in the old leaves (Maynard 1979), Fe-chlorosis in the young leaves (Mengel and Kirkby 2001), P-induced Fe-chlorosis and heavy metals (e.g.- Mn, Zn and Cu)-induced Fe-chlorosis (Mengel and Kirkby 2001).

Reddish color of the roots of As-treated plants was most probably due to increase of Fe. Iron may form plaque on the root surface resulting in the reddish color. Iron may effectively form complex with As in the root surface. It is reported that 12 nm size crystal of Fe-oxide form complex with As of contaminated water, this Fe-As complex could be removed from the water by a simple magnate and the concentration of As come much below to the permissible limit (Yavuz et al. 2006).

Shoot DW decreased by almost 48, 34 and 34% in the shoots in As-treated and additional citrate-Fe^{3+} or additional EDTA-Fe^{3+} plants, respectively. The values were 17, 6 and 6% in the roots for the same. Shoot DW increased by 27% in both the additional citrate-Fe^{3+} and additional EDTA-Fe^{3+} plants as compared to As-treated plants. Similar results were obtained in root length and shoot height. Additional citrate-Fe^{3+} and EDTA-Fe^{3+} increased the DW significantly as compared to the As-treated plants, indicating that additional citrate-Fe^{3+} and EDTA-Fe^{3+} could overcome the As-toxicity partially. The difference between the activity of citrate-Fe^{3+} and EDTA-Fe^{3+} was not pronounced **(Fig. 6.3a)**. It is reported that after 14 weeks, up to 40 mg As kg^{-1} soil, the yield of vegetative material of barley (stalks and leaves) did not vary very much but at higher As concentrations (80, 160, and 320 mg As kg^{-1} soil) the amount of above ground matter was greatly reduced (Lambkin and Alloway 2003).

In presence of additional citrate-Fe^{3+} or EDTA-Fe^{3+}, P concentration seemed to be decreased in shoots but increased in roots as compared to the others **(Table 6.1)**. Similar result was also obtained in case of accumulation in additional citrate-Fe^{3+} and EDTA-Fe^{3+} plants. We

know that P could make complex with Fe, then the enhancement of P accumulation in root might be possible because Fe accumulation increased. The reduction of Mg accumulation might be due to reduction of growth by As-toxicity.

Lower concentration of Fe in As-treated shoots tissues was mostly responsible for the induction of whitish chlorosis in the young leaves. The CDL of Fe in the leaves are 30-50 μg g^{-1} DW (Bergmann 1988) which induces Fe-deficiency symptom. Iron concentration in the shoots of our As-treated plants was within the CDL, resulting in whitish chlorosis. In citrate-Fe^{3+} treated plants, Fe concentration increased a little as compared to As-treated plants but the increase was not sufficient for overcoming the whitish chlorosis. In EDTA-Fe^{3+} treated plants, shoots contained higher concentrations of Fe as compared to additional citrate-Fe^{3+} plants. Our result showed that higher Fe in the shoots of EDTA-Fe^{3+} plants results in the alleviation of Fe-chlorosis. This result also indicated that EDTA-Fe^{3+} was more effective to ameliorate Fe-chlorosis than the citrate-Fe^{3+}. Among the macro and micronutrients, Fe-translocation was the most affected with As-treatment **(Table 6.1)**. Arsenic-toxicity reduced almost 60% translocation of Fe **(Table 6.1)**. We believe that this is the first report showing that As-induced chlorosis can not be ameliorated with additional citrate-Fe^{3+} but it could be ameliorated with additional EDTA-Fe^{3+}. To the best of our knowledge, there was no report regarding the effectivity of citrate-Fe^{3+} and EDTA-Fe^{3+} to ameliorate As-induced Fe-chlorosis, resulting from Fe-deficiency. The result may be caused by the fact that citrate may be degraded by microorganisms in the medium more easily than EDTA.

Higher concentration of Fe in roots of As-treated plants was most probably due to the fact that in presence of As, citrate-Fe^{3+} was mostly concentrate in roots, resulting in lower translocation from roots to shoots **(Table 6.1)**. Higher Fe was concentrated in the roots of additional citrate-Fe^{3+} plants and, therefore, may not be able to overcome whitish chlorotic symptom in shoots. In case of EDTA-Fe^{3+} treated plants, the concentration in roots was lowered compared to citrate-Fe^{3+} treated plants, but in shoots it was higher and therefore, resulting in the more efficient result for overcoming the whitish chlorosis.

Manganese concentration was also decreased in shoots like the Fe concentration. Lower concentration of Mn in shoots might also be involved for the induction of chlorosis in the As-treated plants. However, in additional citrate-Fe^{3+} plants the Mn concentration was higher than the As-treated plants but the plants were chlorotic. Additionally, Mn concentration in additional

EDTA-Fe^{3+} plants was lower compared to additional citrate-Fe^{3+} plants but the plants were green in EDTA-Fe^{3+} plants. Therefore, it could be postulated that lower concentration of Mn in As-treated plants was not involved for the induction of whitish chlorosis in the fully developed young leaves **(Fig. 6.1)**. Similar to the Mn concentration, Zn and Cu also were not be involved for the induction of chlorosis in the As-treated plants.

Reduction of As concentration both in shoots and roots was most probably due to the dilution effect as the DW was higher in additional citrate-Fe^{3+} and EDTA-Fe^{3+} treatment as compared to As-treated plants.

6.5 CONCLUSIONS

Arsenic induced chlorosis in the fully developed young leaves could be alleviated with additional EDTA-Fe^{3+}. The effectivity of additional EDTA-Fe^{3+} was much higher as compared to citrate-Fe^{3+} to alleviate As-induced Fe-chlorosis. Our result further proved that As-induced chlorosis was Fe-chlorosis caused by Fe-deficiency.